CHAPTER 4

Results and discussion

4.1 Algal extract

In this study, anti-HSV activity of the freshwater green macroalgae, *Spirogyra* spp. (Tao) was determined, which was selected based on much usefulness and commonly consumed in Northern of Thailand. This green macroalgae was cultivated and collected from the natural pond at Ban Nakooha, Sankhun district, Amphoe Muang, Phrae province.

4.2 Extraction of *Spirogyra* spp.

Spirogyra spp.. was extracted by water, ethanol and methanol. The extracts were evaporated and lyophilized to yield the crude extracts. The percentage yield of each extract was calculated by the dry weight as follows:

$$\text{dry weight (\%)} = \frac{\text{Dry weight} \times 100}{\text{Fresh algal weight}}$$

It was found that the highest percentage yield of 23.52% was the methanolic extract of *Spirogyra* spp. Whereas, the aqueous and ethanolic extracts showed the lower percentage yield of 6.78 and 12.43 % as shown in Table 4.1.

All of crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solution at concentration of 100 mg/ml. The appearance of crude extracts of the ethanolic and methanolic extracts showed similar color of deep green. On the other hand, the appearance of crude extracts from the aqueous extract showed different color of black-brown (Figure 4.1).

Table 4.1 Percentage yield of aqueous, ethanolic and methanolic extracts of *Spirogyra spp.*.

Crude extract	Fresh algal weight (g)	Dried weight (g)	(%)
Aqueous extract (AE)	250.00	16.95	6.78
Ethanolic extract (EE)	250.00	31.08	12.43
Methanolic extract (ME)	250.00	58.79	23.52

(a) (b) (c)

Figure 4.1 The appearance of crude extracts; aqueous extract (a), ethanolic extract (b) and methanolic extract (c) from *Spirogyra* spp.

Steriled distilled water, 95% ethanol and methanol were used as solvent for extraction because these solvents were able to solubilize compounds depending on their polarity. Water is universal solvent, which can extract the polar constituents of natural substance while ethanol and methanol can extract unpolar compound. Thus, the solvents were able to extract the algal constituents such as active, inactive or inert compounds (Osonwa *et al*., 2012; Tiwari *et al*., 2011). Many prior reports were used these solvents for extraction of constituent substances of microalgae and macroalgae that showed anti-microorganism, antioxidant and anti-acethycholinesterase activities (Zhu *et al*., 2003; Ghosh *et al*., 2004; Kartal *et al*., 2009).

Moreover, some natural compounds were sensitive to chemical degradation. The validation of procedure, temperature and periods of extraction were necessary in extraction protocol (Tiwari *et al*., 2011). Additionally, different extraction step also affected quantity composition of extracts. In the same way, many factors were influence

on the extract such as the natural geographical location, collection period, drying methods and storage condition (Das *et al.*, 2010; Odey *et al.,* 2012; Osonwa *et al*., 2012).

4.3 Cytotoxicity of algal extract by MTT assay

The evaluation of cytotoxicity is an important section for safety margin of antiviral agent. Thus, the cytotoxicity of algal extracts were tested before determination of the efficacy of algal extract against HSV infection.

In this study, the investigation of cell viability assay was performed by MTT assay. Cytotoxicity doses of aqueous, ethanolic and methanolic extracts of *Spirogyra* spp. were reported as 50% cytotoxic dose (CD_{50}), which was the concentration of the extract that caused cell toxicity by 50% as compared to the cell control.

The extracts of *Spirogyra* spp. were evaluated for cytotoxicity on Vero cells by MTT assay after reconstitution the algal extracts by DMSO. Cytotoxicity of DMSO on Vero cells was also determined. The result showed that CD_{50} value of DMSO on Vero cells was 3.60±0.00%. Therefore, percentage of DMSO that used to reconstitute the algal extract had no impact on the antiviral activity. Accordingly, some similar documents were shown that DMSO, could be used as solvent to reconstitute the compound (Georges *et al* , 2002; Spruance *et al*., 1983). Moreover, acyclovir (ACV) anti-viral drug was used as positive control for the examination of anti-HSV infection. Thus, the toxicity of ACV on Vero cells was also performed by MTT assay. The CD_{50} value of ACV was 1,025.15±0.00 µg/ml.

The results showed that 50% cytotoxicity dose (CD_{50}) of the aqueous extract of *Spirogyra* spp. exerted lower toxicity than ethanolic and methanolic extract with CD_{50} values of 4,363.30, 356.57 and 250.80 µg/ml, respectively (Table 4.2). Cytotoxicity effects from the other extracts of marine and freshwater algae such as the polysaccharide extract of red seaweed macroalgae; *Gracilaria corticata* (Mazumder *et al.*, 2002), the aqueous extract of brown seaweed macroalgae; *Sargassum patens* (Zhu *et al.*, 2003), hot water extract of green seaweed macroalgae; *Caulerpa racemosa* (Ghosh *et al.*, 2004) and ethanolic extract from freshwater green macroalgae; *Spirogyra*

gratiana (Kartal *et al.*, 2009) were also determined. The CD_{50} values ranged from 1,525-3,460 µg/ml.

In this study, the non-toxic concentrations of *Spirogyra* spp. extracts were used to determine anti-HSV activity. Anti-HSV activity of this algal extracts on standard HSV-1(F) and HSV-2(G) and HSV-1 resistant strains were tested before, during and after viral adsorption using a plaque reduction assay.

Table 4.2 The 50% Cytotoxicity doses (CD_{50}) of aqueous extract, ethanolic extract and methanolic extract of *Spirogyra* spp. determined by MTT assay.

Crude extract of *Spirogyra* spp.	CD_{50} ± SD (µg/ml)*	The highest of non-toxic concentration used (µg/ml)
Aqueous extract (AE)	4,363.30 ± 11.05	1,250
Ethanolic extract (EE)	356.57 ± 8.03	300
Methanolic extract (ME)	250.80 ± 7.04	150

* The values in table are presented as means ± standard deviation (SD) of three independent experiments

4.4 Plaque titration assay of herpes simplex virus and inhibitory activity of acyclovir

The quantitation of standard HSV-1F, HSV-2G and clinical ACV-resistant HSV-1 isolates 1A, 1B, 11, 12 and 22 were investigated by plaque titration assay. The result showed that the titers of HSV-1F and HSV-2G were 9.18 x 10^8±0.32 and 2.40 x 10^6±0.26 PFU/ml (Table 4.3). Additionally, The titers of five samples of ACV-resistant HSV-1 isolates ranged from 1.51 x 10^6 - 5.28 x 10^8 PFU/ml.

Inhibitory activity of ACV on HSV infection was determined by plaque reduction assay. The concentration of ACV which inhibited viral growth or plaque formation were evaluated and expressed as 50% Inhibitory concentration (IC_{50}).

The result showed that, ACV could inhibit HSV-1F and HSV-2G with IC_{50} values of 1.93 ± 0.10 and 3.81 ± 0.21 μg/ml (Table 4.3). Accordingly, the dose that inhibit HSV infection by 50% (IC_{50}) values of ACV, were used as drug positive control in antiviral assay. IC_{50} values of ACV on the clinical ACV-resistant HSV-1 isolates were 6.80 - 11.19 μg/ml (Table 4.3).

Table 4.3 The viral titers and the 50% inhibitory concentration (IC_{50}) of acyclovir.

HSV strains	**Viral titers (PFU/ml)***	**IC_{50} of acyclovir (μg /ml)***
Standard HSV-1(F)	$9.18 \times 10^8 \pm 0.32$	1.93 ± 0.10
Standard HSV-2(G)	$2.40 \times 10^6 \pm 0.26$	3.81 ± 0.21
HSV-1 isolate 1A	$1.51 \times 10^6 \pm 0.12$	8.89 ± 0.24
HSV-1 isolate 1B	$2.25 \times 10^7 \pm 0.16$	7.41 ± 0.32
HSV-1 isolate 11	$5.24 \times 10^7 \pm 0.24$	6.80 ± 0.26
HSV-1 isolate 12	$1.60 \times 10^7 \pm 0.15$	10.85 ± 0.22
HSV-1 isolate 22	$5.28 \times 10^8 \pm 0.20$	11.19 ± 0.16

* The values in table are presented as means ± standard deviation (SD) of three independent experiments

4.5 Determination of mechanism of *Spirogyra* spp. extracts on inhibition of HSV inhibition

The efficacy of *Spirogyra* spp. extracts on many stages of standard HSV-1(F), HSV-2(G) and clinical ACV-resistant HSV-1 isolates multiplication cycles were elucidated. Standard HSV strains and ACV-resistant HSV strains were treated with *Spirogyra* spp. extracts before, during and after viral attachment on Vero cells. Moreover, the measurement of viral titers was investigated using plaque titration assay. Direct Inhibition of viral particles and viral replication were also determined. The inhibitory effect of *Spirogyra* spp. extracts on viral DNA and protein synthesis was also investigated.

4.5.1 Effect of *Spirogyra* spp. extracts on standard HSV and ACV-resistant HSV-1 isolates when treatment with the extracts before viral attachment

In this present study, the inhibitory effect of *Spirogyra* spp. extracts on HSV infection was determined when the algal extract was treated before viral attachment to Vero cells. Vero cells were pretreated with various non-toxic concentrations of *Spirogyra* spp. extracts before attachment of standard HSV-1F, HSV-2G and ACV-resistant HSV-1 (Table 4.4).

The result showed that efficacy of these algal extracts on HSV infection and concentration of extracts, which reduced number of plaques by 50% when comparing to virus control was determined and expressed as effective dose at 50% (ED_{50}) (Robin *et al*., 2002). The low value of ED_{50} exerted the high efficacy of algal extracts on HSV (Kartal *et al.,* 2009; Tolo *et al.,* 2006). The ED_{50} value of algal extract was evaluated from calculation of graphic plot, which was determined by the linear regression equation, $Y = mx + C$; whereas Y was the percentage of inhibition plaques formation, X was the concentrations of algal extract, while value of m and c were the constant values. In addition, the therapeutic index; TI value was calculated from the ratio of CD_{50} and ED_{50} value. TI value demonstrated the therapeutic potential of the algal extracts since the high TI value reflected high therapeutic potential effect of the extracts.

The results showed that plaques formation of HSV-2G on Vero cells was highly inhibited by aqueous extract of *Spirogyra* spp. with ED_{50} value of 271.4±0.80 µg/ml and TI value of 16.08±0.65 (Table 4.4). In contrast, the result found that the aqueous extract of *Spirogyra* spp. could protect the cell from HSV-1F infection with percentage of inhibition by 42.40±0.50. Moreover, the ethanolic extract of *Spirogyra* spp. inhibited HSV-1F and HSV-2G with percentage of inhibition by 24.30±1.05 and 20.57±2.00, respectively. Furthermore, the methanolic extract of *Spirogyra* spp. inhibited HSV-1F and HSV-2G with percentage of inhibition by 19.96±1.70 and 24.35±1.52, respectively (Table 4.4).

Moreover, the efficacy of *Spirogyra* spp. extracts was evaluated before attachment of HSV-1 resistant strains. The methanolic extract showed the strongest protection of the cell from infection by HSV-1 resistant strains as observed by ED_{50} and

TI values. The results showed that the ED_{50} values of methanolic extract ranged from 56.09-85.14 µg/ml and TI values ranged from 2.95-4.47. The methanolic extract had the highest efficacy to inhibit HSV-1 resistant strains isolate No. 1A, 1B, 22, 12, 11 by 66.50, 64.20, 61.80, 51.40 and 50.50 %, respectively (Table 4.4). However, the aqueous and ethanolic extracts were able to inhibit HSV-1 resistant strains less than methanolic extract. The aqueous extract of *Spirogyra* spp. could inhibit HSV-1 resistant strains with percentage of inhibition ranging from 20.95-23.55%. Similarity, the ethanolic extract of *Spirogyra* spp. could inhibit HSV-1 resistant strains infection on Vero cells with percentage of inhibition ranging from 24.75-45.88% (Table 4.4). Thus, aqueous and ethanolic extracts had low effect on HSV-1 resistant strains and could protect the cells from viral infection less than 50%. For this reason, ED_{50} of these extracts; aqueous extract (AE), ethanolic extract (EE) could not be calculated (Table 4.4).

Therefore, anti-HSV activities of *Spirogyra* spp. extracts on HSV-1F activity when treatment before viral attachment were ranged from the highest to lowest activities in the following order; aqueous extract (AE) > ethanolic extract (EE) > methanolic extract (ME), respectively. On the other hand, the highest inhibition when treatment before HSV-2G attachment was revealed from extracts of aqueous extract (AE) > methanolic extract (ME) > ethanolic extract (EE), respectively. The algal extracts demonstrated the inhibitory effects on HSV-2G higher than HSV-1F except EE extract.

Moreover, anti-HSV activities of *Spirogyra* spp. extracts on five samples of HSV-1 resistant strains infection when treatment before viral attachment on Vero cells were ranged from the highest to lowest activities in the following order; methanolic extract (ME) > ethanolic extract (EE) > aqueous extract (AE), respectively. *Spirogyra* spp. (Tao) might be the new source of anti-HSV agents that has been recognized.

The interaction between the active compounds and viral outer surface proteins were from specific attachment of the pathogen to the host cell membrane (Gescher *et al*., 2011; Wagner *et al*., 2008). Hence, inhibition of this process may prevent or decrease the severity of viral infection (Spear and Roizman *et al.,* 1972). These reports could support that the natural compounds of *Spirogyra* spp. extracts had prophylaxis effect against HSV infections, which might block viral entry or penetration process of

viral particle into cell culture (Adhikari *et al*., 2006; Mazzanti *et al.,* 2008). The crude 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 initial entry to the host cell culture was found from the study of Mazumder *et al.* (2002), which reported that the extract of polysaccharides from red seaweed macroalgae; *Gracilaria corticata* could inhibit HSV-1 and HSV-2 infection. Additionally, the report of Adhikari *et al.* (2006) illustrated the sulfated fucoidan from brown seaweed macroalgae; *Stoechospermum marginatum* that inhibited virus entry and adsorption.

Table 4.4 Inhibition of standard HSV and ACV-resistant HSV-1 infection by aqueous, ethanolic and methanolic extract of *Spirogyra* spp. when treatment before viral attachment on Vero cells.

HSV strain	Crude extract (*Spirogyra* spp.)								
	Aqueous extract (AE)			Ethanolic extract (EE)			Methanolic extract (ME)		
	% Inhibition*	ED_{50} ± SD (μg /ml)*	TI±SD*	% Inhibition*	ED_{50} ± SD	TI±SD*	% Inhibition*	ED_{50} ± SD (μg /ml)*	TI±SD*
Standard HSV									
HSV-1(F)	42.40±0.50[a]	0 [a]	0 [a]	24.30±1.05	0 [a]	0 [a]	19.96±1.70[a]	0 [a]	0 [a]
HSV-2(G)	51.24±1.05[a]	271.4±0.80[a]	16.08±0.65[b]	20.57±2.00[a]	0 [a]	0 [a]	24.35±1.52	0 [a]	0 [a]
ACV-resistant HSV-1									
Isolate 1A	22.81±1.00	0 [a]	0 [a]	25.35±1.09	0 [a]	0 [a]	66.50±1.00[a]	85.14±0.50[a]	2.95±0.61[a]
Isolate 1B	20.95±1.23[a]	0 [a]	0 [a]	45.88±1.20[a]	0 [a]	0 [a]	64.20±1.05[a]	80.59±0.93[a]	3.11±0.50[a]
Isolate 11	23.55±1.35	0 [a]	0 [a]	26.33±0.90	0 [a]	0 [a]	50.50±0.89	58.11±0.80[a]	4.32±0.52[b]
Isolate 12	21.91±2.20[a]	0 [a]	0 [a]	30.45±1.89	0 [a]	0 [a]	51.40±0.95	56.09±1.14[a]	4.47±0.86[b]
Isolate 22	22.46±1.28	0 [a]	0 [a]	24.75±1.56	0 [a]	0 [a]	61.80±1.59[a]	75.40±0.85[a]	3.33±1.04[a]

The measurement of TI value; TI= CD_{50}/ED_{50}

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.5.2 Effect of *Spirogyra* spp. extracts on standard HSV and ACV-resistant HSV-1 isolates when treatment with the extracts during viral attachment

The inhibitory effect of *Spirogyra* spp. extracts during viral attachment to cell culture was evaluated. Various non-toxic concentrations of *Spirogyra* spp. extracts, standard HSV-1F, HSV-2G and ACV-resistant HSV-1 were applied together on monolayer of Vero cells. Plaque reduction assays were also performed to determine the efficacy of *Spirogyra* spp. extracts on inhibition of HSV attachment and infection to host cell.

The result demonstrated the potential efficacy of these algal extracts on HSV infection by ED_{50} and TI values. The inhibition of plaques formation of HSV-2G on Vero cells was the highest when treatment with methanolic extract (ME) of *Spirogyra* spp. Effective dose at 50% (ED_{50}) was 75.03 ±1.12 µg/ml and therapeutic indices (TI) was 3.34±0.90 (Table 4.5). Likewise, the high efficacy of ME extract of *Spirogyra* spp. could inhibit HSV-1F infection with ED_{50} and TI values of 108.16±1.05 µg/ml and 1.25±0.94, respectively. The result found that the aqueous extract (AE) and ethanolic extract (EE) of *Spirogyra* spp. could protect the cell during HSV-1F and HSV-2G attachment on Vero cells lower than the ME extract.

Moreover, the high inhibitory effect of EE extract during HSV-2G attachment was shown by the ED_{50} values of 72.65 ±0.96 µg/ml and therapeutic index (TI) of 4.91±0.70 (Table 4.5). Similarity, the AE extract could inhibit HSV-2G with the ED_{50} and TI values of 159.07±1.20 and 27.43±1.00, respectively. Furthermore, the results of inhibitory effect of EE extract when treatment during HSV-1F attachment was shown. The ED_{50} value was 164.20±1.26 µg/ml and therapeutic index (TI) was 2.17±0.85. Similarity, the AE extract could inhibit HSV-1F with the ED_{50} and TI values of 310.88±0.94[a] and 14.04±0.60, respectively (Table 4.5).

In addition, the study was investigated the inhibitory efficacy of *Spirogyra* spp. extracts when treatment during HSV-1 resistant strains attachment on Vero cells. The methanolic extract was shown the highest protection of the cell from infection of HSV-1 resistant strains infection . The ME extract had efficacy to inhibit five samples of HSV-1 resistant strains isolate No. 1A, 1B, 22, 12, 11 with 75.94, 52.68, 62.10, 55.65 and

70.14% inhibition, respectively (Table 4.5). The ED_{50} values of ME extract, ranged from 70.45-148.09 µg/ml and therapeutic index (TI) ranged from 1.69-3.56. However, the aqueous (AE) and ethanolic (EE) extracts showed inhibitory effect on HSV-1 resistant strains lower than ME extract. The result was similar to the result of treatment before viral attachment to host cell. In addition, the EE extract of *Spirogyra* spp. could inhibit HSV-1 resistant strains infection on Vero cells with percentage of inhibition ranging from 24.98-55.46% (Table 4.5). Particularly, the EE extract had efficacy to inhibit HSV-1 resistant strains isolate No. 12 with the ED_{50} and TI values of 284.16±1.20 and 1.25±0.94, respectively. The AE extract of *Spirogyra* spp. could inhibit HSV-1 resistant strains with percentage of inhibition ranging from 20.04-38.19%. Thus, the AE extracts had the lowest effect on HSV-1 resistant strains and could protect the cells from viral infection less than 50%. Thus, the ED_{50} value of this extracts could not be calculated (Table 4.5).

Accordingly, anti-HSV activities of *Spirogyra spp.* extracts on HSV-1F activity when treatment during viral attachment were ranged from the highest to lowest activities in the following order; ethanolic extract (EE) > methanolic extract (ME) > aqueous extract (AE), respectively. The inhibition when treatment HSV-2G before attachment was revealed from extracts of methanolic extract (ME) > ethanolic extract (EE) > aqueous extract (AE), respectively. The algal extracts had inhibitory effects on HSV-2G higher than HSV-1F when treatment during viral attachment.

Therefore, anti-HSV-1 resistant strains activities of *Spirogyra* spp. extracts when treatment of *Spirogyra* spp. extracts during viral attachment on Vero cells were ranged from the highest to lowest activities as follows; methanolic extract (ME) > ethanolic extract (EE) > aqueous extract (AE), respectively. These results related to the treatment of *Spirogyra* spp. extracts during viral attachment on host cell (Table 4.5).

These results could suggest that several types of compounds in *Spirogyra* spp. extracts might have potential to inhibit HSV infection by blocking or direct interfering and/or competitive binding of during viral particles to specifically cellular receptor on host cell surface. The interference of initially virion attachment on host cells might be occurred and 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 algal extract could obstruct the fusion process between virion envelope and cell surface receptor that required for penetration (Manservigi *et al*., 1977; Wagner *et al*., 2008). Moreover, the extract might interfere with gD and oligomers of gH and gL from interaction with cell receptor (Roizman and Spear, 1996). In addition, various compounds in algal 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 *Spirogyra* spp. 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; Wagner *et al*., 2008). Moreover, viral particles might be destroyed directly by *Spirogyra* spp. extracts.

Table 4.5 Inhibition of standard HSV and ACV-resistant HSV-1 infection by aqueous, ethanolic and methanolic extract of *Spirogyra* spp. when treatment during viral attachment on Vero cells.

HSV strain	Crude extract (*Spirogyra* spp.)								
	Aqueous extract (AE)			Ethanolic extract (EE)			Methanolic extract (ME)		
	% Inhibition*	ED_{50} ± SD (μg /ml)*	TI±SD*	% Inhibition*	ED_{50} ± SD (μg /ml)*	TI±SD*	% Inhibition*	ED_{50} ± SD (μg /ml)*	TI±SD*
Standard HSV									
HSV-1(F)	50.31±1.10[a]	310.88±0.94[a]	14.04±0.60[a]	81.40±1.52	164.20±1.26	2.17±0.85[a]	71.23±1.65[a]	108.16±1.05[a]	1.25±0.94[a]
HSV-2(G)	87.54±058[a]	159.07±1.20[a]	27.43±1.00[a]	94.52±0.70[a]	72.65 ±0.96[a]	4.91±0.70[a]	96.25±0.39	75.03 ±1.12[a]	3.34±0.90[a]
ACV-resistant HSV-1									
Isolate 1A	21.04±1.10	0 [a]	0 [a]	24.98±1.00	0 [a]	0 [a]	75.94±1.15[a]	70.45±0.80[a]	3.56±0.72[a]
Isolate 1B	20.04±1.50[a]	0 [a]	0 [a]	42.11±0.90[a]	0 [a]	0 [a]	52.68±1.00[a]	148.09±1.25[a]	1.69±0.80[-a]
Isolate 11	21.86±1.09[a]	0 [a]	0 [a]	29.25±0.65	0 [a]	0 [a]	62.10±0.85	137.51±0.93[a]	1..82±0.75[a]
Isolate 12	38.19±1.45[a]	0 [a]	0 [a]	55.46±1.19	284.16±1.20[a]	1.25±0.94[a]	55.65±0.91	145.88±0.70[a]	1.72±1.00[a]
Isolate 22	20.90±1.70	0 [a]	0 [a]	25.04±1.03	0 [a]	0 [a]	70.14±1.50[a]	106.41±0.90[a]	2.36±1.02[a]

The measurement of TI value; TI= CD_{50}/ED_{50}

*****These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.5.3 Effect of *Spirogyra* spp. extracts on standard HSV and ACV-resistant HSV-1 isolates when treatment with the extracts after viral attachment

The inhibitory effects on HSV infection by *Spirogyra* spp. extracts was also investigated when treatment after viral attachment to cell culture. After viral adsorption for 1 hour, each concentration of algal extracts was applied onto the infected cells. This step demonstrated antiviral 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; Mandal *et al*., 2008).

The inhibitory effects of *Spirogyra* spp. extracts on standard HSV-1F, HSV-2G and and ACV-resistant HSV-1 isolates were demonstrated using plaque reduction assay when treatments with the extract after viral attachment. The result demonstrated the potential of these algal extracts on inhibition of HSV infection by calculation of ED_{50} and TI values.

The results showed that the highest inhibitory effect of HSV-1F on Vero cells was from ethanolic extract (EE) of *Spirogyra* spp. with ED_{50} values of 166.40±1.12 µg/ml and TI value of 2.14±0.50 (Table 4.6). In contrast, the result found that the aqueous extract (AE) and ethanolic extract (EE) of *Spirogyra* spp. could inhibit HSV-1F infection lower than the ME extract. The efficacy of AE and ME extract of *Spirogyra* spp. was low and they could inhibit HSV-1F infection with percentage of inhibition as 23.11±0.70 and 30.72±1.10, respectively. Thus, AE and ME extracts had little effect on HSV-1F standard strain, and could inhibit viral infection less than 50%. So that, the ED_{50} value of this extracts could not be calculated (Table 4.6). Moreover, inhibitory effect after HSV-2G attachment by AE extract showed ED_{50} value of 621.58±0.80 µg/ml and TI value of 7.02±0.71 (Table 4.6). On the other hand, the EE and ME extracts could inhibit HSV-2G infection lower than AE extract with the percentage of inhibition of 20.14±0.40 and 22.51±0.23, respectively (Table 4.6).

Additionally, the study demonstrated the inhibitory effect of *Spirogyra* spp. extracts on ACV-resistant HSV-1 strains after viral attachment on Vero cells. The result revealed that the EE extract showed the highest inhibition from infection of five samples of ACV-resistant HSV-1 strains as reported by ED_{50} and TI values. The ED_{50}

values of EE extract ranged from 125.60-137.25µg/ml and TI values ranged from 2.60-2.84. Furthermore, the ME extract had an efficacy to inhibit five samples of HSV-1 resistant strains isolate No. 1A, 1B, 22, 12, 11 with the percentage of inhibition as 54.20, 51.08, 48.02, 46.55 and 52.45, respectively. However, the aqueous (AE) extract showed the lowest inhibitory effect on HSV-1 resistant strains with the percentage of inhibition of 28.40, 21.25, 23.60, 20.65 and 28.53% inhibition, respectively (Table 4.6). The result of inhibition effect of AE extracts on HSV-1 resistant strains was less than 50%. Then, the ED_{50} value of this extracts could not be calculated

Consequently, anti-HSV activities of *Spirogyra* spp. extracts on HSV-1F activity when treatment after HSV attachment were ranged from the highest to lowest activities in the following order; ethanolic extract (EE) > methanolic extract (ME) > aqueous extract (AE), respectively. In contrast, the highest inhibition when treatment after HSV-2G attachment was revealed from extracts of aqueous extract (AE) > methanolic extract (ME) > ethanolic extract (EE), respectively. Besides, the EE and ME extracts showed the inhibitory effects against ACV- resistant HSV-1 higher than the AE extract (Table 4.6).

The anti-HSV activities obtained from the macroalgae 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. *Spirogyra* spp. extracts might inhibit viral production and prevent immediate early (α)-genes transcription including viral gC and gD expressions and viral DNA synthesis (Adhikari *et al*., 2006; Kiani *et al*., 2010). The similar researches on antiviral activity of *Spirogyra* spp. extract were reported. The extract of various marine macroalgae could inhibit HSV-1 and HSV-2 after viral attachment by polysaccharides from the extract of red marine algae *Gracilaria corticata*, (Mazumder *et al*., 2002). The polysaccharide from red marine algae, *Scinaia hatei* had potent anti-HSV activity (Mandal *et al*., 2008). Some reports showed that HSV adsorption to the cells was inhibited by sulfated fucans from brown marine macroalgae, *Stoechospermum marginatum* (Adhikari *et al*., 2006). The sulfated xylogalactofucan from marine brown algae, *Laminaria angustata* appeared to be an important hallmark of inhibition of HSV-1 attachment to cells and direct interaction of viral particles with the polysaccharides (Saha *et al.*, 2012).

Table 4.6 Inhibition of standard HSV and ACV-resistant HSV-1 infection by aqueous, ethanolic and methanolic extract of *Spirogyra* spp. when treatment after viral attachment on Vero cells.

HSV strain	Crude extract (*Spirogyra* spp.)								
	Aqueous extract (AE)			Ethanolic extract (EE)			Methanolic extract (ME)		
	% Inhibition*	ED_{50} ± SD (µg /ml)*	TI±SD*	% Inhibition*	ED_{50} ± SD (µg /ml)*	TI±SD*	% Inhibition*	ED_{50} ± SD (µg /ml)*	TI±SD*
Standard HSV									
HSV-1(F)	23.11±0.70[a]	0 [a]	0 [a]	52.24±0.81	166.40±0.70[a]	2.14±0.50[b]	30.72±1.10[a]	0 [a]	0 [a]
HSV-	51.60±0.90[a]	621.58±0.80[a]	7.02±0.71[b]	20.14±0.40[a]	0 [a]	0 [a]	22.51±0.23	0 [a]	0 [a]
ACV-resistant HSV-1									
Isolate 1A	28.40±1.20	0 [a]	0 [a]	61.52±1.10	137.25±1.03[a]	2.60±0.90[a]	54.20±0.90[a]	152.04±0.65[a]	1.65±0.80[a]
Isolate 1B	21.25±1.10[a]	0 [a]	0 [a]	54.64±0.89[a]	125.60±0.90[a]	2.84±0.85[a]	51.08±1.00[a]	148.25±0.90[a]	1.69±1.04[a]
Isolate 11	23.60±1.05	0 [a]	0 [a]	65.50±0.90	132.14±0.70[a]	2.70±0.65[a]	48.02±0.50	0 [a]	0 [a]
Isolate 12	20.65±1.14[a]	0 [a]	0 [a]	64.60±1.50	130.09±0.90[a]	2.74±1.00[a]	46.55±0.90	0 [a]	0 [a]
Isolate 22	28.53±1.00	0 [a]	0 [a]	67.54±1.04	128.18±1.00[a]	2.78±0.95[a]	52.45±1.00[a]	155.72±0.95[a]	1.61±1.15[a]

The measurement of TI value; TI= CD_{50}/ED_{50}

*****These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.5.4 Inactivation kinetics

In this study, viruses were incubated with the algal extracts and the mixtures were added to the cells. The mixtures of viruses and algal extracts were incubated at room temperature for 60 minutes interval up to 240 minutes. Direct inactivation of standard HSV-1F, HSV-2G and ACV-resistant HSV-1 particles was shown after treatment with aqueous (AE), ethanolic (EE) and methanolic (ME) extracts of *Spirogyra* spp. The viral particles were directly inactivated by all of the extracts to negligible amount within 2-4 hours. The result revealed that the ME extract had an ability to inhibit HSV-1F and HSV-2G particles (Table 4.7). Especially, HSV-2G was directly inactivation by ME extract to completely inhibition within 2 hours, while the inhibition of amount of HSV-1F was inactivated to negligible amounts within 4 hours. Thus, the inhibitory effect of ME extract on HSV-2G particles was the highest.

Moreover, virucidal effect of *Spirogyra* spp. 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 (Wijesekara *et al*., 2011). Log amount of HSV-1F was drastically reduced within 2 hours after incubation with the aqueous extracts. Thus, the aqueous extract of *Spirogyra* spp. showed significantly the highest inhibition of HSV-1F by reduction of virus titer to 2.51±0.40 PFU/ml (Table 4.7). The antiviral activities of extracts on viral particles were increased with a time-dependent manner. Moreover, at 2 hours of inactivation, HSV-2G was inactivated by significantly reduction of log virus titer (PFU/ml) to 5.14±0.09 when treatment with aqueous extract of *Spirogyra* spp. (Table 4.7).

Log amount of ACV-resistant HSV-1 strains was drastically reduced at 4 hours after incubation with the extracts. AE extract of *Spirogyra* spp. showed significantly the highest inhibition of ACV-resistant HSV-1 strains reduction of virus titter. The viral isolate No.1A, 1B, 11, 12 and 22 was inhibited and the titer were 3.05±0.10, 3.05±0.10, 2.73±0.11, 3.58±0.20 and 2.36±0.05 PFU/ml, respectively (Table 4.7). The antiviral activities of extracts on viral particles were increased by the time.

Table 4.7 Direct inactivation of standard HSV and ACV-resistant HSV-1 isolates by aqueous extract of *Spirogyra* spp.

HSV strain	Concentration of aqueous extract (µg/ml)[a]	Reduction of log virus titer (PFU/ml) ±SD*			
		Time of inactivation (h)			
		1	2	3	4
Standard HSV					
HSV-1(F)	500	0.65±0.40 [a]	0.76±0.20 [a]	0.84±0.15 [a]	1.08±0.20 [b]
HSV-2(G)	500	0.13±0.12 [a]	0.26±0.14 [a]	0.34±0.11 [a]	0.49±0.40 [a]
ACV-resistant HSV-1					
Isolate 1A	500	2.10±0.16 [b]	2.20±0.05 [b]	2.39±0.15 [b]	3.05±0.10 [b]
Isolate 1B	500	2.19±0.20 [c]	2.15±0.30 [c]	3.00±0.20 [c]	3.98±0.06 [c]
Isolate 11	500	1.08±0.05 [a]	1.12±0.04 [a]	1.55±0.09 [a]	2.73±0.11 [a]
Isolate 12	500	1.56±0.10 [c]	1.99±0.28 [c]	2.60±0.10 [c]	3.58±0.20 [c]
Isolate 22	500	1.44±0.09 [a]	1.60±0.50 [a]	1.95±0.08 [a]	2.36±0.05 [a]

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

Interestingly, HSV-1F and HSV-2G were completely inactivated after treatment with ethanolic (EE) extracts of *Spirogyra* spp. at a concentration of 250 µg/ml as shown in Table 4.8. Thus, the EE 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. The result showed that maximum reduction of plaque number was observed after treatment with EE extract at concentration of 250 µg/ml with significantly reduction of log HSV-1F titer by 7.05±0.11 PFU/ml at 4 hours after treatment. Furthermore, EE extract at concentration of 250 µg/ml were completely reduced HSV-2G titer by the reduction of virus titer by 8.95±0.14 log PFU/ml at 4 hours after treatment (Table 4.8).

On the other hand, EE extract at concentration of 250 µg/ml could highly reduce ACV-resistant HSV-1 isolate No.1B and No.12 titer by 5.94±0.06 and 5.25±0.08 log PFU/ml, respectively (Table 4.8). Moreover, the ability of extract on directly inactivation of ACV-resistant HSV-1 strains was significantly increased by the time. In addition, virucidal effect of the extracts on HSV particle was not caused by DMSO that was used to dissolve the extracts.

Thus, both aqueous and ethanolic extracts of *Spirogyra* spp. 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 (Saha *et al*., 2012; 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 (Table 4.7 and 4.8). This result suggested that the bioactive compounds could be isolated by ethanol and were able to inhibit HSV particle better than aqueous compound. Moreover, suitable solvent should be selected after consideration of low price and extraction potency. These results showed that these selected algal extracts showed strongly inhibition of HSV-1F, HSV-2G and ACV-resistant HSV-1 particles. Thus, the fresh green macroalgae *Spirogyra* spp. should be used for treatment of HSV infection.

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 (Saha *et al*., 2012). Moreover, compounds of algal 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 crude extract (Mazzanti *et al*., 2008; Yoosook *et al.,* 1999). Thus, it could indicate that *Spirogyra* spp. extracts might prevent re-infections with the newly produced viruses and also inhibited extracellular virus (Adhikari *et al*., 2006; Saha *et al*., 2012).

In this study, the ME extracts of *Spirogyra* spp. had strong inhibitory effect on HSV-2G viral particles when incubation period of virus and extracts was increased (Table 4.9). The result showed that maximum reduction of plaque number was observed after treatment with ME extract at concentration of 500 µg/ml with significantly reduction of HSV-2G titer by 7.62±0.11 log PFU/ml at 4 hours after treatment. The reduction of plaque number was shown after treatment with ME extract at concentration of 500 µg/ml with significantly reduction of HSV-1F titer by 5.97±0.03 log PFU/ml at 4 hours of treatment (Table 4.9). Furthermore, ME extract at concentration of 500 µg/ml were completely reduced the ACV-resistant HSV-1 isolate No.1A, 1B, 11, 12 and 22 titer by 7.05±0.05, 4.86±0.05, 4.67±0.07, 5.64±0.05 and 5.80±0.09 log PFU/ml, respectively, at 4 hours (Table 4.9). Moreover, the potential ability of extract on directly inactivation of ACV-resistant HSV-1 strains was significantly increased by the time.

Similar results of other algal extracts on inactivation of viral particles were shown by reduction of virus titers. Methanolic extract of various algae extracts such as red marine algae could inhibit cell free virus by virucidal effect and inhibited replication of herpes simplex virus (Chattopadhyay *et al*., 2007; Gonzalez *et al.,* 1987).

Table 4.8 Direct inactivation of standard HSV and ACV-resistant HSV-1 isolates by ethanolic extract of *Spirogyra* spp.

HSV strain	Concentration of ethanolic extract (µg/ml)[a]	Reduction of log virus titer (PFU/ml) ±SD*			
		Time of inactivation (h)			
		1	2	3	4
Standard HSV					
HSV-1(F)	250	1.04±0.10 [a]	1.42±0.30 [a]	3.72±0.14 [b]	5.63±0.20 [b]
HSV-2(G)	250	2.94±0.11 [c]	3.21±0.20 [c]	4.86±0.09 [b]	5.24±0.10 [b]
ACV-resistant HSV-					
Isolate 1A	250	2.06±0.10 [b]	2.21±0.07 [b]	2.94±0.08 [b]	3.68±0.12 [b]
Isolate 1B	250	2.41±0.13 [b]	2.65±0.10 [b]	3.46±0.09 [b]	5.94±0.06 [b]
Isolate 11	250	2.11±0.05 [b]	2.30±0.09 [b]	3.24±0.14 [b]	3.54±0.10 [b]
Isolate 12	250	2.33±0.07 [b]	2.49±0.12 [b]	3.36±0.08 [b]	5.25±0.08 [b]
Isolate 22	250	2.20±0.10 [b]	2.28±0.05 [b]	2.78±0.09 [b]	3.51±0.10 [b]

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

Table 4.9 Direct inactivation of standard HSV and ACV-resistant HSV-1 isolates by methanolic extract of *Spirogyra* spp.

HSV strain	Concentration of methanolic extract (µg/ml)[a]	Reduction of log virus titer (PFU/ml) ±SD*			
		Time of inactivation (h)			
		1	2	3	4
Standard HSV					
HSV-1(F)	500	5.28±0.10 [b]	5.40±0.21 [b]	5.90±0.13 [b]	5.93±0.10 [b]
HSV-2(G)	500	6.11±0.09 [c]	6.69±0.10 [c]	6.86±0.20 [c]	6.54±0.12 [c]
ACV-resistant HSV-					
Isolate 1A	500	2.50±0.02 [b]	2.68±0.06 [b]	3.95±0.09 [b]	7.05±0.05 [c]
Isolate 1B	500	2.05±0.10 [b]	2.29±0.10 [b]	3.10±0.04 [b]	4.86±0.05 [b]
Isolate 11	500	2.11±0.09 [b]	2.47±0.08 [b]	2.99±0.05 [b]	4.67±0.07 [b]
Isolate 12	500	2.38±0.07 [b]	2.46±0.05 [b]	3.31±0.10 [b]	5.64±0.05 [b]
Isolate 22	500	2.10±0.05 [b]	2.27±0.03 [b]	3.45±0.07 [b]	5.80±0.09 [b]

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.6 Effect of *Spirogyra* spp. extracts on HSV and ACV-resistant HSV-1 multiplication

The effect of *Spirogyra* spp. extracts on viral multiplication was performed in this study. Efficacy of these algal 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 IC_{50} values of 1.93 and 3.81 µg/ml for HSV-1F and HSV-2G. Moreover, ACV was used as positive control at IC_{50} values of 8.89, 7.41, 6.80, 10.85 and 11.19 µg/ml for ACV-resistant HSV-1 isolates No. 1A, 1B, 11, 12 and 22, respectively.

Anti-HSV effects of *Spirogyra* spp. extracts on viral multiplication were observed from the extracts that inhibited virus by reduction more than 1 log of virus yield (Yoosook *et al*., 1999). At 30 hours after treatment the infected cell with these algal extracts, HSV-1F yield was inhibited as observed by reduction of log virus titer (PFU/ml).

The result revealed that HSV-1F yields were reduced more than HSV-2G after treatment with aqueous extract of *Spirogyra* spp. by 5.72±0.30 and 2.63±0.10 log PFU/ml, respectively when compared to untreated viral control (Figure 4.2, 4.3). Whereas, yield of ACV-resistant HSV-1 isolates was similarly reduced after treatment with aqueous extract of *Spirogyra* spp. (Figure 4.4, 4.5, 4.6, 4.7, 4.8).

The result showed that HSV-1F yields were reduced after treatment with the extracts. The HSV-1 titers after treatment with aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. were 5.72±0.30, 5.36±0.20 and 5.51±0.10 log PFU/ml, respectively when compared to untreated viral control (Figure 4.2). The HSV-2G yield were also reduced after treatment with aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. to 2.63±0.10, 3.10±0.20 and 2.88±0.10 log PFU/ml, respectively when compared to untreated viral control (Figure 4.3). HSV-2G yield were reduced more than HSV-1F by 3.87±0.10 and 4.02±0.10 when treatment with ACV at IC_{50} values. The highest reduction of HSV-1F titer after treatment with ethanolic extract (EE) of *Spirogyra* spp. was 5.36±0.20 log PFU/ml, at 36 hours (Figure 4.2). While, yields of HSV-2G were reduced with the

highest reduction after treatment with aqueous extract (AE) to 2.63±0.10 log PFU/ml at 36 hours (Figure 4.3).

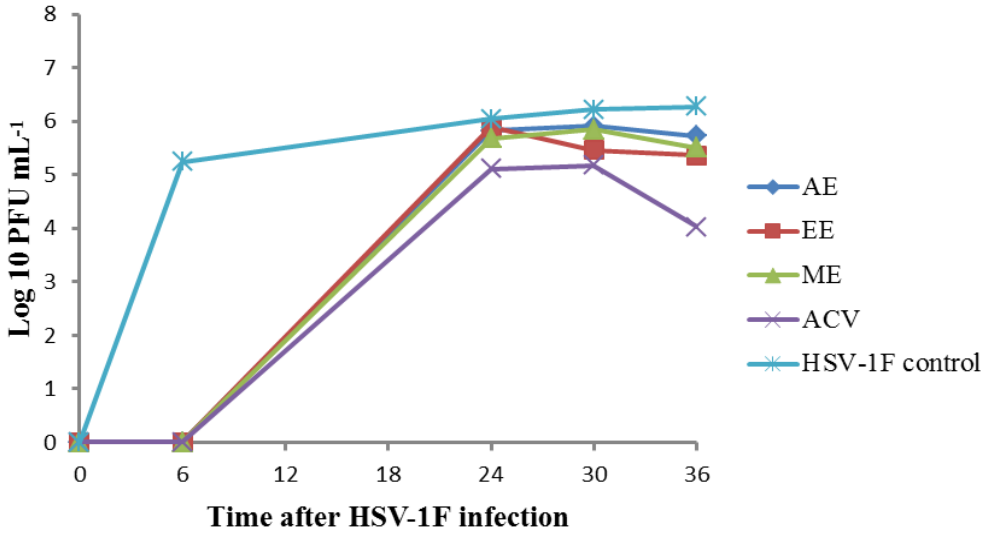


Figure 4.2 Log of HSV-1F titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 1.93 µg/ ml and viral control.

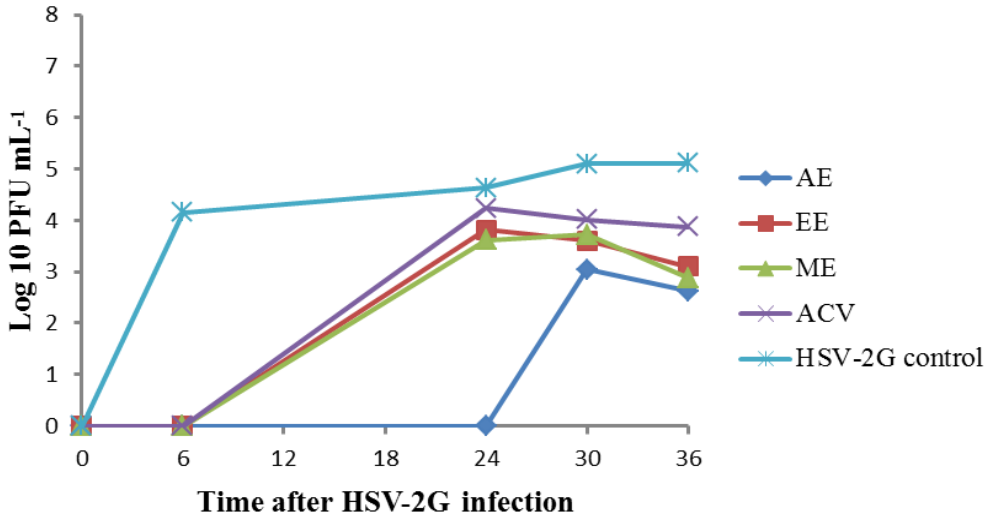


Figure 4.3 Log of HSV-2G titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 6.81 µg/ ml and viral control.

The viral multiplication of five samples of ACV-resistant HSV-1 isolates was studied when treatment with crude extracts of *Spirogyra* spp. The viral titer at different time 0, 6, 24, 30 and 36 hours after viral multiplication were determined. ACV was used as positive control at IC_{50} values of 8.89, 7.41, 6.80, 10.85 and 11.19 µg/ml for ACV-resistant HSV-1 isolate No. 1A, 1B, 11, 12 and 22, respectively (Figure 4.4 - 4.8).

The results showed that all of ACV-resistant HSV-1 isolates were inhibited as observed by reduction of log virus titer (PFU/ml) when treatment with these algal extracts after 30 hours. Especially, the viral yields of ACV-resistant HSV-1 isolate No. 11, 12 and 22 were reduced by 4.78±0.20, 4.91±0.20, 5.01±0.50 log PFU/ml, respectively with after treatment with ethanolic extract (EE) of *Spirogyra* spp. more than ACV at IC_{50} dose when compared to untreated viral control (Figure 4.6, 4.7, 4.8). ACV-resistant HSV-1 isolate No. 1A and 1B yields were reduced after treatment with crude extract of *Spirogyra* spp. less than ACV at IC_{50} dose when compared to untreated viral control (Figure 4.4, 4.5).

The determination of ACV-resistant HSV-1 isolate No. 1A showed that the yield of virus were reduced after treatment with aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. by 5.30±0.20, 4.00±0.60 and 4.51±0.30 log PFU/ml, respectively when compared to untreated viral control (Figure 4.4). Similarity, the yields of ACV-resistant HSV-1 isolate No. 1B were reduced after treatment with aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. by 5.02±0.30, 4.40±0.50 and 4.81±0.30 log PFU/ml, respectively when compared to untreated viral control (Figure 4.5).

The results of high potential reduction of ACV-resistant HSV-1 isolate No. 11 yields were observed after treatment with aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. were 5.60±0.30, 4.78±0.20 and 4.80±0.10 log PFU/ml, respectively when compared to untreated viral control (Figure 4.6). Whereas, the reduction of ACV-resistant HSV-1 isolate No. 12 yields was reduced after treatment with aqueous extract (AE), ethanolic extract (EE) and ethanolic extract (ME) of *Spirogyra* spp. by 5.52±0.20, 4.91±0.20 and 5.30±0.40 log PFU/ml, respectively when compared to untreated viral control (Figure 4.7). In addition, the viral yields of ACV-resistant HSV-1 isolate No. 22 were reduced after treatment with

aqueous extract (AE), ethanolic extract (EE) and methanolic extract (ME) of *Spirogyra* spp. by 5.60±0.20, 5.04±0.50 and 5.55±0.50 log PFU/ml, respectively when compared to untreated viral control (Figure 4.8).

The ethanolic extract (EE) showed the highest efficacy to inhibit HSV-1F and all of ACV-resistant HSV-1 isolates No. 1A, 1B, 11, 12 and 22 at 36 hours after viral multiplication (Figure 4.4, 4.5, 4.6, 4.7, 4.8). These results are related to that reported by Chaliewchalad 2013, who observed that the ethanolic extract of Thai herbs *Houttuynia cordata* Thunb. showed the highest direct inactivation of both the types of HSV at 20 minutes of treatment. It was revealed viral multiplication was inhibited with the highest reduction of log HSV-1 and HSV-2 titers at 30 hours after treatment with the ethanolic extract of *H. cordata*. On the other hand, the methanolic extract of *Spirulina* exerts antiviral effect on HSV-1 with IC_{50} value of 25.1 µg/ml (Chirasuwan *et al.* 2009). This effect is due to the presence of a compound called sulphoquinovosyl diacylglycerol (SQDG). It was reported that SQDG of some cyanobacteria possessed both antiviral (human immunodeficiency virus type 1 (HIV-1) and antitumor activity (Shirahashi *et al*., 1993; Loya *et al*., 1998). Moreover, the methanolic extract of *Spirulina maxima* exhibited antiviral activity against HSV-2 with EC_{50} 6.9 mg/ml, and IC_{50} 0.13 mg/ml (Corona *et al*., 2002). The results suggested that the antiviral activity could be due to highly polar compounds present in methanolic extract.

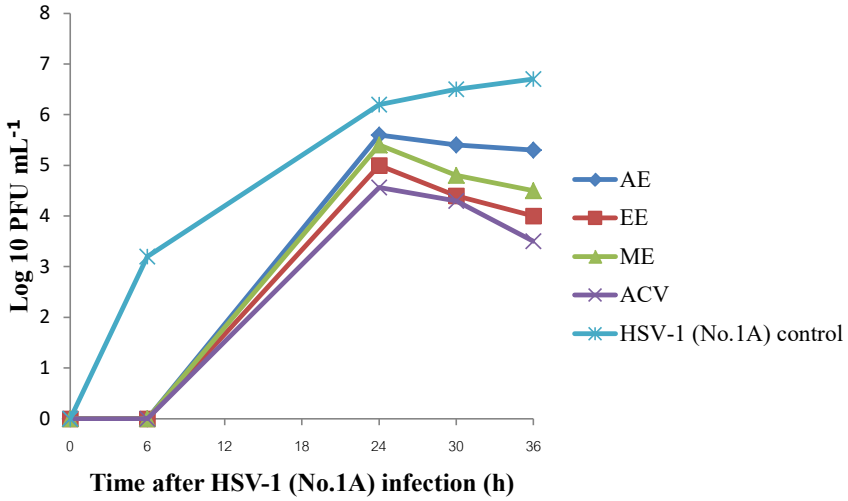


Figure 4.4 Log of ACV-resistant HSV-1 isolate 1A titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 8.89 µg/ ml and viral control

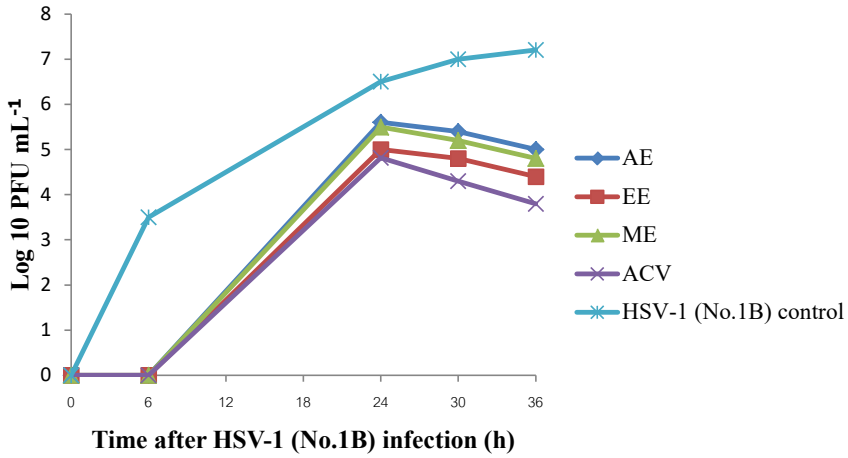


Figure 4.5 Log of ACV-resistant HSV-1 isolate 1B titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 7.41 µg/ ml and viral control

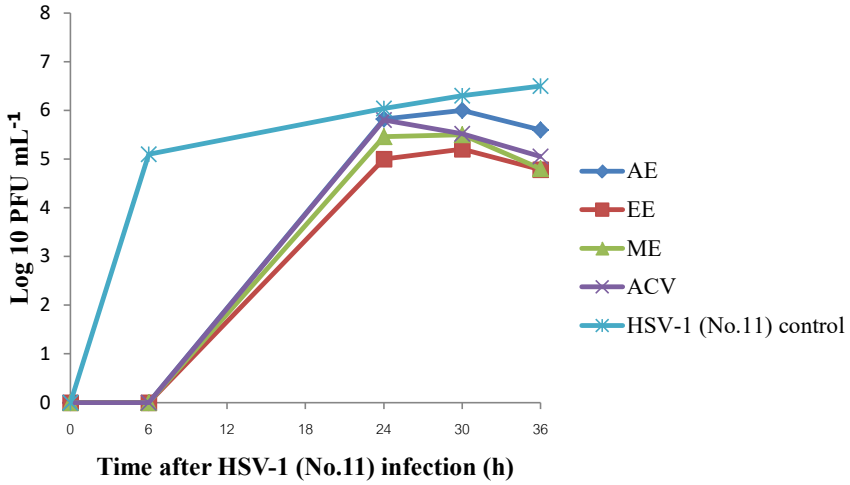


Figure 4.6 Log of ACV-resistant HSV-1 isolate 11 titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 6.80 µg/ ml and viral control

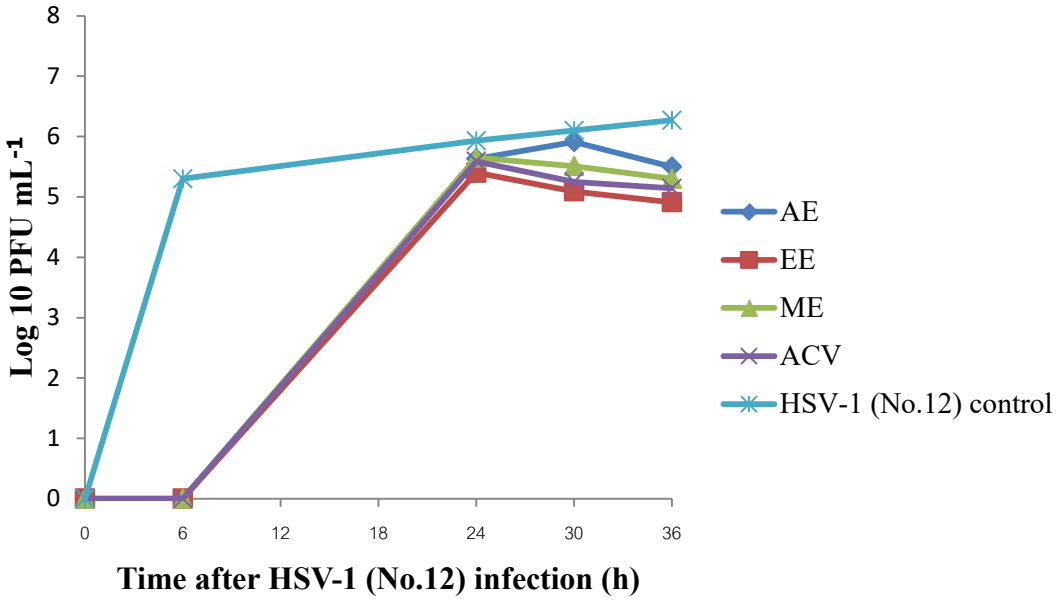


Figure 4.7 Log of ACV-resistant HSV-1 isolate 12 titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 10.85 µg/ ml and viral control

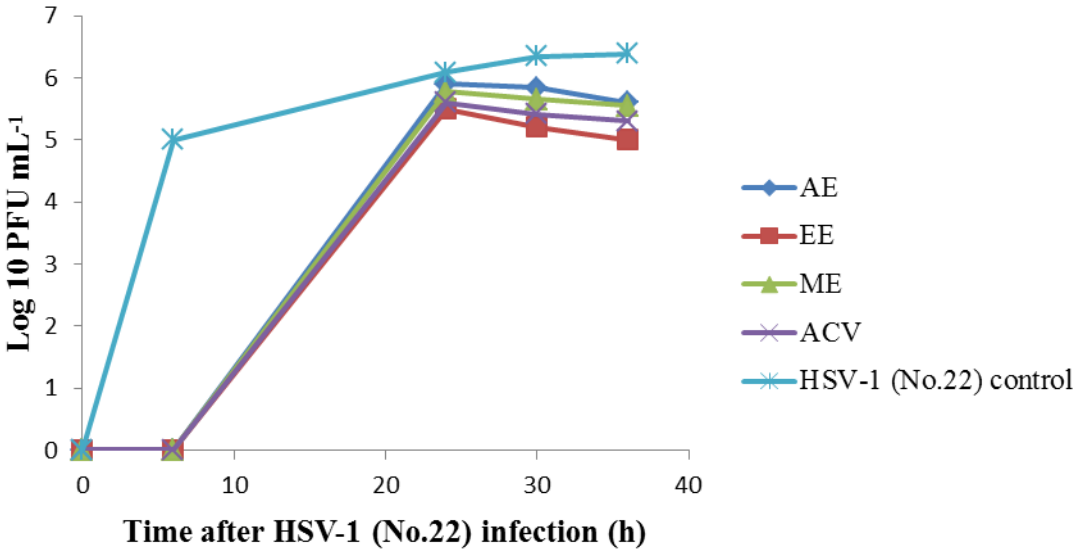


Figure 4.8 Log of ACV-resistant HSV-1 isolate 22 titer at 0, 6, 24 30 and 36 hours after treatment with crude extract of *Spirogyra* spp. when compared to antiviral agent, ACV 11.19 µg/ ml and viral control

4.7. Effect of *Spirogyra* spp. extracts on HSV after partition extraction

In this study, separation and partial purification of *Spirogyra* spp. extracts were performed. The methodology of separation and purification was used to separate the natural active compound in algal materials by the selective solvents in standard extraction method. In addition, separation and purification process should be efficient, simple, rapid and inexpensive.

The potential inhibitory effects of *Spirogyra* spp. extracts against the standard HSV-1F, ACV-resistant HSV-1 isolates and standard HSV-2G infection were investigated to clarify their antiviral activity. The ethanolic extracts of *Spirogyra* spp. showed anti-HSV-1F activity better than the aqueous and methanolic extracts. Whereas, the aqueous extracts of *Spirogyra* spp. showed anti-HSV-2G activity better than the ethanolic and methanolic extracts. Therefore, the aqueous and ethanolic extracts of *Spirogyra* spp. were selected to be further fractionated to determine their composition of

bioactive compounds that affected HSV-1 and HSV-2 infection by plaque reduction assay. Phytochemical groups were also evaluated by phytochemical screening assay. Furthermore, the fraction that showed the highest anti-HSV activity was analyzed to determine the extract pattern on thin layer chromatography (TLC). The similar fractions were selected to further isolate by column chromatography technique. The anti-HSV efficacy of isolated fraction against HSV infection was compared to the algal crude extract.

4.7.1 Separation of ethanolic extract of *Spirogyra* spp.

4.7.1.1 Separation of ethanolic extract of *Spirogyra* spp. by partition technique

Crude ethanolic extract of *Spirogyra* spp. was selected to separate by partition technique and eluted with two different solvent to separate the constituent of the extracts bases on their polarity from low to high polarity to give the partial fractions using the concentration of 5%, 10%, 20%, 40%, 60, 80% and 100% of methanol in dichloromethane.

4.7.1.2 Column chromatography of ethanolic extract of *Spirogyra* spp.

In this study, the active compound of ethanolic extract of *Spirogyra* spp. against HSV infection were selected by column chromatography using siliga gel as stationary phase and eluted with suitable solvent; hexane, hexane:ethyl acetate, ethyl acetate and methanol, consecutively. Each fraction was collected at volume of 20-30 ml. The similar patterns of fraction were combined together in accordance on the result of their TLC pattern after visualization with ultraviolet light at 365 nm. After that, the solvent of each fraction was removed by evaporation and reconstituted by DMSO as the stock concentration.

The results showed that after crude ethanolic extract of *Spirogyra* spp. was separated by various solvent and column chromatography using silica gel as stationary phase, seven major fractions, EE01-EE07 were obtained. These fractions showed the different percentage yield ranging from 0.805-15.741% (Table 4.10). The

percentage yield of EE04 fraction was recovered the most by 15.741%, while the lowest percentage yield was obtained from EE03 by 0.805%.

Table 4.10 Percentage yield of each fraction isolated from ethanolic extract of *Spirogyra* spp. by column chromatography using silica gel as a stationary phase

Fractions	Yield (%)
EE01	0.864
EE02	1.067
EE03	0.805
EE04	15.741
EE05	9.359
EE06	4.310
EE07	5.436

4.7.1.3 TLC screening of ethanolic extract of *Spirogyra* spp. 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. In this study, seven fractions were analyzed on TLC pattern after the small spot of sample was applied on the plate. Then, TLC plate was put in a solvent or solvent mixture system as mobile phase. Different compounds in the sample mixture were moved at different rates. Visualization of chromatogram under UV light at 365 nm showed separated bands for active compound (Males and Medic-Saric, 2001).

The TLC pattern of the fractions after fractionation by column chromatography were further observed. The results of TLC patterns were illustrated seven similar groups of separated fractions. Seven groups of fractions showed the best separation using hexane: ethyl acetate (90:10) as a mobile phase. The result of color appearance and number of spots were observed under UV light at 365 nm. The chemical patterns on TLC plate of EE01 (Spot No.1, 4, 7), EE02 (Spot No. 10, 13), EE03 (Spot No. 14, 16, 19, 22), EE04 (Spot No. 25, 28, 31, 34, 37, 40, 43), EE05 (Spot No. 45, 49, 52, 55), EE06 (Spot No. 56, 58, 61, 64, 67,) and EE07 (Spot No. 68, 70, 73, 77, 81, 84, 87) fractions were clearly demonstrated the same R_f value (Figure 4.9). Hence, seven major fractions were combined from the fractions that had the same TLC profile were combined. After combination, the fractions EE01-EE07 were selected to further evaluated their cytotoxicity and efficacy against HSV infection by plaque reduction assay.

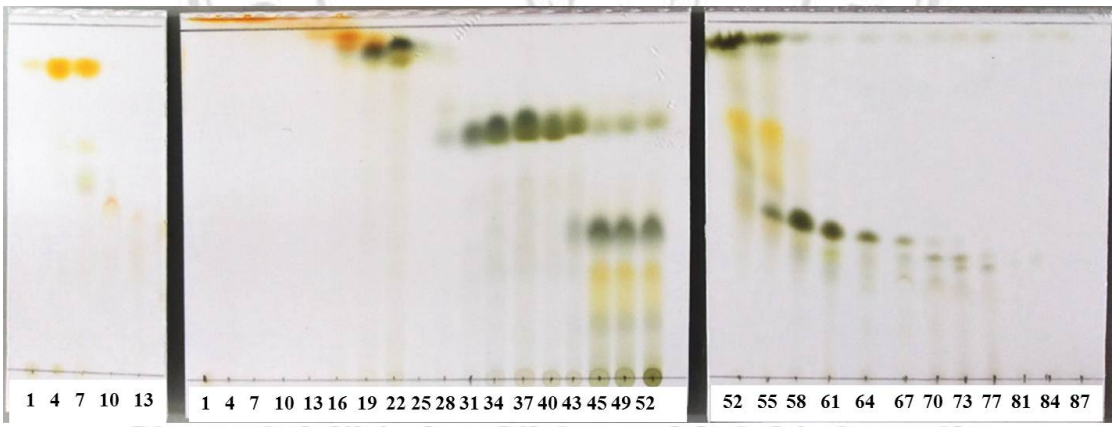


Figure 4.9 The partial purified fractions of ethanolic extract of *Spirogyra* spp.by TLC analysis using hexane: ethyl acetate (90:10) as mobile phase; EE01 (Spot No.1, 4, 7), EE02 (Spot No. 10, 13), EE03 (Spot No. 14, 16, 19, 22), EE04 (Spot No. 25, 28, 31, 34, 37, 40, 43), EE05 (Spot No. 45, 49, 52, 55), EE06 (Spot No. 56, 58, 61, 64, 67,) and EE07 (Spot No. 68, 70, 73, 77, 81, 84, 87)

4.7.1.4 Cytotoxicity of ethanolic extract of *Spirogyra* spp. fractions isolated from column chromatography on Vero cells

This study was carried out to determine the efficacy of fractions which separated from ethanolic extract of *Spirogyra* spp. by column chromatography using silica gal as stationary phase. Seven reconstituted fractions, EE01-EE07 fractions were selected to investigate their cytotoxicity on Vero cells by MTT assay. 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-1F and HSV-2G infection.

The color appearance of ethanolic extract of *Spirogyra* spp. separated fractions after reconstitution by DMSO were green to deep green. Cytotoxicity of seven fractions were determined and exhibited cytotoxicity by dose dependent value (Table 4.11). The result showed that seven fractions had the toxicity on Vero cells with CD_{50} values ranging from 12.50-117.23 µg/ml. Moreover, the highest toxicity of fractions isolated from ethanolic extract of *Spirogyra* spp. was EE06. However, the lowest toxicity from seven fractions was EE01. The result of high CD_{50} value reflected low toxicity of algal fractions on Vero cells. Additionally, non-toxic concentrations at 10-80 µg/ml of each fraction were used to further investigate antiviral activity against HSV infection by plaque reduction assay (Table 4.11).

Table 4.11 The cytotoxicity dose, 50% (CD_{50}) and non-toxic concentrations of *Spirogyra* spp. fractions

Fractions	CD_{50} (µg/ml)*	The highest non-toxic concentrations (µg/ml)
EE01	117.23±1.10	80.00
EE02	96.85±1.40	80.00
EE03	51.20±0.96	30.00
EE04	36.64±1.20	30.00
EE05	14.85±2.20	10.00
EE06	12.50±0.86	10.00
EE07	19.11±1.84	10.00

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

4.7.1.5 Antiviral activity of ethanolic extract of *Spirogyra* spp. fraction isolated from column chromatography

The investigation of antiviral activity against HSV-1F, ACV-resistant HSV-1 isolated and HSV-2G infection by non-toxic concentration of seven fractions; EE01-EE07 which isolated from ethanolic extract of *Spirogyra* spp. by column chromatography was examined by plaque reduction assay.

The result showed that HSV-1F was the highest sensitive to EE02 fraction with percentage of inhibition by 98.03%, ED_{50} value of 45.62±0.40 µg/ml and TI value of 2.12±0.06. EE01, EE03 to EE07 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 seven fractions after viral attachment, EE01 fraction was the highest ability to inhibit HSV-2G with percentage of inhibition by 70.36±0.65%, ED_{50} value of 64.42±0.60 µg/ml and TI value of 1.82±0.54 (Table 4.12).

From our result showed that both types of HSV-1F and HSV-2G were affected similarity by seven fractions of ethanolic extract of *Spirogyra* spp. It was suggested that the main active compounds in each fraction may affect the inhibitory effect against HSV infection. The results revealed the efficacy of EE02 fraction against HSV-1F infection was increased when compared to their crude ethanolic extract. Since the crude extract showed the ED_{50} value of 166.40±0.70 µg/ml and TI value of 2.14±0.50. These may be resulted from various beneficial bioactive compounds in algal 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). However, the efficacy against HSV-2G infection from these fractions was decreased when compared to their crude ethanolic extract.

The result also confirmed that all phytochemical compounds in this algal crude extract had the effective antiviral agent. Thus, further determination of phytochemical groups in the fraction was performed.

Table 4.12 Antiviral activity of seven fractions of ethanolic extract of *Spirogyra* spp. on standard HSV-1F and HSV-2G infection.

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
Standard HSV				
HSV-1(F)	EE01	42.51 ±0.85 [a]	0 [a]	0 [a]
	EE02	98.03 ±0.50 [a]	45.62±0.40 [a]	2.12±0.06 [a]
	EE03	43.55 ±1.00 [a]	0 [a]	0 [a]
	EE04	45.60 ±1.00 [a]	0 [a]	0 [a]
	EE05	33.29 ±0.95 [a]	0 [a]	0 [a]
	EE06	40.54 ±0.99 [a]	0 [a]	0 [a]
	EE07	35.72 ±0.85 [a]	0 [a]	0 [a]
HSV-2(G)	EE01	70.36 ±0.65 [a]	64.42±0.60 [a]	1.82±0.54 [a]
	EE02	53.10 ±0.50 [a]	71.54±0.57 [a]	1.35±0.09 [a]
	EE03	49.55 ±1.00 [a]	0 [a]	0 [a]
	EE04	40.24 ±0.90 [a]	0 [a]	0 [a]
	EE05	31.59 ±0.80 [a]	0 [a]	0 [a]
	EE06	39.62 ±0.70 [a]	0 [a]	0 [a]
	EE07	31.50 ±0.95 [a]	0 [a]	0 [a]

TI = CD_{50}/ED_{50}

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

The determination of antiviral activity against five isolates of ACV-resistant HSV-1 by non-toxic concentration of seven fractions after viral attachment was performed by plaque reduction assay. The result showed that all of ACV-resistant HSV-1 isolates were similarly sensitive to EE01 and EE02 fraction (Table 4.13). In particular, EE02 fraction showed the highest efficacy to inhibit ACV-resistant HSV-1 isolates No.12, with percentage of inhibition by 95.60 ±0.54%, ED_{50} value of 39.24±0.70 µg/ml and TI value of 2.75±0.90.

Moreover, the high inhibitory effect of EE01 fraction could inhibit ACV-resistant HSV-1 isolates No. 1A, 1B, 11, 12 and 22 after viral attachment showed that the ED_{50} values of 70.54±0.50, 69.55±0.90, 68.64±0.70, 65.01±0.50 and 64.39±0.46 µg/ml, respectively. Similarity, EE02 fraction also inhibited ACV-resistant HSV-1 isolates No. 1A, 1B, 11, 12 and 22 after viral attachment by ED_{50} values of 41.05±0.40, 40.56±0.60, 43.58±0.30, 39.24±0.70 and 41.50±0.20 µg/ml, respectively (Table 4.13). On the other hand, EE03-EE04 fractions had low ability to inhibit five isolates of ACV-resistant HSV-1 infected cell by percentage of inhibition ranging from 29.50- 41.42 % (Table 4.13).

From the results, the natural active compound in each fraction may affect the inhibitory effect against ACV-resistant HSV-1 isolates infection. It was suggested that the inhibitory effect of seven fractions were occurred by inhibition of ACV-resistant HSV-1 infection. The substances of EE01 and EE02 fractions could interfere the viral replication process of ACV-resistant HSV-1 isolates (Table 4.13). Other study showed that, the active compounds of brown marine algae; *Stoechospermum marginatum* extracts might destroy or block the mechanism of viral replication (Adhikari *et al*., 2006). Thus, it could indicate that the fraction of ethanolic extract of *Spirogyra* spp. had potential to inhibit ACV-resistant HSV-1 isolates. The active phytochemical compounds in fractions of *Spirogyra* spp., which had the effective antiviral agent was further study.

Table 4.13 Antiviral activity of seven fractions of ethanolic extract of *Spirogyra* spp. on ACV-resistant HSV-1 isolates.

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
ACV-resistant HSV-1 isolates				
No.1A	EE01	55.60 ±0.65 [a]	70.54±0.50 [a]	1.44±0.20 [a]
	EE02	87.42 ±0.50 [a]	41.05±0.40 [a]	2.23±0.35 [a]
	EE03	35.61 ±1.10 [a]	0 [a]	0 [a]
	EE04	36.84 ±1.00 [a]	0 [a]	0 [a]
	EE05	31.20 ±0.90 [a]	0 [a]	0 [a]
	EE06	37.81 ±0.59 [a]	0 [a]	0 [a]
	EE07	32.50 ±0.75 [a]	0 [a]	0 [a]
No.1B	EE01	51.50 ±0.40 [a]	69.55±0.90 [a]	1.62±0.54 [a]
	EE02	82.11 ±0.50 [a]	40.56±0.60 [a]	2.34±0.409 [a]
	EE03	34.15 ±0.55 [a]	0 [a]	0 [a]
	EE04	40.54 ±0.90 [a]	0 [a]	0 [a]
	EE05	30.83 ±0.50 [a]	0 [a]	0 [a]
	EE06	35.62 ±0.65 [a]	0 [a]	0 [a]
	EE07	32.40 ±0.70 [a]	0 [a]	0 [a]

Table 4.13 (continued)

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
ACV-resistant HSV-1 isolates				
No.11	EE01	52.80 ±0.85 [a]	68.64±0.70 [a]	1.13±0.50 [a]
	EE02	89.13 ±0.50 [a]	43.58±0.30 [a]	2.59±0.24 [a]
	EE03	33.50 ±1.00 [a]	0 [a]	0 [a]
	EE04	41.42 ±1.00 [a]	0 [a]	0 [a]
	EE05	32.30 ±0.95 [a]	0 [a]	0 [a]
	EE06	34.54 ±0.99 [a]	0 [a]	0 [a]
	EE07	33.63 ±0.85 [a]	0 [a]	0 [a]
No.12	EE01	56.31 ±0.30 [a]	65.01±0.50 [a]	1.47±0.44 [a]
	EE02	95.60 ±0.54 [a]	39.24±0.70 [a]	2.75±0.90 [a]
	EE03	36.75 ±0.90 [a]	0 [a]	0 [a]
	EE04	38.44 ±0.95 [a]	0 [a]	0 [a]
	EE05	29.50 ±0.81 [a]	0 [a]	0 [a]
	EE06	31.26 ±1.20 [a]	0 [a]	0 [a]
	EE07	31.15 ±0.90 [a]	0 [a]	0 [a]

Table 4.13 (continued)

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
ACV-resistant HSV-1 isolates				
No.22	EE01	55.40 ±0.45 [a]	64.39±0.46 [a]	1.41±0.54 [a]
	EE02	94.18 ±0.50 [a]	41.50±0.20 [a]	2.66±0.25 [a]
	EE03	35.40 ±0.90 [a]	0 [a]	0 [a]
	EE04	36.70 ±0.60 [a]	0 [a]	0 [a]
	EE05	31.56 ±0.50 [a]	0 [a]	0 [a]
	EE06	33.44 ±1.00 [a]	0 [a]	0 [a]
	EE07	32.52 ±0.70 [a]	0 [a]	0 [a]

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

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.7.2 Separation of aqueous extract of *Spirogyra* spp. by partition technique

Crude aqueous extract of *Spirogyra* spp. was selected to separate by partition technique and eluted with two different solvent to separate the constituent of the extracts bases on their polarity from low to high polarity to give the partial fractions using the concentration of 5%, 10%, 15%, 20%, 40%, 60, 80% and 100% of methanol in dichloromethane.

4.7.2.1 Column chromatography of aqueous extract of *Spirogyra* spp.

In this study, the active compound of aqueous extract of *Spirogyra* spp. against HSV infection were selected by column chromatography using siliga gel as stationary phase and eluted with suitable solvent; hexane, hexane:ethyl acetate, ethyl acetate and methanol, consecutively. Each fraction was collected at volume of 20-30 ml. The similar patterns of fraction were combined together in accordance on the result of their TLC patterns after visualization with ultraviolet light at 365 nm. After that, the solvent of each fraction was removed by evaporation and reconstituted by DMSO as the stock concentration.

The results showed that after crude aqueous extract of *Spirogyra* spp. was separated by various solvents and column chromatography using silica gel as stationary phase, seven major fractions, AE01-AE03 were obtained. These fractions showed the different percentage yield ranging from 6.055-28.062% (Table 4.14). The percentage yield of AE01 fraction was recovered the most by 28.062%, while the lowest percentage yield was obtained from AE02 by 6.055%.

Table 4.14 Percentage yield of each fraction isolated from aqueous extract of *Spirogyra* spp. by column chromatography using silica gel as a stationary phase

Fractions	Yield (%)
AE01	28.062
AE02	6.055
AE03	17.184

4.7.2.2 TLC screening of aqueous extract of *Spirogyra* spp. after separation by column chromatography

In this study, three fractions were analyzed on TLC pattern after the small spot of sample was applied on the plate. Then, TLC plate was put in a solvent or solvent mixture system as mobile phase. Different compounds in the sample mixture were moved at different rates. Visualization of chromatogram under UV light at 365 nm showed separated bands for active compounds (Males and Medic-Saric, 2001).

The TLC patterns of the fractions after fractionation by column chromatography were further observed their patterns. The results of TLC patterns were illustrated three similar groups of separated fractions. Seven groups of fractions showed the best separation using dichloromethane: methanol (90:10) as a mobile phase. The result of color appearance and number of spots were observed under UV light at 365 nm. The chemical patterns on TLC plate of AE01, AE02 and AE03 fractions were clearly demonstrated the same R_f value. Hence, the fractions AE01-AE03 were selected to further evaluate their cytotoxicity and efficacy against HSV infection by plaque reduction assay.

4.7.2.3 Cytotoxicity of aqueous extract of *Spirogyra* spp. fractions isolated from column chromatography on Vero cells

This study was carried out to determine the efficacy of fractions which separated from aqueous extract of *Spirogyra* spp. by column chromatography using silica gal as stationary phase. Three reconstituted fractions, AE01-AE07 fractions were selected to investigate their cytotoxicity on Vero cells by MTT assay. 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-1F and HSV-2G infection.

The color appearance of aqueous extract of *Spirogyra* spp. fractions after reconstitution by DMSO was green to deep green. Cytotoxicity of three fractions were determined and exhibited cytotoxicity by dose dependent value (Table 4.15). The result showed that three fractions had the toxicity on Vero cells with CD_{50} values ranging from

58.56-67.81 µg/ml. Moreover, the highest toxicity of fractions isolated from aqueous extract of *Spirogyra* spp. was AE02. However, the lowest toxicity of three fractions was AE01. The result of high CD_{50} value reflected low toxicity of algal fractions on Vero cells. Additionally, non-toxic concentrations at 50 µg/ml of each fraction were used to further investigate antiviral activity against HSV infection by plaque reduction assay (Table 4.15).

Table 4.15 The cytotoxicity dose, 50% (CD_{50}) and non-toxic concentrations of *Spirogyra* spp. fractions

Fractions	CD_{50} (µg/ml)*	the highest non-toxic concentrations (µg/ml)
AE01	67.81±1.20	50.00
AE02	58.56±1.55	50.00
AE03	62.10±1.70	50.00

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

4.7.2.4 Antiviral activity of aqueous extract of *Spirogyra* spp. fraction isolated from column chromatography

The investigation of antiviral activity against HSV-1F, ACV-resistant HSV-1 isolated and HSV-2G infection by non-toxic concentration of three fractions; AE01-AE03 which isolated from aqueous extract of *Spirogyra* spp. by column chromatography was examined by plaque reduction assay.

The result showed that HSV-1F was the highest sensitive to AE03 fraction with percentage of inhibition by 49.62 %. AE01 and AE02 were weakly

effective against HSV-1F infection, which could inhibit HSV infection less than 40% (Table 4.16). On the other hand, HSV-2G infected cells were treated with three fractions after viral attachment, AE02 fraction was the highest ability to inhibit HSV-2G with percentage of inhibition by 45.50 ±0.90% (Table 4.16).

From our result showed that HSV-1F, ACV-resistant HSV-1 isolates and HSV-2G were weakly affected similarity by three fractions of aqueous extract of *Spirogyra* spp., which could inhibit HSV infection less than 50% (Table 4.16). The results revealed the efficacy of AE01, AE02 and AE03 fraction against HSV infection were similar when compared to their crude aqueous extract. Since the crude aqueous extract showed the inhibitory effect on HSV infection with percentage of inhibition ranging from 20.65-51.60%. However, the efficacy against HSV-2G infection from these fractions was decreased when compared to their crude aqueous extract.

From the results, AE01, AE02 and AE03 fractions had low inhibitory effect on HSV-1F, ACV-resistant HSV-1 isolates and HSV-2G infected cell. Then, the EE01 and EE02 fractions had the highest inhibitory effect on HSV-1F, ACV-resistant HSV-1 isolates and HSV-2G infected cell when compared to their fractions. Therefore, the result also confirmed that all phytochemical compounds in this algal crude extract had the effective antiviral agent. Thus, further determination of phytochemical groups in the fraction was performed.

Table 4.16 Antiviral activity of three fractions of aqueous extract of *Spirogyra* spp. on standard HSV-1F, HSV-2G and five isolates of ACV-resistant HSV-1 infection.

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
Standard HSV				
HSV-1(F)	AE01	33.51 ±0.90	0 [a]	0 [a]
	AE02	35.80 ±0.60	0 [a]	0 [a]
	AE03	49.62 ±1.10 [a]	0 [a]	0 [a]
HSV-2(G)	AE01	30.64 ±0.75	0 [a]	0 [a]
	AE02	45.50 ±0.90 [a]	0 [a]	0 [a]
	AE03	38.51 ±1.00	0 [a]	0 [a]
ACV-resistant	AE01	25.56 ±0.70	0 [a]	0 [a]
HSV-1 No.1A	AE02	26.80 ±0.50	0 [a]	0 [a]
	AE03	34.63 ±1.00	0 [a]	0 [a]
ACV-resistant	AE01	22.19 ±0.50	0 [a]	0 [a]
HSV-1 No.1B	AE02	30.90 ±0.70	0 [a]	0 [a]
	AE03	32.24 ±0.60	0 [a]	0 [a]

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

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

Table 4.16 (Continues).

Viruses	Fractions	Inhibition (% ± SD*)	ED_{50}±SD (µg/ml)*	TI±SD*
ACV-resistant	AE01	24.66 ±0.50	0 [a]	0 [a]
HSV-1 No.11	AE02	27.50 ±0.90	0 [a]	0 [a]
	AE03	30.16 ±1.10	0 [a]	0 [a]
ACV-resistant	AE01	20.46 ±0.85	0 [a]	0 [a]
HSV-1 No.12	AE02	24.40 ±0.70	0 [a]	0 [a]
	AE03	28.71 ±1.00	0 [a]	0 [a]
ACV-resistant	AE01	20.50 ±0.90	0 [a]	0 [a]
HSV-1 No.22	AE02	21.97 ±0.90	0 [a]	0 [a]
	AE03	29.40 ±0.50	0 [a]	0 [a]

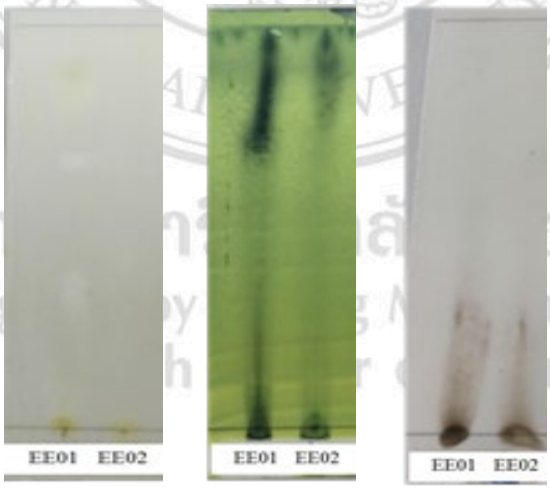
$TI = CD_{50}/ED_{50}$

*These result are presented as mean ± standard deviation (SD) of three independent experiments. Tested groups are compared using statistical analysis (SPSS statistic 17.0) by randomized complete blocks (RCB) and Post hoc Tukey's b test. The different alphabets in each group show significantly different value ($P<0.05$).

4.7.3 Preliminary determination of phytochemical groups of ethanolic extract of *Spirogyra* spp. fraction isolated from column chromatography

Crude ethanolic extract of *Spirogyra* spp. were selected to further elucidate the major phytochemical constituents profile by phytochemical analysis. From the results, EE01 and EE02 fractions had high inhibitory effect on HSV-1F, ACV-resistant HSV-1 isolates and HSV-2G infected cell. Therefore, the EE01 and EE02 fractions were selected to evaluate their phytochemical constituent. Phytochemical analysis was performed by standard methods for identification of biological activity; alkaloids, antraquinone, lactone, essential oil, terpenoid, flavonoid and tannin.

The phytochemicals compound of fractions of *Spirogyra* spp. extract and EE01 and EE02 of *Spirogyra* spp. revealed many groups of chemical constituents. Alkaloids, essential oil and terpenoid were found in these two fractions (Table 4.17). These results showed positive profiles as follows; Dragendorff's test for alkaloids, phosphomolydic acid test for essential oil and sulfuric acid test for terpenoid (Figure 4.10). EE01 and EE02 fractions were similarity presented the natural active compound (Table 4.17).



(A) (B) (C)

Figure 4.10 The positive results of active compound of EE01 and EE02 partial fractions by phytochemical analysis; Dragendorff's test for alkaloids (A), Phosphomolydic acid test for essential oil (B) and Sulfuric acid test for terpenoid (C)

The efficacy against HSV-1F, ACV resistant and HSV-2G infection by EE01 and EE02 fraction suggested that the action of these algal extract on antiviral activity might be from many groups of phytochemical compounds. It could act together and affected at different mode of antiviral action by additive or synergistic action of many compounds (Adhikari *et al*., 2006; Yucharoen *et al*., 2012). In addition, this result indicated that *Spirogyra* spp. extract had several secondary products that presented in combination. Therefore, activity of each fraction was unique to particular phytochemical groups (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 evaporate (Mohamed *et al*., 2010). Furthermore, active constituents that were present in algal extract depended on geographical distribution, season of collection and also ecological condition at collection site (Rajbhandari *et al*., 2001).

Table 4.17 Phytochemical groups of *Spirogyra* spp. ethanolic extract fraction

Phytochemical groups	Test	*Spirogyra* spp. ethanolic extract fractions	
		EE01	EE02
Alkaloid	Dragendorff's reagent	+ + +	+ + +
Antroquinone	Potassium hydroxide's test	- - -	- - -
Lactone	Kedd's test	- - -	- - -
Essential oil	Phosphomplydic acid's test	+ + +	+ + +
Terpenoid	Sulfuric acid's test	+ + +	+ + +
Flavonoid, tannin	Furric chloride	- - -	- - -

(-) = negative reaction

(+) = positive reaction

* The data in table are given as the result of reaction of triplicate experiments.

4.8 Effect of *Spirogyra* spp. extracts on viral DNA

Viral DNA was determined in order to elucidate the effect of algal extracts on standard HSV-1(F), ACV-resistant HSV-1 isolates and standard HSV-2(G) DNA synthesis.

The results revealed that percentage of HSV-1F DNA inhibition after treatment with the ethanolic extracts of *Spirogyra* spp. was 81.24±1.49. Furthermore, percentages of ACV-resistant HSV-1 isolate No. 1A, 1B, 11, 12 and 22 DNA remaining after treatment with the ethanolic extracts of *Spirogyra* spp. was 75.16±2.49, 70.95±1.64, 62.13±3.88, 69.81±3.43 and 58.14±2.26, respectively. In addition, percentages of HSV-2G DNA remaining after treatment with the aqueous extract *Spirogyra* spp. was 84.36±2.85 (Table 4.18). Thus, ethanolic extract of *Spirogyra* spp. showed the highest inhibition of ACV-resistant HSV-1 isolate No. 22 DNA synthesis from infected cell when compared to other isolates, which observed by lowest DNA remaining. On the other hand, the ethanolic extract of *Spirogyra* spp. had the potential inhibitory effect on HSV-1F DNA synthesis similar as the effect of aqueous extract of *Spirogyra* spp. on HSV-2G DNA synthesis which showed high DNA remaining (Table 4.18).

In the present study, the result showed that viral DNA was decreased after treatment with *Spirogyra* spp. extracts compared to untreated viral control. This result suggested that the extract might interfere viral DNA synthesis, viral replication directly or essential enzymes for viral DNA synthesis. Accordingly, 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; Wagner *et al*., 2006). 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 *Spirogyra* spp. 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*., 200). 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 4.18 Percentage of HSV DNA remaining after treatment with *Spirogyra* spp. extracts

HSV strains	*Spirogyra* spp. extract	Mean DNA inhibition (% ± SD*)
Standard HSV		
HSV-1(F)	Ethanolic extract	81.24±1.49
HSV-2(G)	Aqueous extract	84.36±2.85
ACV-resistant HSV-1		
Isolate 1A	Ethanolic extract	75.16±2.49
Isolate 1B	Ethanolic extract	70.95±1.64
Isolate 11	Ethanolic extract	62.13±3.88
Isolate 12	Ethanolic extract	69.81±3.43
Isolate 22	Ethanolic extract	58.14±2.26

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

4.8.1 Effect of *Spirogyra* spp. extracts on viral DNA detection by quantitative real-time Polymerase chain reaction (PCR)

In this study, the determination of HSV DNA replication in the presence or absence of the *Spirogyra* spp. extract was performed by real time PCR using primer specific to HSV DNA polymerase gene, PCR products were detected at 1350 base pair. It was indicated that high cycle threshold, Ct reflected many cycles of PCR to generate DNA product. Thus, it implied low amount of the template DNA. The amount of DNA after amplification HSV DNA polymerase gene and Ct of HSV-1 and HSV-2 DNA were displayed in Figure 51 and Table 14. It was showed that average Ct of standard HSV-1F DNA control, ACV-resistant HSV-1 isolate No.1A, 1B, 11, 12 and 22 DNA control were 12.33±2.25, 11.80±3.40, 11.24±1.62, 13.59±1.94, 12.70±2.09 and 12.05±3.67, respectively. In addition, the results revealed the average Ct of standard HSV-1F DNA, ACV-resistant HSV-1 isolate No.1A, 1B, 11, 12 and 22 DNA after treatment with ethanolic extract were 16.91±2.14, 22.62±3.47, 18.50±3.10, 24.75±3.54, 23.11±2.29 and 23.5±2.16, respectively. While, average Ct of HSV-2G DNA after treatment with aqueous extract and HSV-2G control were 18.86±3.79 and 12.65±1.46. Therefore, HSV DNA after treatment with the extracts was less than HSV DNA control (Figure 4.11, Table 4.19).

In this study, ACV-resistant HSV isolates contained mutations by substitutions of nucleotides in the *UL30* gene. *UL30* gene encodes the activating/phosphorylating TK enzyme, and/or in the *UL30* gene, which encodes the viral target DNA polymerase enzyme (Schnipper and Crumpacker, 1980). The marked function of HSV DNA polymerase is required for replication, whereas the viral thymidine kinase (TK) is dispensable in cultured cells and certain mammalian tissues (Sauerbrei *et al*., 2010; Wagner *et al*., 2008). Thus, in this study, the results of Ct values were exhibited the efficacy effect of *Spirogyra* spp. extract on functional HSV DNA polymerase. In particular, it was a higher inhibition of ACV-resistant HSV-1 DNA by natural active substances of *Spirogyra* spp. extract.

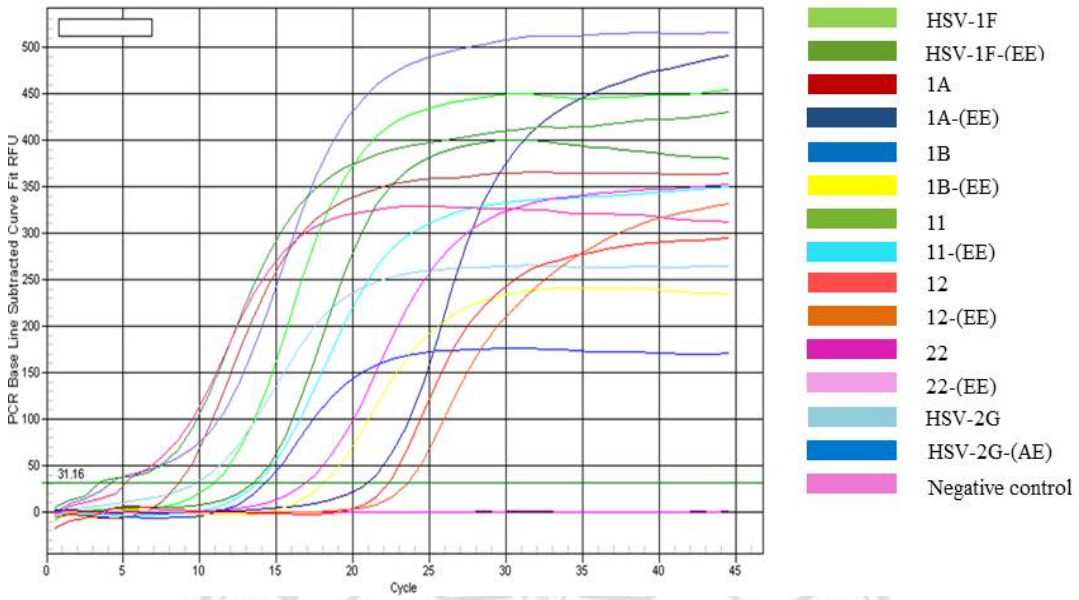


Figure 4.11 Fluorescence curve from SYBR Green detection of HSV-1F DNA, HSV-1F DNA after treatment with ethanolic extract; HSV-1F-(EE), ACV-resistant HSV isolate No.1A DNA, ACV-resistant HSV isolate No.1A DNA after treatment with ethanolic extract; 11-(EE), ACV-resistant HSV isolate No.1B DNA, ACV-resistant HSV isolate No.1B DNA after treatment with ethanolic extract; 1B-(EE), ACV-resistant HSV isolate No.11 DNA, ACV-resistant HSV isolate No.11 DNA after treatment with ethanolic extract; 11-(EE), ACV-resistant HSV isolate No.12 DNA after treatment with ethanolic extract; 12-(EE), ACV-resistant HSV isolate No.22 DNA after treatment with ethanolic extract; 22-(EE), HSV-1F DNA, HSV-2G DNA after treatment with aqueous extract; HSV-2G-(AE) and Vero cells negative control

Table 4.19 Amount of DNA and cycle threshold (Ct) values of HSV DNA polymerase gene after amplification by real-time polymerase chain reaction

HSV strains	DNA concentration ± SD* (µg/ml)	Mean cycle threshold (Ct) ± SD*
Standard HSV		
HSV-1F	48.45±2.41	12.33±2.25
HSV-1F-(EE)	2.90±0.50	16.91±2.14
HSV-2G	45.68±3.55	12.65±1.46
HSV-2G-(AE)	1.04±0.59	18.86±3.79
ACV-resistant HSV-1		
Isolate 1A	41.88 ±3.10	11.80±3.40
Isolate 1A – (EE)	0.16±0.99	22.62±3.47
Isolate 1B	50.17 ±2.01	11.24±1.62
Isolate 1B – (EE)	0.73±0.45	18.50±3.10
Isolate 11	47.33 ±2.87	13.59±1.94
Isolate 11 – (EE)	0.09±0.84	24.75±3.54
Isolate 12	44.15 ±1.09	12.70±2.09
Isolate 12 – (EE)	0.16±0.25	23.11±2.29
Isolate 22	48.19 ±1.71	12.05±3.67
Isolate 22 – (EE)	0.14±0.12	23.5±2.16

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

4.9 Effect of *Spirogyra spp.* extracts on viral proteins

The different protein extraction methods and buffers in proteomic approaches can vary widely in the reproducibility and representation of the global proteome. The chemical composition in buffers and protein extraction method comparison is the first

important step in proteomic studies. In this study, three types of extraction buffers were compared for the protein extraction and protein solubilisation of herpes simplex virus infection on Vero cells. The extraction buffers consisted of CHAP-Urea-Thiourea buffer, Nonidet P-40 (NP-40) buffer and NP-40-Urea-Thiourea buffer. Proteins from each of extraction buffer were determined by 1-dimensional gel electrophoresis (1-DE) to examine the range of proteins molecular weight (MW) and then 2-dimensional gel electrophoresis (2-DE) was performed to investigate the reproducibility and reliability of the extraction buffers.

However, the unique protein spots identification was performed by MS/MS spectroscopy. The results showed that CHAP-Urea-Thiourea buffer was a suitable protein extraction buffer for HSV-1 and HSV-2 infected cell proteins due to its reproducibility, representation of total proteome and higher MW protein solubilization.

In order to obtain the appropriate protein extraction buffer for gel-based proteomic studies of Vero cells infected HSV-1, the solubility of total proteins was considered. The effective solubilization depended on the alternative of cell disruption methods and detergents. In this study, Vero cells were infected with HSV-1F at MOI 1.0 for 24 hours. Then infected pellet cells were collected by cell scrapers. After that, three types of validated extraction buffers were subsequently used for total protein extraction, denaturation and solubilized protein. The total protein concentrations which obtained from three extraction buffers were measured by Bradford assay and bovine serum albumin (BSA) was used to generate an accurate standard curve. It was found that the total protein concentrations were determined between 1.2 - 2.0 mg per $3x10^5$ cells. The NP-40-Urea-Thiourea buffer showed the most effective solubilized buffer which resulted in highest yield (1.90 µg/µl) of protein of HSV-1F infected cell after extraction when compared with NP-40 buffer (1.45 µg/µl) or CHAP-Urea-Thiourea buffer (1.17 µg/µl).

Total proteins from each extraction buffers were loaded on 12.5% SDS-PAGE gels to determine the molecular weights (MW) of proteins range and to evaluate the contamination in protein samples. The total proteins from all three extraction buffers showed proteins patterns with a wide range of MW from 170 kDa to 10 kDa and same minor protein bands. All gels showed protein bands with similar patterns.

Interestingly, some proteins bands with MW between 10 and 17 kDa were clearly observed when using CHAP-Urea-Thiourea and NP-40-Urea-Thiourea buffers (Figure 4.12).

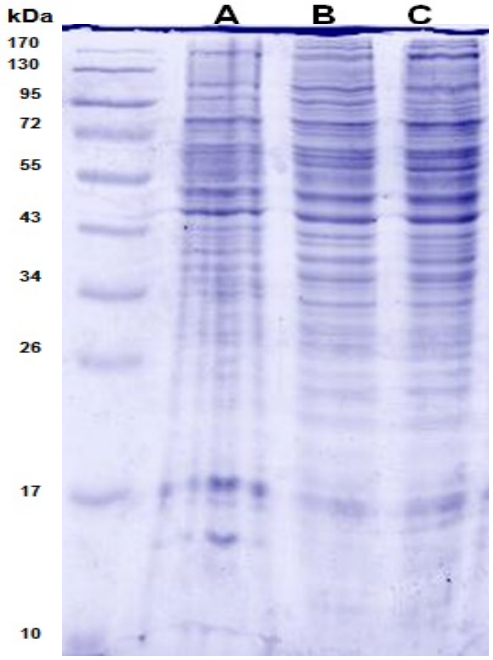


Figure 4.12 12.5% SDS-PAGE gel of proteins of HSV-1F infected cells which were extracted by CHAP-Urea-Thiourea buffer (A), NP-40 buffer (B), NP-40-Urea-Thiourea buffer (C). Ten micrograms of total proteins from each of extraction buffer were loaded on gel and stained with Coomassie Brilliant Blue R-250.

The analysis of 2-DE (2-dimentional polyacrylamide gel electrophoresis) was performed for further investigation of the differences in global protein profiles and reproducibility after using all three extraction buffers. In the first dimensional electrophoresis, the protein samples were separated according to their isoelectric points (pI) on the IPGphor II (GE Healthcare). The second dimensional electrophoresis was carried out using 12.5% SDS-PAGE to separate protein samples according to their MW. The differences of protein patterns were observed when using different three extraction buffers (Figure 4.12). It was observed that the proteins in basic area were incompletely focused on 2-DE gels in all three extraction buffer (Figure 4.13).

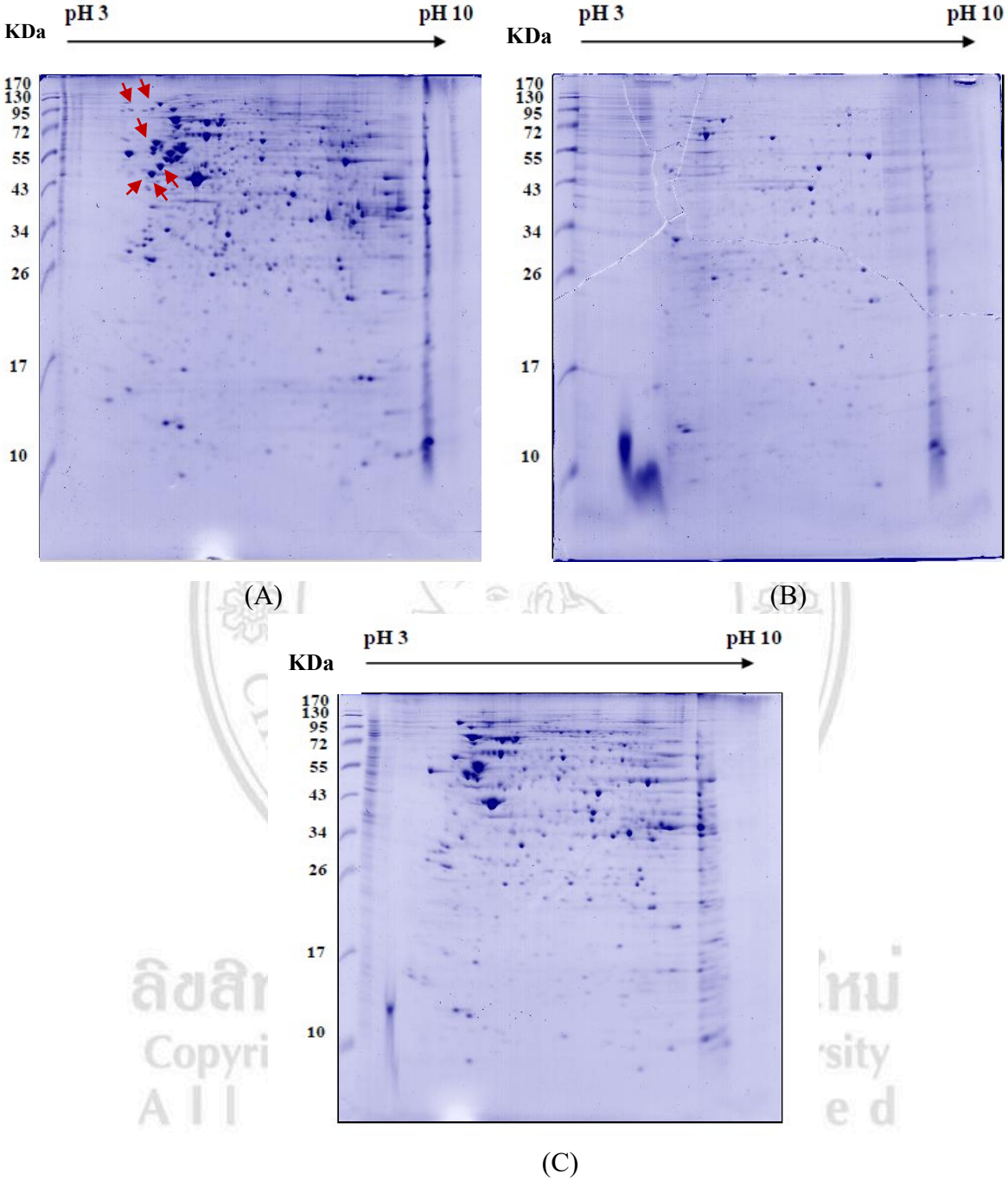


Figure 4.13 A comparison of HSV-1F infected protein profiles after extracted by CHAP-Urea-Thiourea buffer (A), NP-40 buffer (B), NP-40-Urea-Thiourea buffer (C). Two hundred microgram of total proteins were separated by IEF (pH 3-10 NL, 13 cm), followed by the 12.5% SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G-250. The numerous highly abundant protein spots were differently extracted by three types of buffer.

However, the results of 2-DE patterns of protein spots from all three extraction buffers were similar with broad range in the neutral to acidic pI which showed numerous different protein spots on the 2-DE gels. The high abundance proteins between 45 kDa and 97 kDa were observed in 2-DE gels when using CHAP-Urea-Thiourea and NP-40-Urea-Thiourea buffer (Figure 4.13). Some unique protein spots between 30 kDa and 97 kDa were found only CHAP-Urea-Thiourea buffer protein sample whereas 2-DE gels of NP-40 and NP-40-Urea-Thiourea buffer displayed horizontal streaking at the same area.

In the experiments, duplicated 2-DE gels from three extraction buffers were analyzed by using the ImageMaster 2D PlatinumTM v.5.0 software to determine the total number of protein spots. The difference of amount of protein spots were observed when examining a merged image 2-DE gels from each extraction buffer. CHAP-Urea-Thiourea buffer showed the best reproducibility and reliability (Figure 4.13). .

In summary, the results showed that CHAP-Urea-Thiourea buffer was an appropriate protein extraction buffer for HSV-1F infected cell proteins due to its reproducibility, representation of total proteome with higher MW protein solubilization. Similarity, the appropriate protein extraction buffer for HSV-2G infected cell proteins was CHAP-Urea-Thiourea buffer (Tragoolchana, 2015).

4.9.1 Protein quantification of sample loading

In this study, normal Vero cells (5×10^6cells), HSV-1F infected cells, HSV-2G infected cells and algal treatment of HSV infected cells were collected at 24 hours of incubation. The control cells and tested cells were treated by lysis buffer and freeze-thaw technique to extract total proteins for study of protein expression by 2-DE analysis. Before 2-DE analysis, protein concentration was measured using the Bradford assay with BSA as the standard sample for normalization and the standard graph of protein was made to determine the protein content of sample**.** The protein content of HSV-1F infected cell were described in Table 4.20, and protein content of HSV-2G infected cell were described in Table 4.21 comparing by proteins of normal Vero cells and algal treatment of HSV infected cell. The protein contents of all experiments were

in the range of 1.67- 2.20 µg/µl. The protein content of HSV infected cell was always higher than HSV treated with *Spirogyra* spp. extract and the uninfected cells negative control.

Table 4.20 Total protein of sample; HSV-1F infected cell, ethanolic extract of *Spirogyra* spp. treated HSV-1F infected cell and negative control as normal Vero cells

Experiment	Sample	Total protein (µg/µl)	Average protein quantity (µg/µl)
Normal Vero cells	Lot 1	1.51	1.67 ± 0.28
	Lot 2	1.50	
	Lot 3	1.99	
HSV-1F infected cell	Lot 1	1.55	1.56 ± 0.43
	Lot 2	1.99	
	Lot 3	1.14	
Ethanolic extract of *Spirogyra* spp. treatment with HSV-1F infected cell	Lot 1	1.73	1.44 ± 0.31
	Lot 2	1.47	
	Lot 3	1.12	

Table 4.21 Total protein of sample; HSV-2G infected cell, aqueous extract of *Spirogyra* spp. treated HSV-2G infected cell and normal Vero cells as negative control

Experiment	Sample	Total protein (µg/µl)	Average protein quantity (µg/µl)
Normal Vero cells	Lot 1	1.51	1.67 ± 0.28
	Lot 2	1.50	
	Lot 3	1.99	
HSV-2G infected cell	Lot 1	2.09	2.20 ± 0.18
	Lot 2	2.41	
	Lot 3	2.11	
Aqueous extract of *Spirogyra* spp. treatment with HSV-2G infected cell	Lot 1	1.89	2.18 ± 0.28
	Lot 2	2.45	
	Lot 3	2.21	

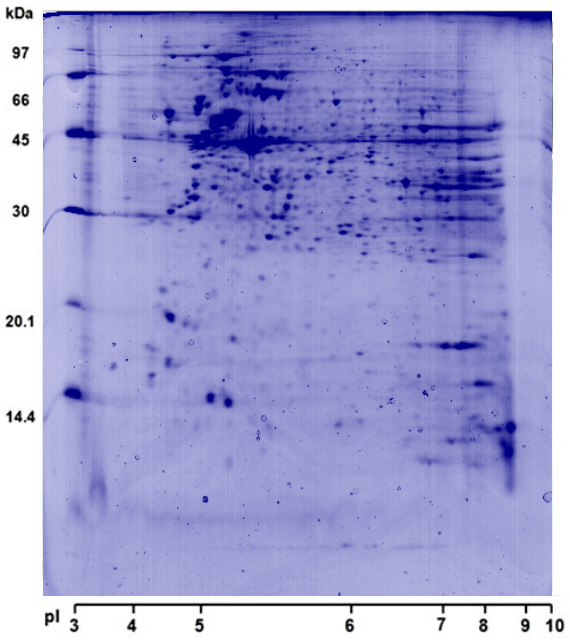
In the method of 2D-PAGE analysis, the protein samples (200 µg) were suspended in rehydration solution and subjected to isoelectric focusing in 13-cm, nonlinear, pH 3–10 immobilized-gradient strips (Immo-biline DryStrips, Amersham Biosciences, Uppsala, Sweden) in an Ettan IPGphor II apparatus (Amersham Biosciences). The second dimension electrophoresis was carried out using 12.5% SDS-PAGE gels. Gels were fixed and subjected to colloidal Coomassie Brilliant Blue G-250 stain.

4.9.2 Protein Analysis by 2-DE technique

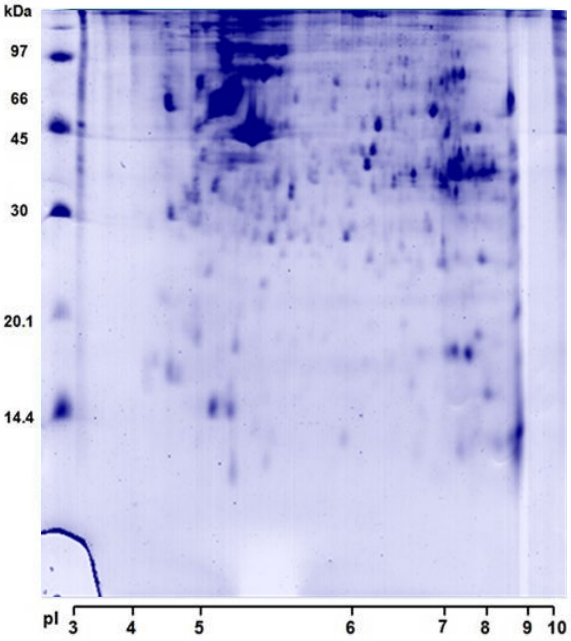
2D gel electrophoresis has been used to determine changes in the cellular proteome upon infection by several different viruses, where protein spots that differ before and after infection are excised and identified by mass spectrometry. The host interactions of three different plant viruses had been studied by this approach. Ventelon-Debout *et al.* (Alexander *et al*., 2004; William, 2000) used this method to study infection by rice yellow mottle virus and found changes in 24 to 40 cellular proteins depending on the cultivar of rice infected. These proteins included several stress response proteins such as salt-induced proteins, heat shock proteins, and superoxide dismutase (SOD) (Merchant *et al*., 2000).

In this study, the 2-DE analysis was demonstrated the reduction of viral proteins after treatment infected cells with *Spirogyra* spp. extract (Figure 4.14). Each 2-DE gels were performed in triplicate. The HSV proteins were revealed range of 545-1638 protein spots. However, the 2-DE patterns of protein spots from both types of HSV were appeared similarly. All pair samples, the number of spots that expressed even though at the significant different level (value $P<0.05$) of expression of HSV-1F and HSV-2G protein, were 27 and 19 spots, respectively (Figure 4.14). These expression-different protein spots will analyze and identify with LC-MS/MS.

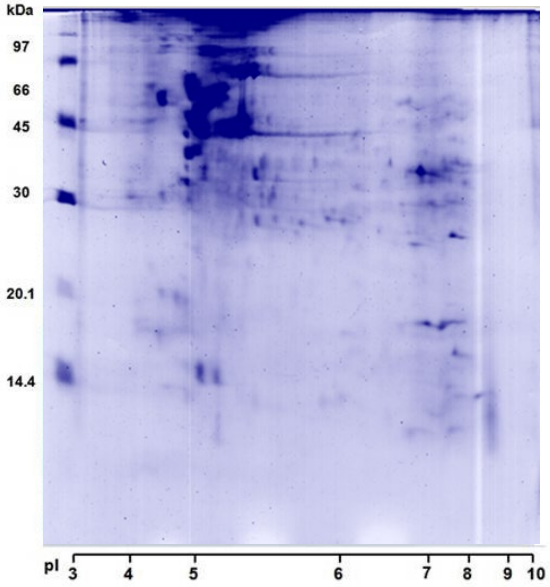
The protein extracts from normal Vero cells (5×10^6cells), HSV-1F and HSV-2G infected cell and algal extract treatment of HSV infected cells were collected at 24 hour of incubation and separated by 2-DE. The protein extracts did not include extracellular proteins. After electrophoresis, the gel images were stained with Coomassie Brillent Blue R-250 and used for data analysis. The protein patterns of two conditions and Vero cells negative control were not much different. The experiments were repeated triplication. The data analysis showed protein spots with different expression in two conditions of HSV-1F and HSV-2G infected cell and HSV infected cells after treatment with *Spirogyra* spp. extract.


kDa
97
66
45
30
20.1
14.4
pI 3 4 5 6 7 8 9 10

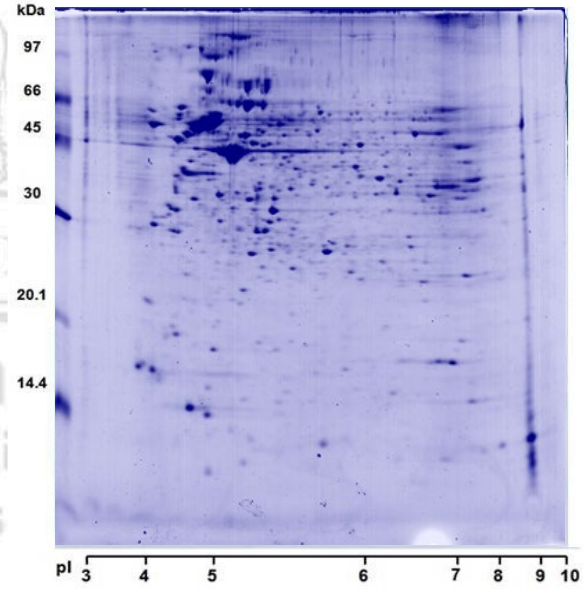
(A)


kDa
97
66
45
30
20.1
14.4
pI 3 4 5 6 7 8 9 10

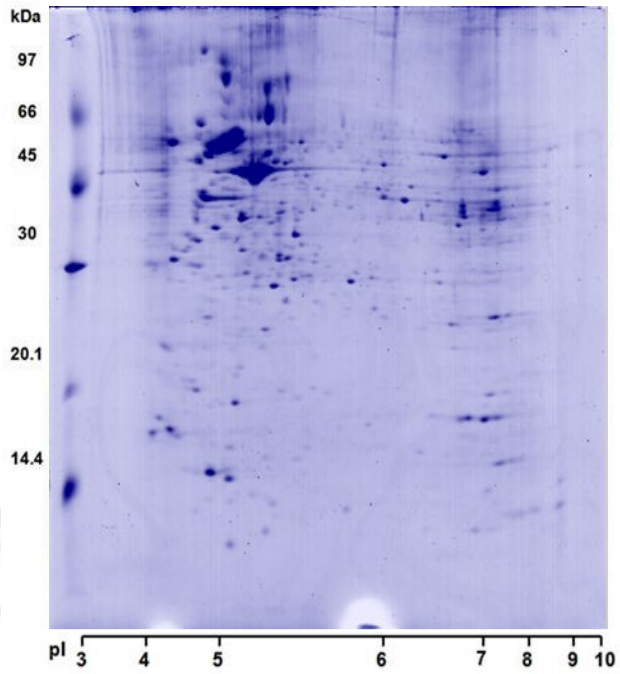
(B)


kDa
97
66
45
30
20.1
14.4
pI 3 4 5 6 7 8 9 10

(C)


kDa
97
66
45
30
20.1
14.4
pI 3 4 5 6 7 8 9 10

(D)



(E)

Figure 4.14 A comparison of normal Vero cells protein profiles (A), HSV-1F non-treated cells protein (B), HSV-1F infected protein after treatment with the ethanolic extract of *Spirogyra* spp. (C), HSV-2G non-treated cells protein (D) and HSV-2G infected protein after treatment with the aqueous extract of *Spirogyra* spp. (E) were identified by 2-DE. Two hundred micrograms of total proteins were separated by IEF (pH 3-10 NL, 13 cm), followed by 12.5% SDS-PAGE and stained with colloidal Coomassie Brilliant Blue G-250.

After separation, proteins in 2D gels are visualized by staining, commonly with a Coomassie Blue stain that is compatible with subsequent MS analysis. The 2D gels are digitized and the resulting gel images are qualitatively and quantitatively analyzed with specialized software programs. In this manner, proteins can be quantified and spot patterns in multiple gels can be matched and compared. Statistical analysis can be performed on groups of features (spots) in sets of gels, and variations, differences, and similarities can be evaluated. Proteins resolved by 2-DE can be identified based on unique attributes that are measured by MS. MS instrumentation techniques have played

a key role in proteomics and in the analysis of peptides and proteins. MALDI-ToF-MS and ESI-QUAD-ToF MS are based on different physicochemical principles and have different characteristics (Malis and White, 1999; Chaurand *et al*., 2001). Therefore, these two methods provide the needed sensitivity and specificty for proteomics research.

The several protein sequence databases are available in the public domain. An excellent annotated database is the SWISSPROT database that is maintained by The Swiss Institute of Bioinformatics and The European Bioinformatics Institute (Cooper *et al*., 2003; *Flajolet* et al., 2000). The main advantages of the SWISSPROT database are low redundancy and a high degree of annotation. Database search programs are often included in commercial software packages that are provided with mass spectrometers. One such example is the SEQUEST program that is used for database searching with uninterpreted product-ion spectra. A number of search engines can also be accessed free-of-charge over the Internet, for example the PeptIdent and MultIdent programs at the ExPASy Molecular Biology server (Alfonso *et al*., 2004; Bruno *et al*., 2014) and/or MASCOT at the Matrix Science server (Baas *et al*., 2006; Qiang *et al*., 2014). These websites also provide additional proteomics software tools, technical information, and links to other resources of proteins identified by 2-DE-based proteomics on a particular spot on the gel images.

The results of this study was illustrated the viral proteins from infected cells and non-treated cells were identified by two-dimensional electrophoresis (2-DE). In gel analysis, the gel images and 3D views of protein spots which were separated by 2-DE gel that had different expression in two conditions including mean pI and MW values from each experiment. The reduction of viral proteins was observed after treatment of infected cells with *Spirogyra* spp. extracts. It was found that the marked area of separated proteins spots in 2-DE gels was preferably in the acidic pI, pH 4-5 and molecular weight of 30-97 kDa (Figure 4.14). It was revealed in a range of 545-1638 protein spots (Table 4.22). Protein spots decreased in *Spirogyra* spp. extract treated cells.

Table 4.22 The total protein spots of HSV-1F and HSV-2G protein were expressed with *Spirogyra* spp. extracts treatment (EE treated HSV-1F and AE treated HSV-2G) and without *Spirogyra* spp. extracts treatment (HSV-1F and HSV-2G), compared with normal Vero cells.

Lot of 2-DE Gel	Tested	Number of protein spots
LOT no.01	Normal Vero cells	998
LOT no.02	Normal Vero cells	1738
LOT no.03	Normal Vero cells	1465
2-DE_lot1_1F	HSV-1F	880
2-DE_lot2_1F	HSV-1F	1638
2-DE_lot3_1F	HSV-1F	1093
2-DE_lot1_1FE	EE treated HSV-1F	573
2-DE_lot2_1FE	EE treated HSV-1F	1101
2-DE_lot3_1FE	EE treated HSV-1F	571
2-DE_lot1_2G	HSV-2G	1011
2-DE_lot2_2G	HSV-2G	1619
2-DE_lot3_2G	HSV-2G	545
2-DE_lot1_2GA	AE treated HSV-2G	1075
2-DE_lot2_2GA	AE treated HSV-2G	1523
2-DE_lot3_2GA	AE treated HSV-2G	531

In addition, the results showed among different viral and host cell proteins showed that the reduction of protein spots were observed after treatment infected cells with the extract of *Spirogyra* spp. The expression-changed protein spots indicated the regulation of viral proteins during HSV infection effect on algal treatment. The three independent repetitions with three paired samples that were prepared. The differential spots should be a reflection for the real protein expression changes *in vitro*. Each spot on the gel is calculated for its relative abundance, and this relative value is used for as a

comparison of the protein expression changes. The results showed specific spots, six of the relative values (three for the control and three for the test) were gained through ImageMaster 2-D Platinum software. In this study, the number of spots differentially expressed even though at significant different level (value $P<0.05$) of expression of HSV-1F and HSV-2G protein, were 19 and 26 spots, including up-regulated and down-regulated proteins (Table 4.23, 4.24)

The results of HSV-1F treated cell after treatment with *Spirogyra* spp. extract from two conditions revealed 19 differentially expressed protein spots (Table 4.23). The different expression of protein spots was shown from the HSV-1F and HSV-1F infected cell after treatment with ethanolic extract of *Spirogyra* spp. There were 13 spots that revealed down-regulation of expression in treated condition. Six spots were highly up-regulation of expressed proteins (Table 4.23). The mean pI and MW of protein spots were calculated from pI and MW of protein spots in every gel images. Then, 19 protein spots were chosen for protein identification by ESI-QUAD-ToF MS analysis.

The HSV-2G treated cell with aqueous extract of *Spirogyra* spp., the comparison of two conditions revealed 26 differentially expressed protein spots which were shown in Table 4.24. The different expression of protein spots was shown from the HSV-2G and HSV-2G infected cell after treatment with aqueous extract of *Spirogyra* spp. There were 15 spots that revealed down-regulation of expression in treated condition and 11 spots were highly up-regulation of expressed proteins (Table 4.24). The mean pI and MW of protein spots were calculated from pI and MW of protein spots in every gel images. Then, 26 protein spots were chosen for protein identification by ESI-QUAD-ToF MS analysis.

Table 4.23 Number of protein spots of HSV-1F protein at the significant different level (value P<0.05) that were expressed after treatment with *Spirogyra* spp. extracts of expression. The expression-different protein spots identified by LC-MS/MS.

Number of protein spot	HSV-1F	
	No. Spot ID of protein	Class of protein expression
1	17836	Down regulated
2	18076	Down regulated
3	18102	Down regulated
4	19173	Down regulated
5	19201	Up regulated
6	19227	Down regulated
7	19241	Down regulated
8	19265	Down regulated
9	19299	Down regulated
10	19419	Down regulated
11	19459	Down regulated
12	19696	Down regulated
13	19840	Down regulated
14	19916	Down regulated
15	19955	Up regulated
16	19960	Up regulated
17	19969	Down regulated
18	20372	Up regulated
19	21224	Up regulated

Table 4.24 Number of protein spots of HSV-2G protein at the significant different level (value P<0.05) that were expressed after treatment with *Spirogyra* spp. extracts of expression. The expression-different protein spots identified by LC-MS/MS.

Number of protein spot	HSV-2G	
	Spot ID of protein	Class of protein expression
1	11612	Down regulated
2	11725	Up regulated
3	12067	Down regulated
4	12087	Down regulated
5	12231	Down regulated
6	12407	Down regulated
7	12455	Up regulated
8	12528	Down regulated
9	12557	Down regulated
10	12608	Up regulated
11	12631	Up regulated
12	12680	Down regulated
13	12716	Down regulated
14	12791	Down regulated
15	12838	Up regulated
16	13020	Up regulated
17	13085	Up regulated
18	13105	Up regulated
19	13107	Down regulated
20	13195	Down regulated

Table 4.24 (continued)

Number of protein spot	HSV-2G	
	Spot ID of protein	Class of protein expression
21	13260	Up regulated
22	13607	Up regulated
23	13736	Up regulated
24	13755	Down regulated
25	13777	Down regulated
26	13879	Down regulated

In present study, the results exhibited 19 differentially expressed protein spots of HSV-1F treated cell with the ethanolic extract of *Spirogyra* spp. extract (Table 4.23). The different expression of protein suggested that six protein spots were highly up-regulation of expressed proteins. There were nesprin-1 protein, Interleukin-25, GATA-type zinc finger protein 1, Protein X and protein FAM162B which were identified by Mascot database programe (Table 4.25). In addition, thirteen spots that revealed down-regulation of expression in treated condition. The results from Mascot showed the down-regulation proteins included the RPGR-interacting protein 1-like protein, FAM162B protein, Interleukin-25, F-box domain, neogenin-like partial protein and fusion protein (Table 4.25).

The results showed 26 differentially expressed protein spots of HSV-2G treated cell with the aqueous extract of *Spirogyra spp.* extract (Table 4.24). The different expression of protein suggested that eleven protein spots were highly up-regulation of expressed proteins such as nesprin-1 protein and protein FAM162B which were identified by Mascot database programe (Table 4.26). In addition, fifteen spots that revealed down-regulation, such as the neogenin-like partial protein, nuclear transcription factor and mitoferrin-2 (Table 4.26). Therefore, all of the identified proteins from were chosen for protein-protein interactions (PPIs) by String database.

Table 4.25 LC-MS/MS identified protein in differentially regulated protein spots when comparison of HSV-1F protein and HSV-1F infected protein after treatment with ethanolic extract of *Spirogyra* spp.

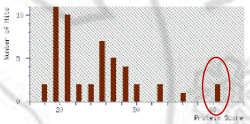
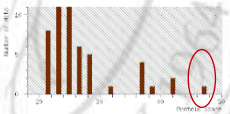
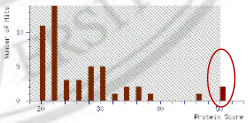
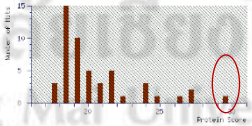
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
1	D_17836	Vero_1F	4.45	89430	5.16	152070	-	primate		41	RPGR-interacting protein 1-like protein	Macaca fascicularis
2	D_18076	Vero_1F	4.49	80740	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii
3	D_18102	Vero_1F	3.97	79290	5.16	152070	-	primate		50	RPGR-interacting protein 1-like protein	Macaca fascicularis
4	D_19173	Vero_1F	5.94	48000	10.77	31379	-	Viruses		30	F-box domain	Pandoravirus salinus

Table 4.25 (continued)

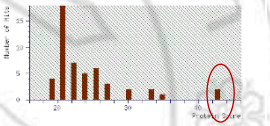
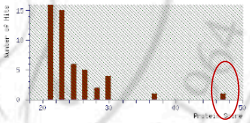
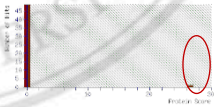
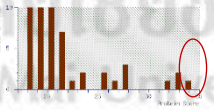
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
5	Up_19201	1F_1FE	6.24	47490	8.70	43388	-	primate		43	nesprin-1	Homo sapiens
6	D_19227	Vero_1F	6.87	46780	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii
7	D_19241	Vero_1F	4.63	46780	8.73	20887	primate	-		27	Interleukin-25	Homo sapiens
8	D_19265	Vero_1F	6.14	44940	6.29	62233	-	Viruses		34	fusion protein	Sendai virus

Table 4.25 (continued)

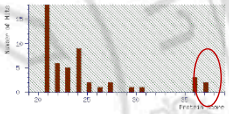
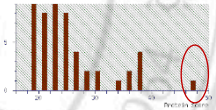
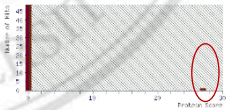
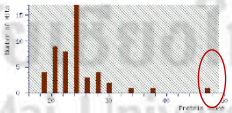
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
9	D_19299	Vero_1F	4.65	44610	4.85	57548	-	primate		37	neogenin-like, partial	Pongo abelii
10	D_19419	Vero_1F	5.73	42970	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii
11	Up_19459	1F_1FE	6.89	41740	8.73	20887	primate	-		27	Interleukin-25	Homo sapiens
12	D_19696	Vero_1F	5.98	38490	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii

Table 4.25 (continued)

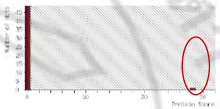
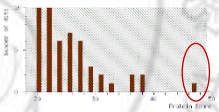
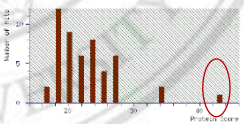
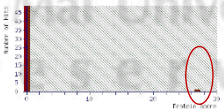
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
13	D_19840	Vero_1F	7.93	36580	8.73	20887	primate	-		28	Interleukin-25	Homo sapiens
14	D_19916	Vero_1F	5.49	35440	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii
15	Up_19955	Vero_1F	6.10	34880	9.03	29133	-	primate		43	LOW QUALITY PROTEIN: GATA-type zinc finger protein 1	Saimiri boliviensis boliviensis
16	Up_19960	1F_1FE	5.53	34830	8.37	17262	Viruses	-		27	Protein X	Hepatitis B virus genotype H (isolate United States/LAS2523/2002)

Table 4.25 (continued)

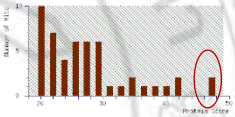
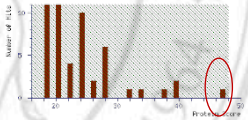
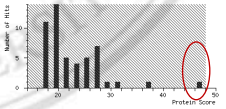
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
17	D_19969	Vero_1F	5.38	34830	4.85	57548	-	primate		47	neogenin-like, partial	Pongo abelii
18	Up_20372	1F_1FE	6.43	27750	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii
19	Up_21224	1F_1FE	6.36	14750	10.68	13819	-	primate		47	protein FAM162B	Otolemur garnettii

Table 4.26 LC-MS/MS identified protein in differentially regulated protein spots when comparison of HSV-2G protein and HSV-2G infected protein after treatment with aqueous extract of *Spirogyra* spp.

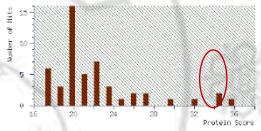
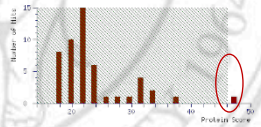
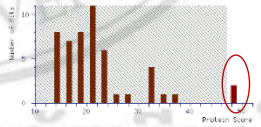
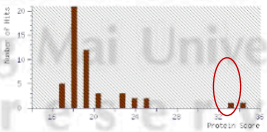
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
1	D_11612	Vero_2G	4.33	54500	5.54	69886	-	N-Vi		35	Regulatory protein E1	Kappapapillomavirus 1
2	Up_11725	2G_2GW	4.65	51240	10.68	13819	-	N-pri		47	FAM162B	Otolemur garnettii
3	D_12067	Vero_2G	5.66	43330	8.70	43388	-	N-Pri		48	nesprin-1	Homo sapiens
4	D_12087	Vero_2G	4.97	42520	6.77	70572	-	N-Vi		33	DNA packaging tegument protein UL25	Aotine herpesvirus 1

Table 4.26 (continued)

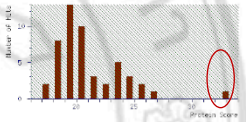
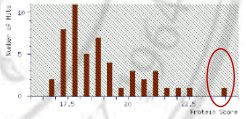
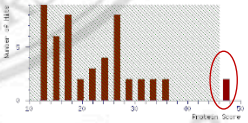
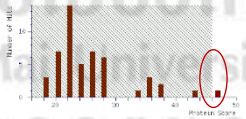
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
5	D_12231	Vero_2G	6.55	39830	6.77	70572	-	Viruses		33	DNA packaging tegument protein UL25	Aotine herpesvirus 1
6	D_12407	Vero_2G	5.77	37040	4.80	30579	-	Viruses		24	gene 9 protein	Enterobacteria phage Sf6
7	Up_12455	2G_2GW	7.48	36290	8.70	43388	-	primate		47	nesprin-1	Homo sapiens
8	D_12528	Vero_2G	5.52	34900	10.68	13819	-	primate		47	FAM162B	Otolemur garnettii

Table 4.26 (continued)

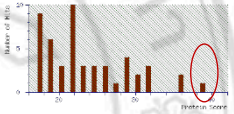
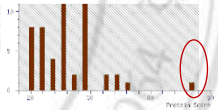
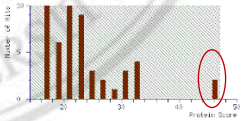
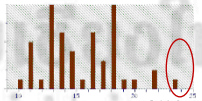
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
9	D_12557	Vero_2G	5.93	34190	4.85	57548	-	primate		39	neogenin-like, partial	Pongo abelii
10	Up_12608	2G_2GW	4.70	33360	10.68	13819	-	primate		47	FAM162B	Otolemur garnettii
11	Up_12631	2G_2GW	5.44	33020	8.70	43388	-	primate		46	nesprin-1	Homo sapiens
12	D_12680	Vero_2G	4.59	32120	8.56	20317	-	Viruses		22	spike protein, partial	Feline coronavirus

Table 4.26 (continued)

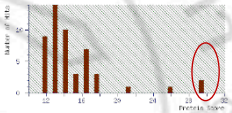
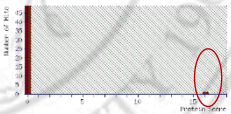
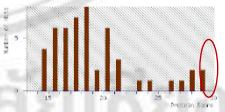
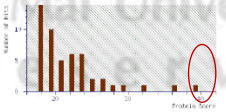
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
13	D_12716	Vero_2G	5.72	31330	5.78	50556	primate	-		25	Nuclear transcription factor Y subunit gamma	Homo sapiens
14	D_12791	Vero_2G	5.28	30350	5.53	88845	primate	-		16	Virion-packaging protein UL17	Psittacid herpesvirus 1 (isolate Amazon parrot/-/97-0001/1997)
15	Up_12838	2G_2GW	5.43	29700	5.54	69886	-	Viruses		29	regulatory protein E1	Kappapapillomavirus 1
16	Up_13020	2G_2GW	6.27	26660	8.70	97993	-	Viruses		37	RNA capping protein VP3	Rotavirus A

Table 4.26 (continued)

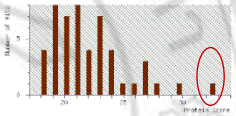
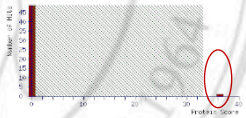
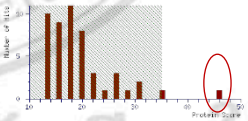
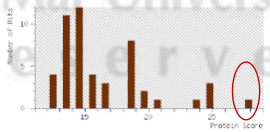
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
17	Up_13085	2G_2GW	5.06	26130	4.89	64492	-	Viruses		29	major tail sheath protein	Staphylococcus phage 812
18	Up_13105	2G_2GW	5.46	25870	5.78	126222	primate	-		36	Apoptosis-stimulating of p53 protein 2	Homo sapiens
19	D_13107	Vero_2G	5.52	31040	5.78	126222	primate	-		46	Apoptosis-stimulating of p53 protein 2	Homo sapiens
20	D_13195	Vero_2G	5.50	24780	10.22	10828	-	N-Vi		28	polyprotein, partial	Hepatitis C virus

Table 4.26 (continued)

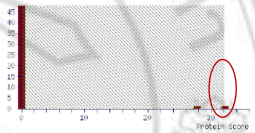
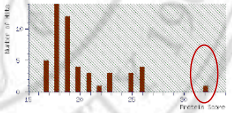
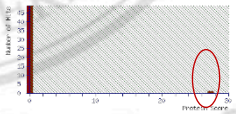
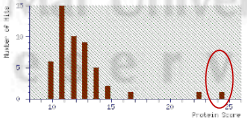
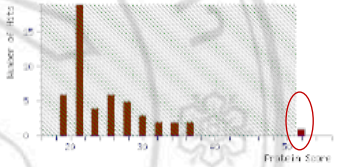
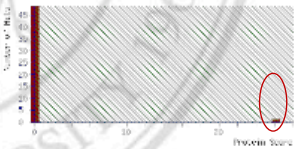
No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
21	Up_13260	2G_2GW	7.82	23570	8.87	66894	Viruses	-		32	Tegument protein UL47 homolog	Psittacid herpesvirus 1 (isolate Amazon parrot/-/97-0001/1997)
22	Up_13607	2G_2GW	7.12	16770	4.89	64492	-	Viruses		32	major tail sheath protein	Staphylococcus phage 812
23	Up_13736	2G_2GW	6.71	14930	4.94	77915	Viruses	-		27	Nucleoprotein	Lake Victoria marburgvirus (strain Angola/2005)
24	D_13755	Vero_2G	8.31	14590	9.98	61607	-	primate		28	mitoferrin-2	Saimiri boliviensis

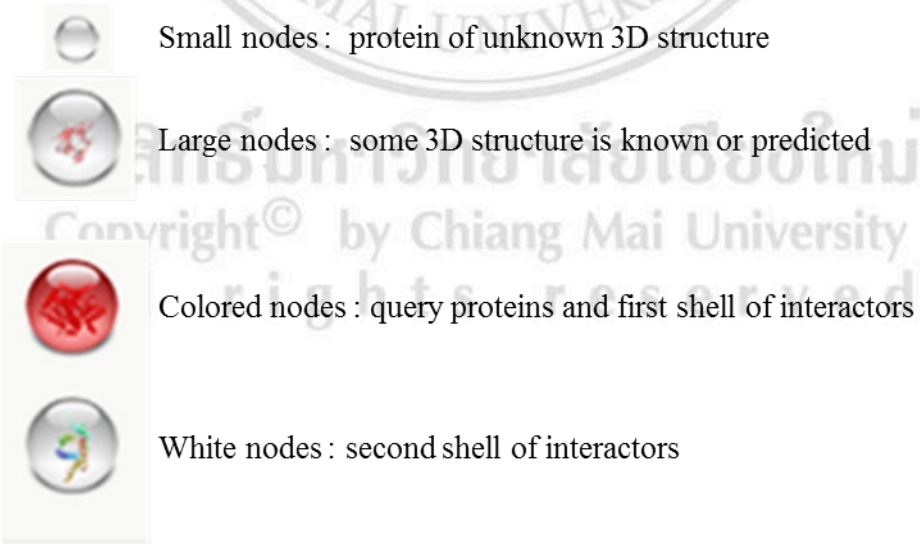
Table 4.26 (continued)

No.	Spot ID	Type	Experiment		Mascot programme		Database		Histogram	Score	Protein	Taxonomy
			pI	MW	pI	MW	Swiss	NCBI				
25	D_13777	Vero_2G	5.15	14500	4.89	64492	-	Viruses		52	major tail sheath protein	Staphylococcus phage 812
26	D_13879	Vero_2G	8.17	14400	9.95	24129	Viruses	-		26	Large delta antigen	Hepatitis delta virus genotype II (isolate Japanese S-1)

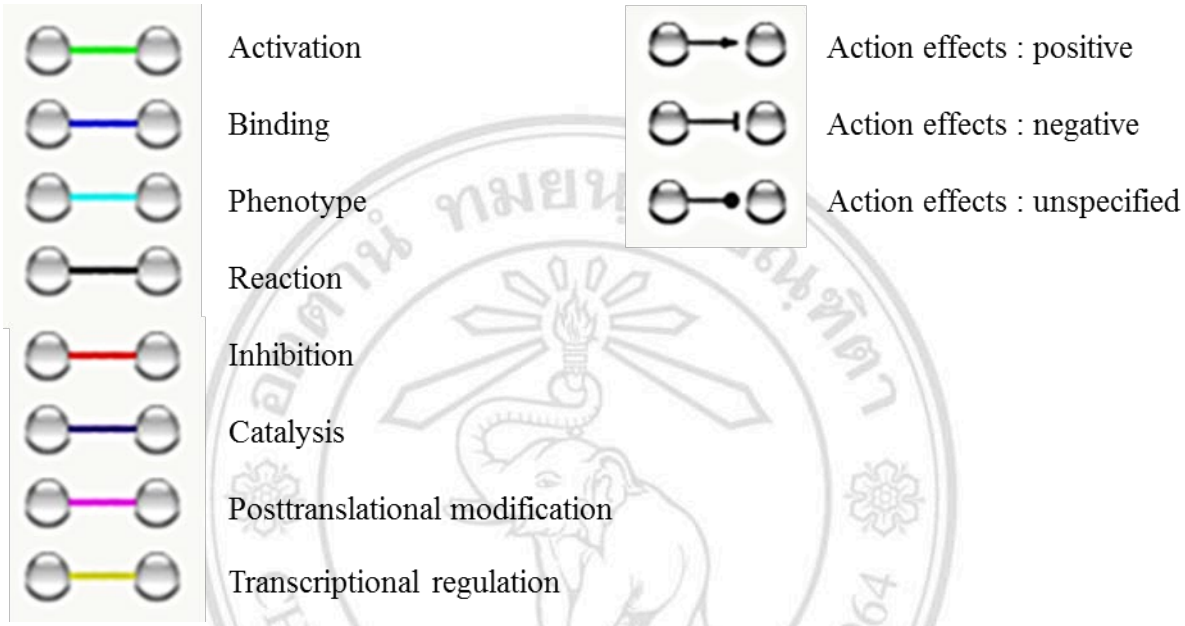
4.9.3 Protein-protein interaction analysis

The results of proteomics analysis could describe for the identification of protein interactions with a large amount of protein data. Therefore, the study of protein had complemented by the computational methods for predicting protein interactions. In this study, the identified protein were illustrated the protein-protein interactions by The String database with the functional interaction networks of globally proteins integrated and scored data (Damian *et al.,* 2011). The STRING website was freely accessible via http://string-db.org, which was a database of known and predicted protein-protein interactions. According to the query of database with global proteins, the resource reported a raw network consisting of the highest scoring interaction partners. This network could convenient to rearrange and cluster directly in the browser window revealing tightly connected functional modules.

The result of network nodes of proteins represented the post-translational proteins produced by a single and protein-coding gene. The String database reported the protein data settings, viewed the association of functional proteins and analysis of protein-protein interaction. The String network nodes analysis could represent proteins including the node size and color as follows:



The result of analysis indicated protein-protein associations. The interaction meant the specific proteins that jointly contributed to share function and physically bind other proteins, which showed in types of action and effect as follows :



4.9.3.1 Protein-protein interaction analysis of HSV-1F treatment with ethanolic extract of *Spirogyra* spp.

The results of protein interaction of the mechanism of HSV-1F after treatment with the ethanolic extract of *Spirogyra* spp. was revealed from the imported STRING network (Figure 4.15). The detail of three networks was largely non-overlapping. The protein-protein complex identification was the crucial step in finding the signal transduction pathways. In particularly, protein-protein interaction complexes after HSV-1F treated with the ethanolic extract of *Spirogyra* spp. mostly consisted of integrin beta-2 (Itgb2), E3 ubiquitin ligase complexes (Cand1) and Nesprin-1 (ENSMUSG00000096054) (Figure 4.15).

The results of functional protein complex from three proteins were suggested. Integrin beta-2 (Integrin alpha-L/beta-2) and E3 ubiquitin ligase complexes promoted the exchange of the substrate-recognition F-box subunit in complexes and

played key role in the cellular protein. The nesprin-1 protein was multi-isomeric modular protein and formed a linking network between organelles and the actin cytoskeleton to maintain the subcellular spatial organization (Bengtsson *et al*., 2008; Miyamoto *et al*., 2012).

The results of expression proteins from the expression-profiling clusters of network protein data were interacted with each other proteins database for description of gene co-expression (Rao *et al*., 2014). In this study, the identified proteins were confirmed to be expressed and co-expression of protein interaction after treatment with the ethanolic extract of *Spirogyra* spp. The results suggested that the mechanism of Nesprin-1 protein expression were up-regulated after HSV-1F infected cells were treated with *Spirogyra* spp. extract. While, the expression of integrin beta-2 and E3 ubiquitin were down-regulated after HSV-1 infected cells were treated with *Spirogyra* spp. extract.

In addition, the component of SUN-protein-containing multivariate complexes called LINC (Linker of Nucleoskeleton and Cytoskeleton) complexes. The LINC linked the nucleoskeleton and cytoskeleton by providing versatile outer nuclear membrane attachment sites for cytoskeletal filaments. It might be involved in the maintenance mechanism of nuclear organization and structural integrity between HSV-1F protein that changed after treatment with the ethanolic extract of *Spirogyra* spp. Therefore, the nuclei connected to the cytoskeleton by interacting with the nuclear envelope and F-actin in the cytoplasm via by protein-protein interaction pathway (Freiberg *et al*., 2001; Pazos *et al*., 2002). Moreover, the inhibitory effect of HSV-1F infection by the ethanolic extract of *Spirogyra* spp. were indicated from these proteins-protein interaction. Upon querying the database from Mascot, the resource consisted of the highest scored of interaction proteins; Itgb2 (integrin beta 2), D17Wsu104e (DNA segment of Chromosome 17), Fam162b (protein from family with sequence similarity 162, member B), Cand1 (cullin associated and neddylation disassociated 1), Doc2b (double C2-beta; Calcium sensor regulated fusion membrane), nesprin-1 and zinc finger; GATA-like protein 1 (Figure 4.15). The results suggested that the HSV-1F proteins may be inhibited by algal substance and biological protein pathway of host cell. Then, HSV-1F may not replicate and assembly to new virion.

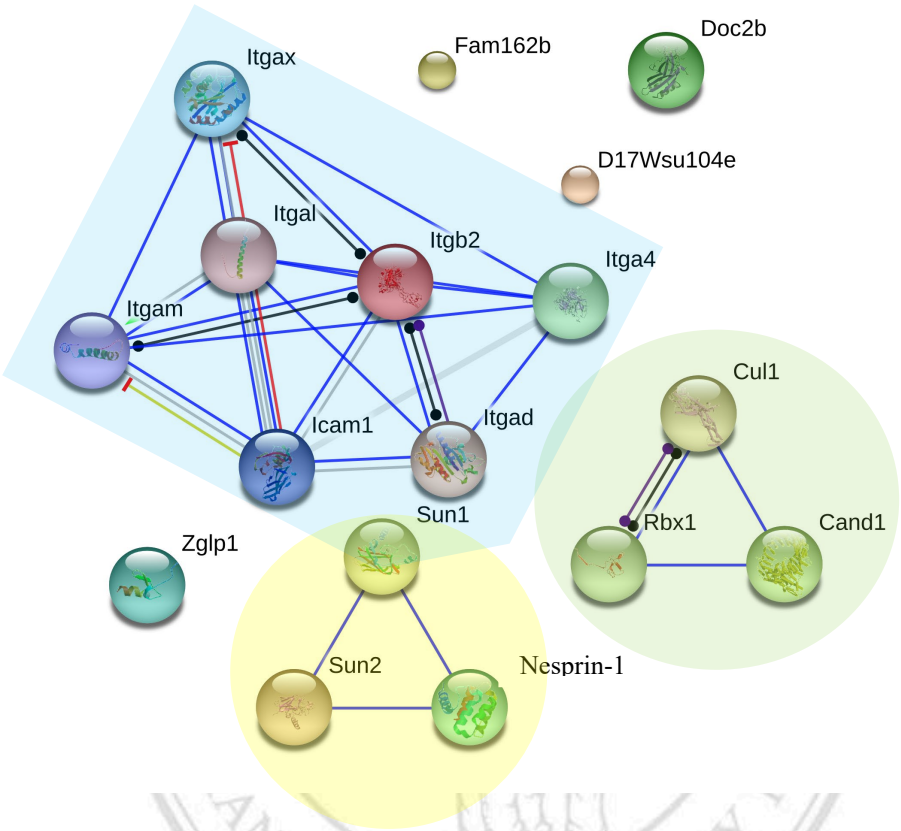


Figure 4.15 Protein network visualization on the STRING website. The interaction focused on a specific protein network in HSV-1F and host cell proteins after treatment with *Spirogyra* spp. extract. Upon querying the database from Mascot, the resource consisted of the highest scored of interaction proteins; Itgb2 (integrin beta 2), D17Wsu104e (DNA segment of Chromosome 17), Fam162b (protein from family with sequence similarity 162, member B), Cand1 (cullin associated and neddylation disassociated 1), Doc2b (double C2-beta; Calcium sensor regulated fusion membrane), ENSMUSG00000096054 (Nesprin-1) and Zglp1 (zinc finger; GATA-like protein 1)

4.9.3.2 Protein-protein interaction analysis of HSV-2G treatment with aqueous extract of *Spirogyra* spp.

In this study, the analysis of protein-protein interaction of the expression mechanism of HSV-2G treated cells after treatment with the aqueous extract of *Spirogyra* spp. were illustrated from the STRING network database (Figure 4.16). The two networks of protein interactions were non-overlapping effect. The identification of expressed protein was found in the cell cycle signaling pathways. Moreover, protein-protein interaction complexes after HSV-2G infected cells treated with the aqueous extract of *Spirogyra* spp. showed proliferating cell nuclear antigen (PCNA), NEDD8 activating enzyme E1 subunit 1 (NAE1) and spectrin repeat containing, nuclear envelope 1 (SYNE1) (Figure 4.16).

The regulated mechanism of HSV-2G inhibition after treatment with the aqueous extract of *Spirogyra* spp. affected two main functional protein pathways, the regulation of cell cycle and intracellular non-membrane bounded organelle. The result suggested that NAE1 was activated with UBA3-NAE1-E1 complex. Then, the ubiquitin-conjugating enzyme was belonged to the ubiquitin-like protein NEDD8 and catalyzed the covalent attachment to other proteins (Goldberg *et al.*, 2003). The regulatory subunit of UBA3-NAE1-E1 comlex was necessary expressed for cell cycle progression through the cell-cycle checkpoint.

In this study, the protein network of the expression of protein interaction after treatment HSV-2G infected cell with the aqueous extract of *Spirogyra* spp. were reported. The results revealed that the mechanism of Nesprin-1 protein expression were up-regulated after treatment of HSV-2G infected cell with *Spirogyra* spp. extract. On the other hand, the regulatory protein E1 and E3 ubiquitin expression were down-regulated after treatment HSV-2G infected cell with *Spirogyra* spp. extract. Upon querying the database from Mascot, the resource consisted of the highest scored of interaction proteins; Itgb2 (integrin beta 2), D17Wsu104e (DNA segment of Chromosome 17), Fam162b (protein from family with sequence similarity 162, member B), Cand1 (cullin associated and neddylation disassociated 1), Doc2b (double C2-beta;

Calcium sensor regulated fusion membrane), nesprin-1 and Zglp1 (zinc finger; GATA-like protein 1 (Figure 4.16).

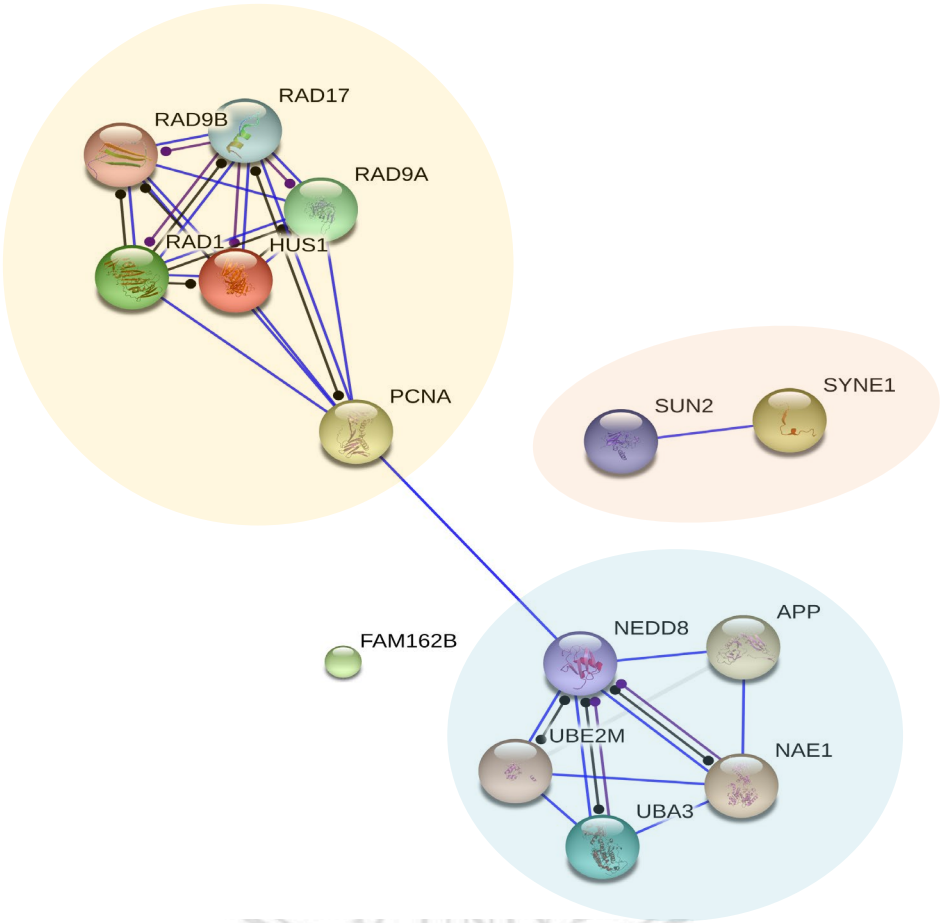


Figure 4.16 Protein network visualization on the STRING website. The interaction focused on a specific protein network in HSV-2G and host cell proteins after treated with *Spirogyra* spp. extract. Upon querying the database from Mascot reported, the resource consisted of the highest scored of interaction proteins; HUS1 (HUS1 checkpoint homolog protein), RAD1 (RAD1 homolog protein), PCNA (prolferating cell nuclear antigen), Fam162b (protein from family with sequence similarity 162, member B), NEDD8 (neural precursor cell expressed protein, developmentally down-regulated 8), APP (amyloid-beta precursor protein), UBA3 (ubiquitin-like modifier activating enzyme 3), NAE1 (NEDD8 activating enzyme E1 subunit 1), SUN2 (Sad1 and UNC84 domain containing 2) and SYNE1 (spectrin repeat containing, nuclear envelope 1)

Additionally, the SUN-protein complexes linked the cytoskeleton and assembled with SYNE1 to arrange transmembrane actin-associated nuclear lines. These complex bounded to F-actin filament and coupled the nucleus to retrograde actin to flow during actin-dependent nuclear movement. Then, it was required for inter-kinetic nuclear migration and affected intracellular non-membrane bounded organelle (Freiberg *et al.,* 2001; Pazos *et al.,* 2002). Therefore, the HSV-2G proteins were inactivated by the up-regulation of SYNE1 protein complex after treatment with the aqueous extract of *Spirogyra* spp. The inhibitory effect of HSV-2G infection by aqueous extract of *Spirogyra* spp. revealed the protein-protein interaction network in Figure 4.16. The expression of HSV-2G and host cell were inhibited by *Spirogyra* spp. extract. Thus, HSV-2G could not replicate and release from host cell.

4.10 Development of gel product for treatment of HSV

From our results, the ethanolic extract of *Spirogyra* spp. had high antiviral efficacy against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection. Thus, in this study the ethanolic extract from *Spirogyra* spp. was selected to develop antiviral gel product for anti-HSV agent.

The *Spirogyra* spp. gel product was evaluated for their efficacy against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by plaque reducing assay. Additionally, the stability and physical properties including pH, color, smell and viscosity of the algal gel product were also studied. Moreover, the efficacy of this algal gel product was also evaluated after heating-cooling 6 cycles and storage at different temperature for 7 months. The skin irritation of algal gel product was also tested on the volunteers.

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

4.10.1 The algal gel product preparation

Nowadays, various products from natural substance have become a new requirement for use in daily life. Gel product was widely used in pharmacological application as carrier for substances that contained the efficacy on various benefit agents

in order to delivery active substances due to its easily spreadable and penetration through mucosal surfaces before absorption to percutaneous layer of skin (Fathy *et al*., 2010; Shivhare *et al.,* 2009). Additionally, the result found that ethanolic extract of *Spirogyra* spp. showed the most effective anti-HSV activity on various stages of HSV multiplication cycle. Therefore, this algal gel product containing ethanolic extract of *Spirogyra* spp. was produced.

The algal gel product was produced by mixed the gel components that composed of carbopol 940, methyl paraben, propylene glycol, triethanolamine, tween 80 and crude ethanolic extract of *Spirogyra* spp.

Carbopol is a agent for gel-forming, which could deform the gel structure, and has bioadhesive properties (Bonacucina *et al.,* 2004). Hence, this carbopol compound also used as thickening agent and also used in pharmaceutical products for drug delivery system (Aminabhavi *et al*., 2004; Asasutjarit *et al.,* 2005). Additionally, methyl parabens are the non-toxic substance that completely absorbed through the skin and usually used as oral and parenteral compound (Soni *et al.,* 2002). Thus, it was used as preservatives, antiseptics and antimicrobial agents against fungi and bacteria (Boukarim *et al.,* 2009). Triethanolamine was used for the emulsifier agent, buffer and surfactant. Moreover, tween 80 was low toxicity agent and used for the surfactants, stabilizer or emulsifier, and enhanced the absorption of drug (Zhang *et al.,* 2003). In addition, propylene glycol was a low toxic agent, which used without non-irritation and increase moisturizing to the skin. Furthermore, the gel base did not show anti-HSV activity.

4.10.2 Cytotoxicity of the algal gel product

Cytotoxicity of algal gel product containing ethanolic extract of *Spirogyra* spp. was determined on Vero cells by MTT assay and CD_{50} values was calculated. In this study, the results showed that CD_{50} value of the gel product was 512.40 µg/ml while crude ethanolic extract of *Spirogyra* spp. showed CD_{50} value of 356.57 µg/ml. Therefore, This result suggested that the combination between *Spirogyra* spp. extract and gel base might reduce the toxicity of this algal gel product.

4.10.3 Stability test of algal gel product

The stability of *Spirogyra* spp. gel product containing ethanolic extract of *Spirogyra* spp. was investigated in this study. Stability study was carried out to determine the quality of active substance under environmental stress (Bajai *et al*., 2012; Daberte *et al.,* 2011). Moreover, stability test was demonstrated to ensure the quality and safety of algal gel product throughout the storage conditions (Vipul and Devesh, 2012).

Our study, the stability test was evaluated after the *Spirogyra* spp. gel product was storage at different condition for short-term and long-term period. Stability test of algal gel was observed on the short-term storage by heating-cooling for 6 cycles while long-term condition was observed by storage the algal gel at 4, 25 and 45ºC for 7 months. Hence, the gel was protected from the light during the storage time.

4.10.3.1 Short-term determination of algal gel stability after heating-cooling cycle

The stability of phytochemical and physiological constituents of pharmaceutical products was very important (Daberte *et al*., 2011; Vipul and Devesh, 2012). Stability of algal gel was analysed by heating and cooling. The algal gel product was placed at 4ºC for 48 hours then the product was placed at 45ºC for 48 hours for 6 cycles. Thus, the gel product was examined for 24 days. Hence, the physical properties including pH, color, smell, viscosity and the forming of emulsion were investigated compared to control of the gel product. In particular, the algal gel was further examined for antiviral activity against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by plaque reduction assay.

In this study, the result revealed that after heating-cooling cycle for 6 cycles, the layer of gel was not separated and the color of pale yellow was retained. Furthermore, *Spirogyra* spp. gel product also retained the natural smell. The viscosity of algal gel was slightly decreased after heating-cooling for 6 cycles. Moreover, pH was increased from 7 to 8 when heating-cooling for 4 cycles (Table 4.27, Figure 4.17).

Algal gel product containing the ethanolic extract of *Spirogyra* spp. was further tested for anti-HSV activity by plaque reduction assay. The result showed that after heating-cooling cycle, the algal gel also retained the antiviral efficacy against HSV-1, HSV-2G and five HSV-1 resistant isolates infection by 70.24-75.56% (Figure 4.18 and 4.19). In this study, the *Spirogyra* spp. gel product changed their physical properties after storage at 4ºC for 48 hours and 45ºC for 48 hours for 6 cycles was the same except increase of pH and decrease of viscosity of algal gel product. Additionally, the algal gel also maintained its stability on antiviral activity against HSV infection.

(a) (b)

Figure 4.17 The algal gel product (a) and algal gel after 6 cycles of heating-cooling (b)

Table 4.27 Characteristics of algal gel product containing ethanolic extract of *Spirogyra* spp.

Gel	Number of heating-cooling	Separation of texture	Color	Smell	Viscosity	pH
Algal gel	0	-	Pale-yellow	+++	+++	7.0
	1	-	Pale-yellow	+++	+++	7.0
	2	-	Pale-yellow	+++	+++	7.0
	3	-	Pale-yellow	+++	+++	7.0
	4	-	Pale-yellow	+++	++	8.0
	5	-	Pale-yellow	+++	++	8.0
	6	-	Pale-yellow	+++	++	8.0

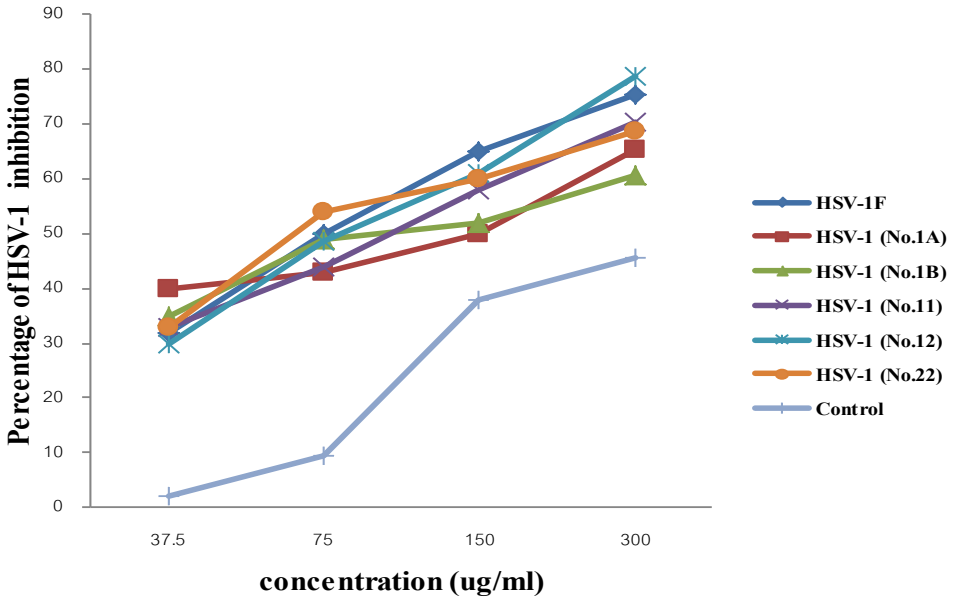


Figure 4.18 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1F, ACV-resistant HSV-1 isolate 1A, 1B, 11, 12 and 22 after heating-cooling for 6 cycles

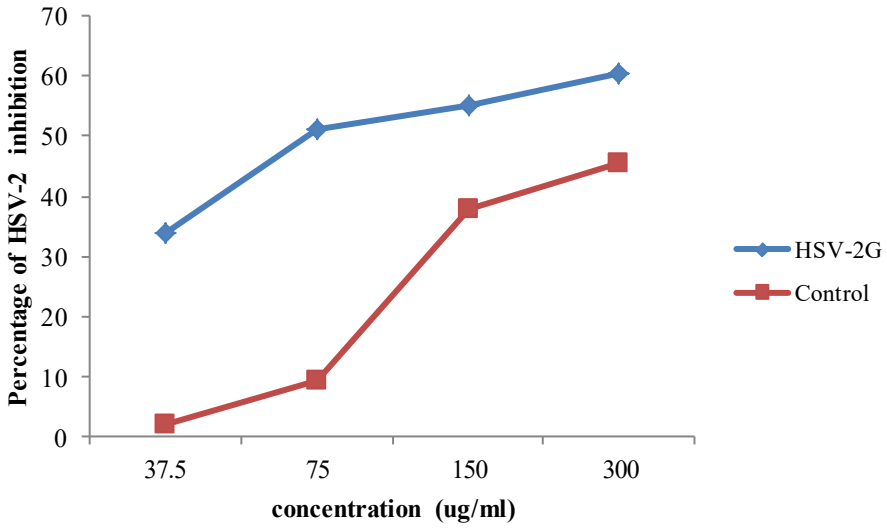


Figure 4.19 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-2G after heating-cooling for 6 cycles

4.10.3.2 Long-term determination of algal gel stability test

The long term stability test of *Spirogyra* spp. gel product was performed in order to determine effect of storage condition on antiviral efficacy against HSV. After gel product was produced, effects of temperature and incubation period on physical properties of the algal gel product such as pH, color, smell, viscosity and emulsion forming were compared to gel base control. Furthermore, the algal gel was also investigated for the efficacy against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by plaque reduction assay.

Stability of algal gel product at different temperature was investigated. Physical properties of algal gel product after storage for 7 months showed that the gel layer was not separated when storage at 4ºC. However, the color of algal gel was changed from pale yellow into deep yellow. The viscosity and emulsion forming of the algal gel product were decreased upon the longer time of storage. Furthermore, the pH was changed from 7 to 8 in 4 months (Table 4.28).

The inhibitory effect of *Spirogyra* spp. gel product against HSV infection by plaque reduction assay was observed. The result revealed that, the algal gel product after storage at 4ºC retained their efficacy to inhibit HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by 55.46-72.10% when testing every month (Figure 4.20-4.26).

Table 4.28 Characteristics of algal gel product containing ethanolic extract of *Spirogyra* spp. after storage at 4°C for 7 months

Test	Month	Separation of texture	Color	Smell	Viscosity	pH
Gel	0	-	Pale yellow	+++	+++	7
at 4°C	1	-	Pale yellow	+++	+++	7
	2	-	Pale yellow	+++	+++	7
	3	-	Pale yellow	+++	++	7
	4	-	Pale yellow	+++	++	8
	5	-	Pale yellow	++	++	8
	6	-	Pale yellow	++	++	8
	7	-	Pale yellow	++	+	8

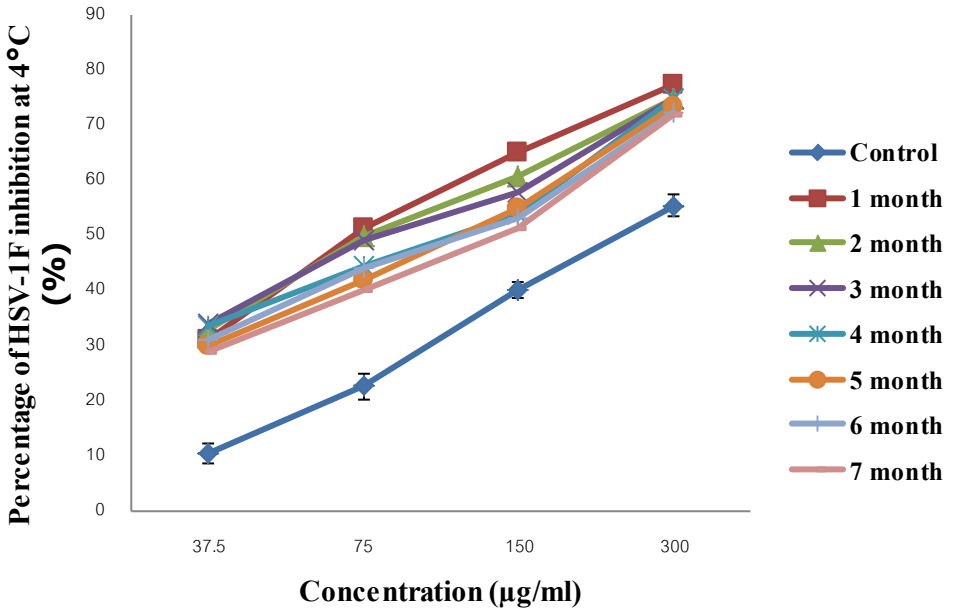


Figure 4.20 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1F after storage at 4ºC for 7 months

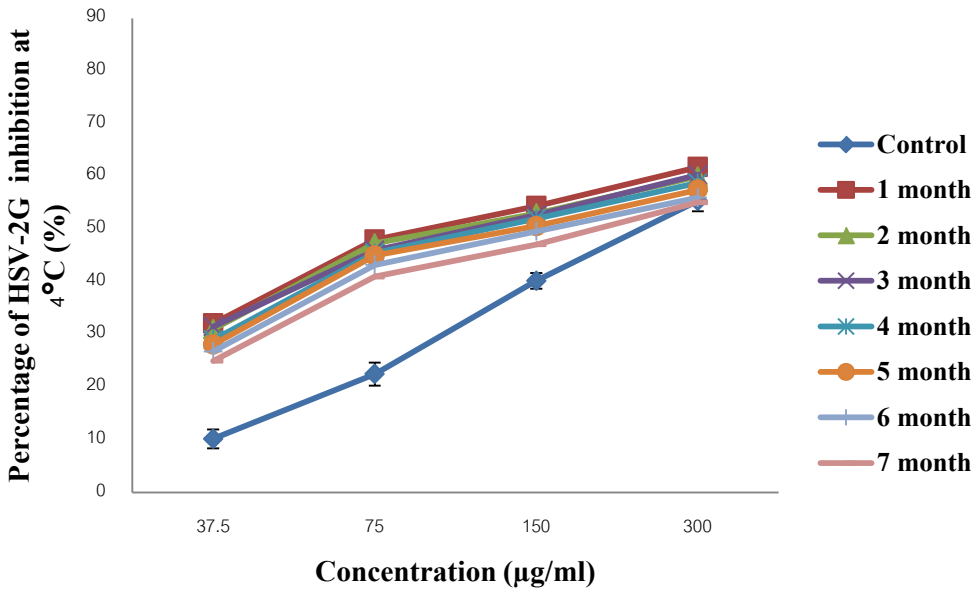


Figure 4.21 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-2G after storage at 4ºC for 7 months

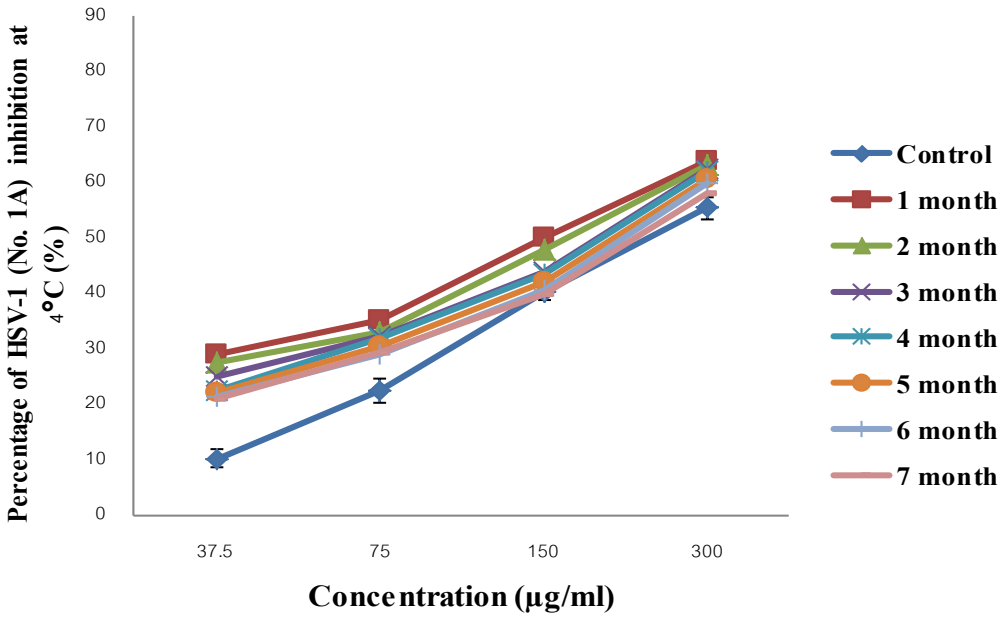


Figure 4.22 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1A) after storage at 4ºC for 7 months

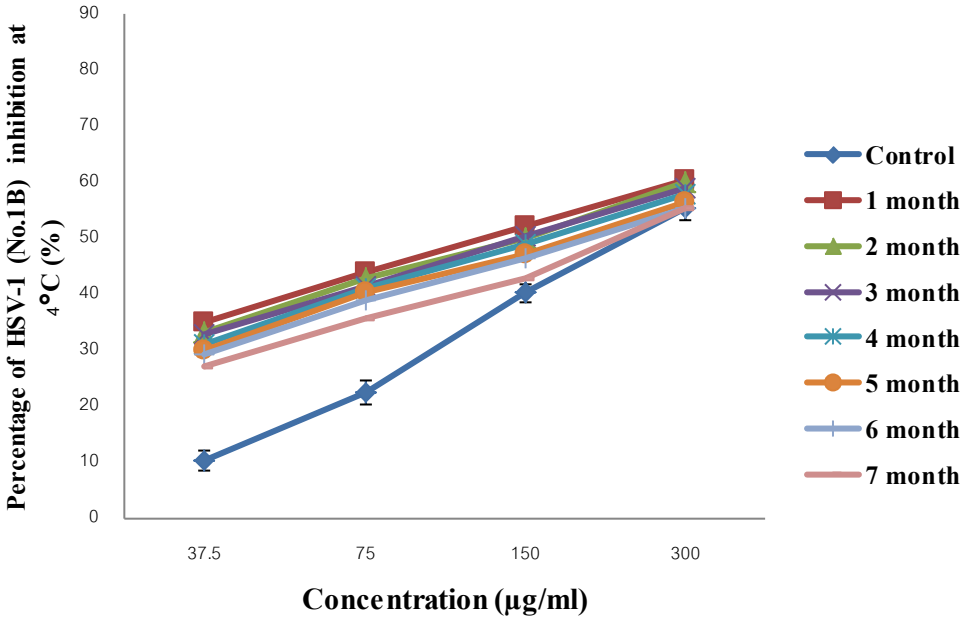


Figure 4.23 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1B) after storage at 4ºC for 7 months

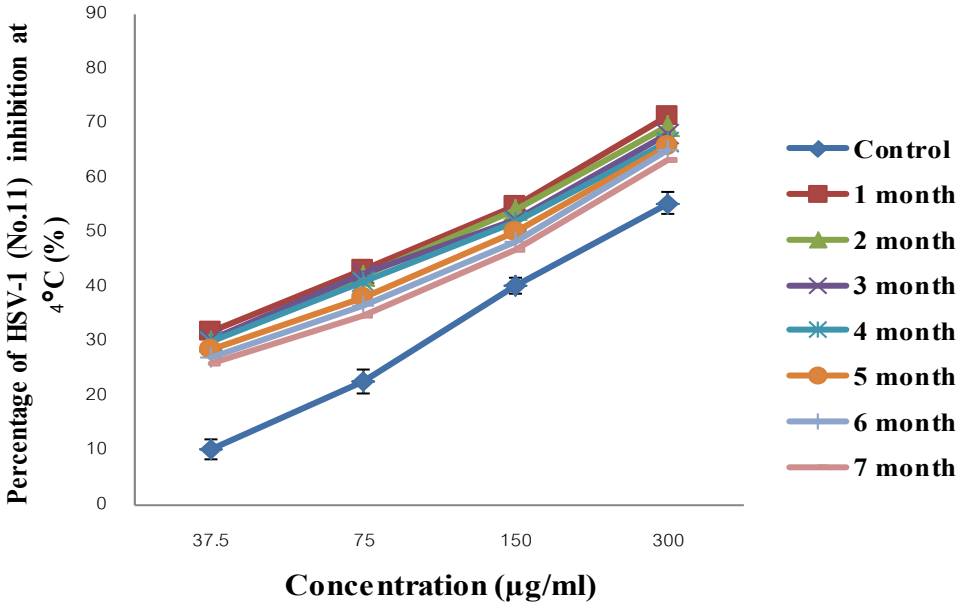


Figure 4.24 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.11) after storage at 4ºC for 7 months

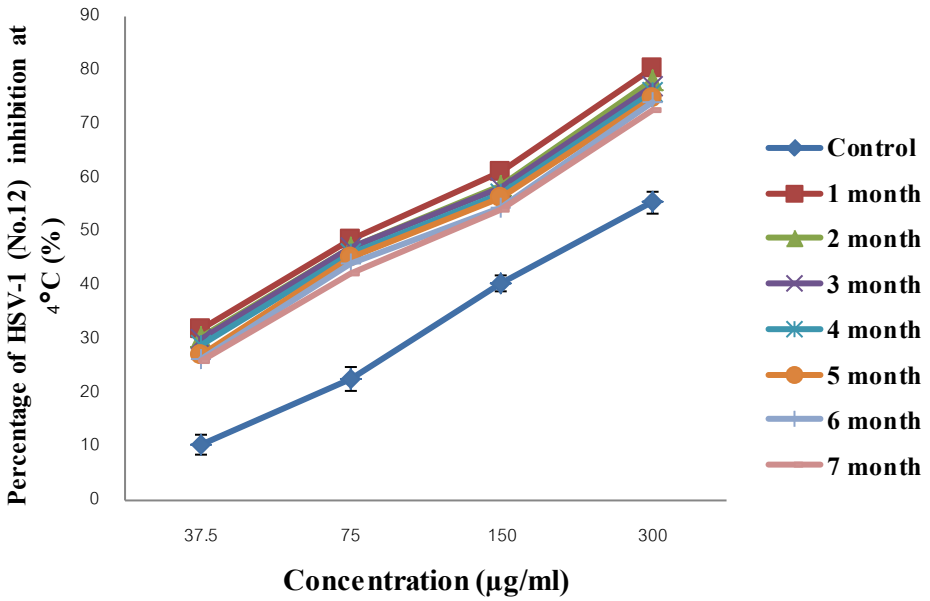


Figure 4.25 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.12) after storage at 4ºC for 7 months

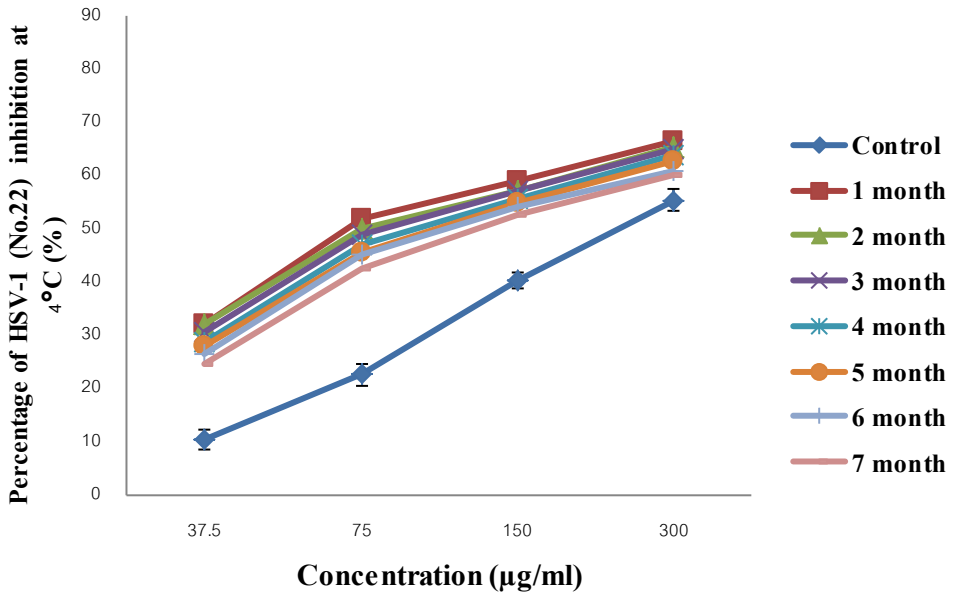


Figure 4.26 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.22) after storage at 4ºC for 7 months

Stability of algal gel when storage at 25ºC was evaluated in this study. The result showed that the layer of algal gel was not separated. However, color of the algal gel was changed from pale yellow into deep yellow after storage for 3 months. The smell of algal 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. Thus, the same result was obtained when the gel was storage at 25ºC (Table 4.29).

Antiviral activity against HSV infection by algal gel containing ethanolic extract of *Spirogyra* spp. when storage at 25ºC was shown. The algal gel retained potent inhibitory effect on HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by 52.11-69.88 % when determined every month by plaque reduction assay (Figure 4.27 - 4.33).

Table 4.29 Characteristics of algal gel product containing ethanolic extract of *Spirogyra* spp. after storage at 25°C for 7 months.

Test	Month	Separation of texture	Color	Smell	Viscosity	pH
Gel	0	-	Pale yellow	+++	+++	7
at 25°C	1	-	Pale yellow	+++	+++	7
	2	-	Pale yellow	+++	+++	7
	3	-	Pale yellow	+++	++	8
	4	-	Pale yellow	+++	++	8
	5	-	Pale yellow	++	++	8
	6	-	Pale yellow	++	++	8
	7	-	Pale yellow	+	+	8

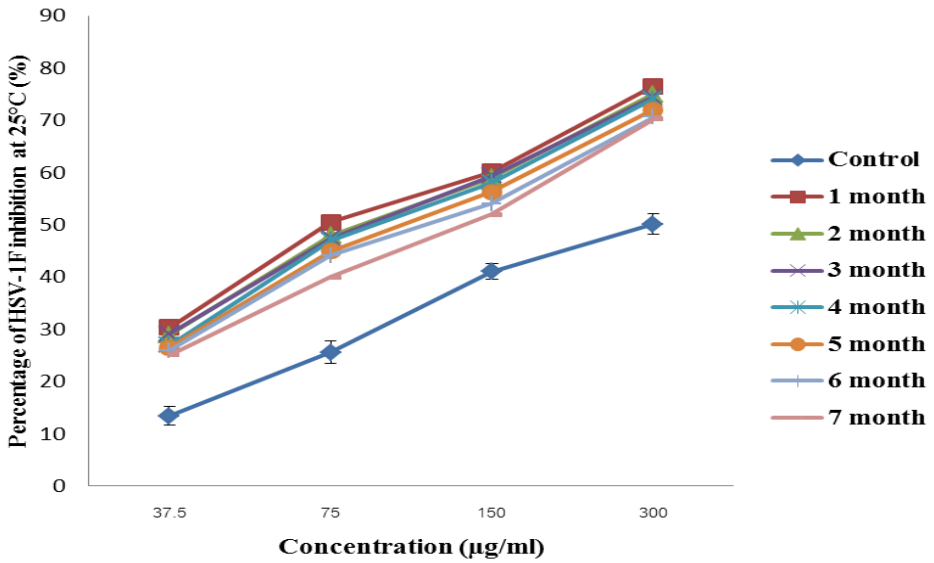


Figure 4.27 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1F after storage at 25ºC for 7 months

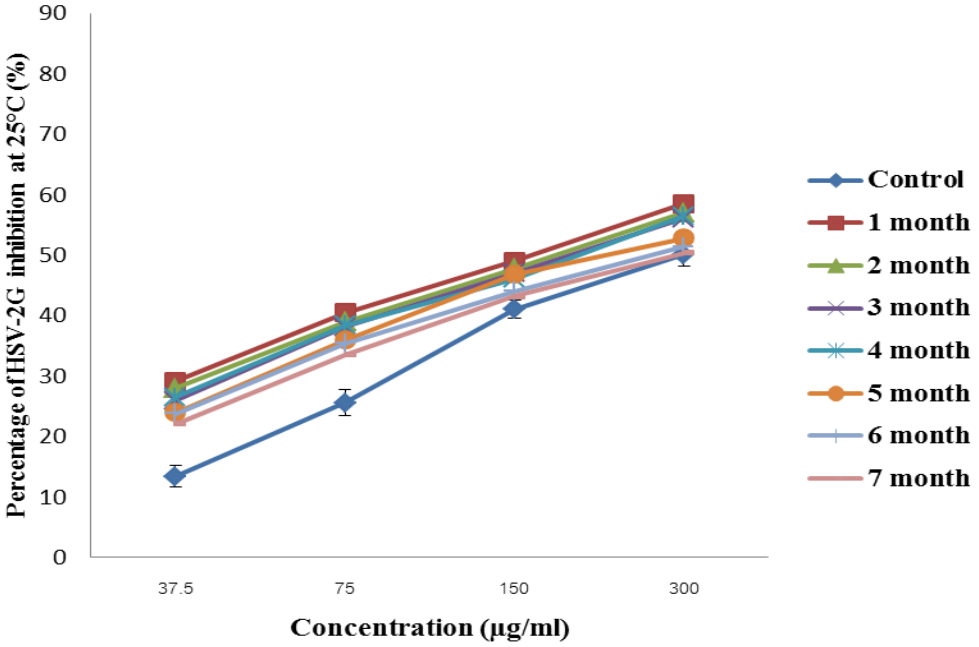


Figure 4.28 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-2G after storage at 25ºC for 7 months

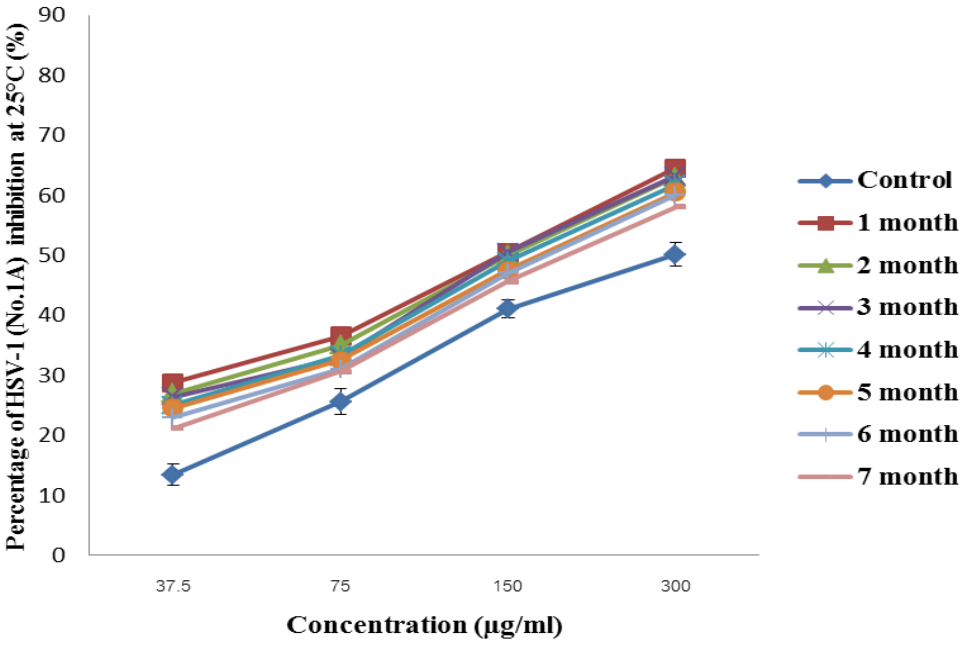


Figure 4.29 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1A) after storage at 25ºC for 7 months

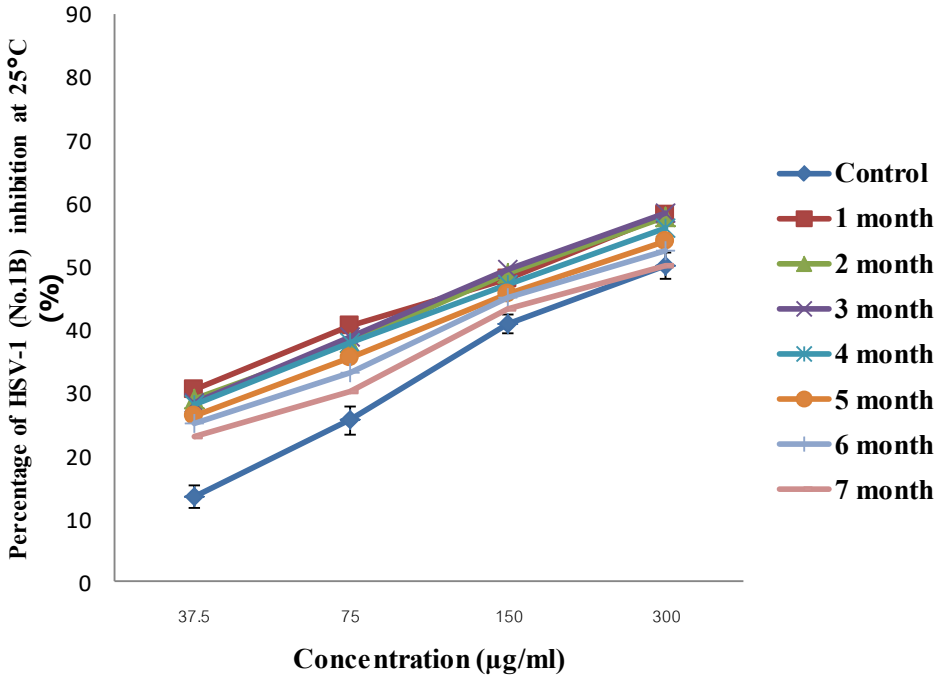


Figure 4.30 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1B) after storage at 25ºC for 7 months

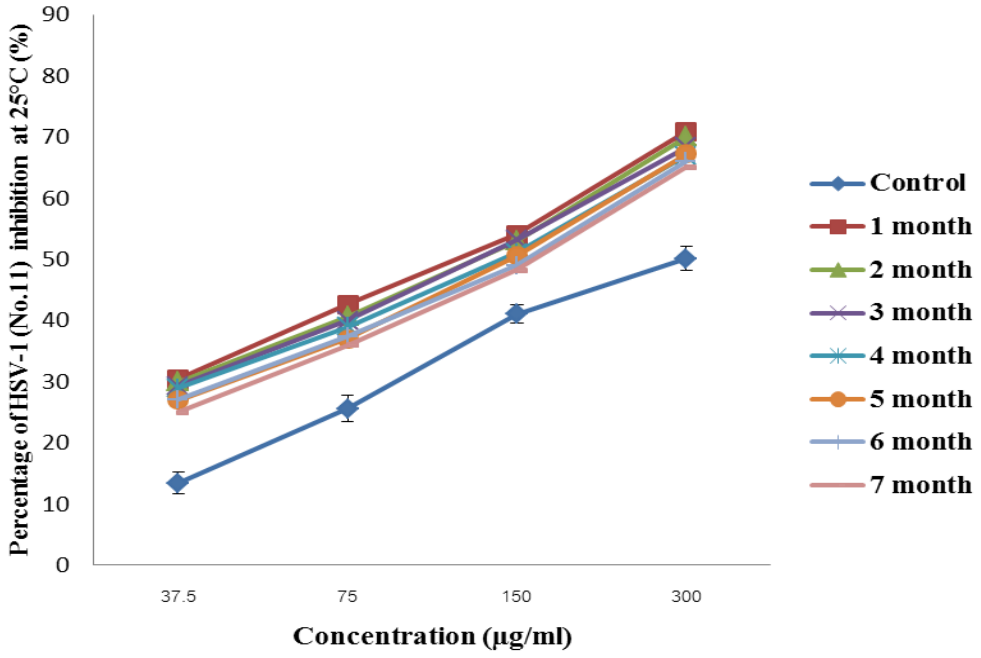


Figure 4.31 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.11) after storage at 25ºC for 7 months

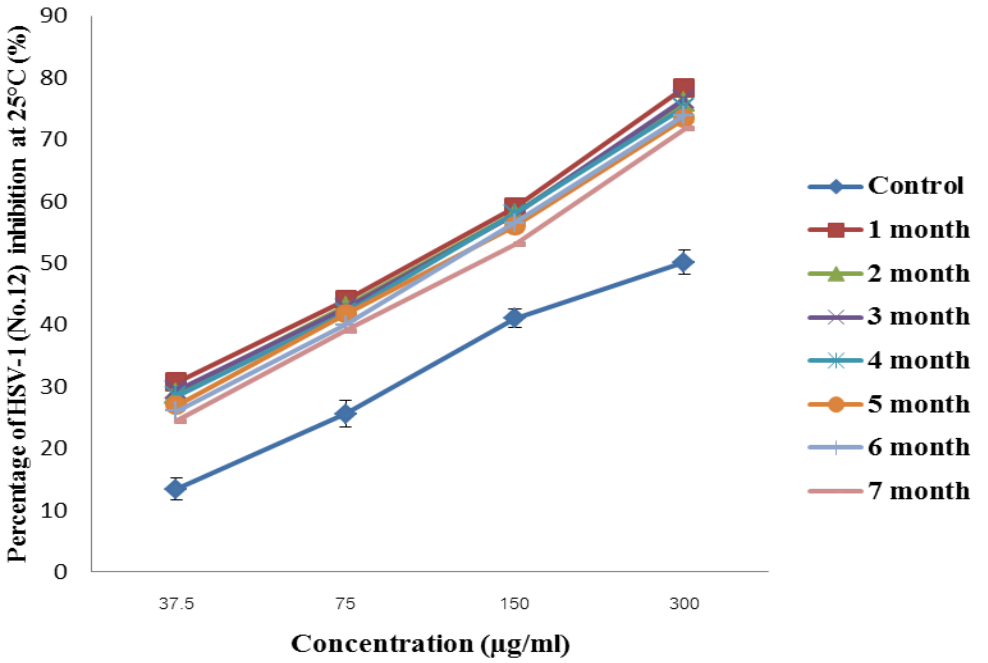


Figure 4.32 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.12) after storage at 25ºC for 7 months

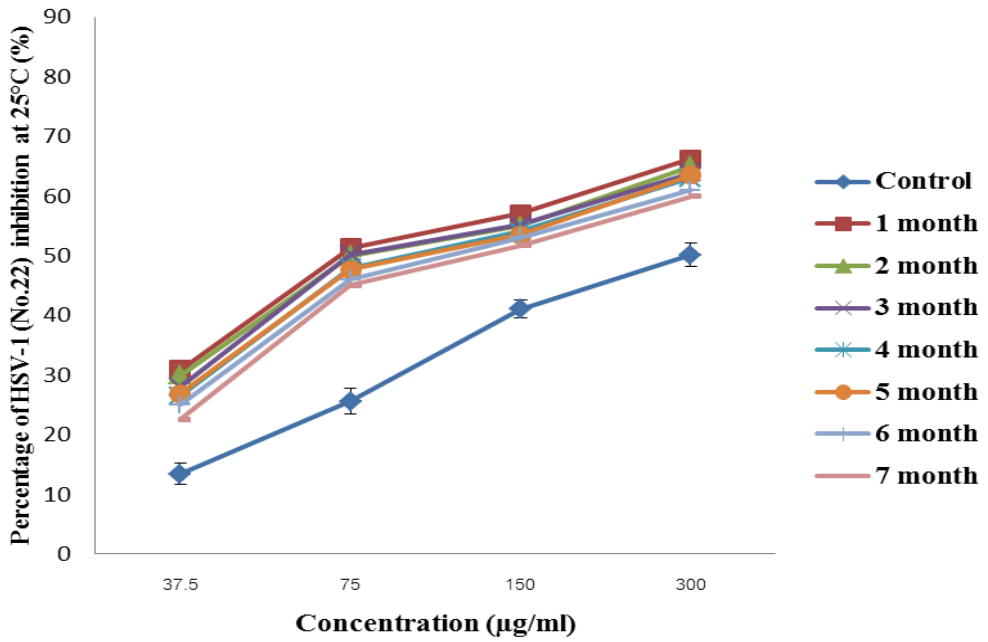


Figure 4.33 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.22) after storage at 25ºC for 7 months

Stability test of algal gel product when storage at 45ºC for 7 months was observed. The physical properties of the algal gel revealed that the gel layer was not separated. Besides, the color of algal gel was changed from pale yellow after storage for 1 month and became deep yellow within 4 months. 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 5 months. Variations of pH was observed when storage at 45ºC since pH was increased to 8 in 3 months. These observation was similar to the pH of algal gel when storage at 4 and 25ºC (Table 4.30).

Antiviral activity against HSV infection by algal gel containing ethanolic extract of *Spirogyra* spp. when storage at 45ºC was presented. The algal gel retained potent inhibitory effect on HSV-1F, HSV-2G and ACV-resistant HSV-1 infection by 44.17-62.84% when determined every month by plaque reduction assay (Figure 4.34 - 4.40).

Table 4.30 Characteristics of algal gel product containing ethanolic extract of *Spirogyra* spp. after storage at 45°C for 7 months

Test	Month	Separattion of texture	Color	Smell	Viscosity	pH
Gel	0	-	Pale yellow	+++	+++	7
at 45°C	1	-	Pale yellow	+++	+++	7
	2	-	Pale yellow	+++	+++	7
	3	-	Pale yellow	+++	++	8
	4	-	deep yellow	++	+	8
	5	-	deep yellow	++	-	8
	6	-	deep yellow	++	-	8
	7	-	deep yellow	+	-	8

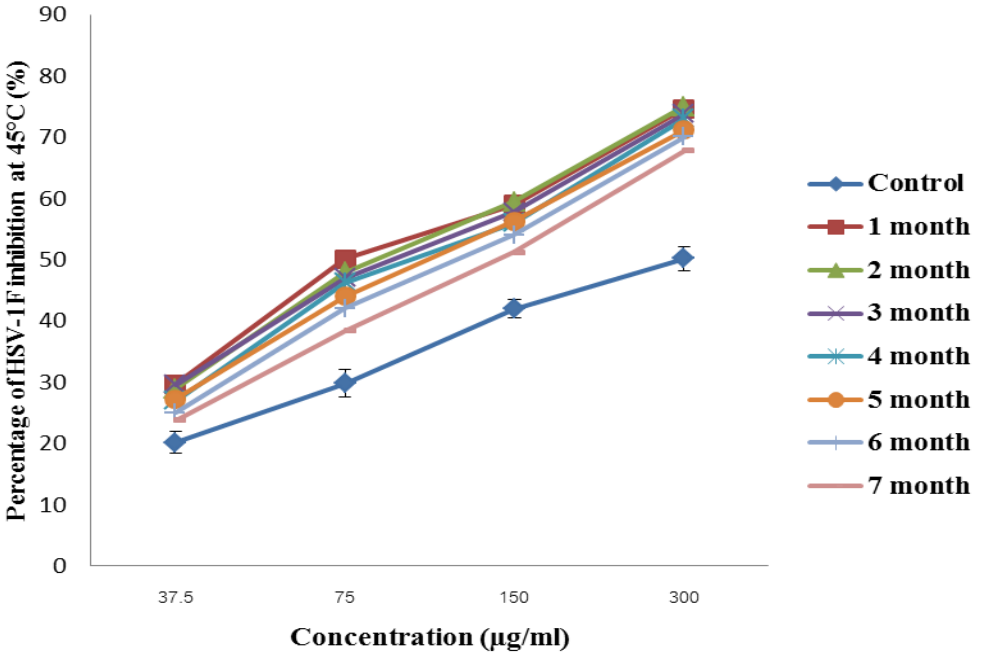


Figure 4.34 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1F after storage at 45ºC for 7 months

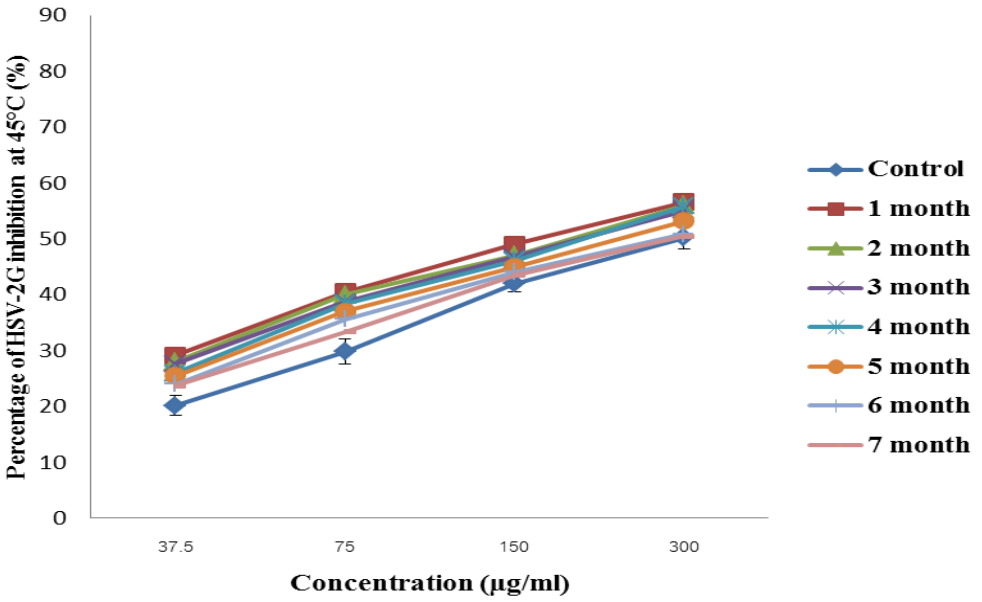


Figure 4.35 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-2G after storage at 45ºC for 7 months

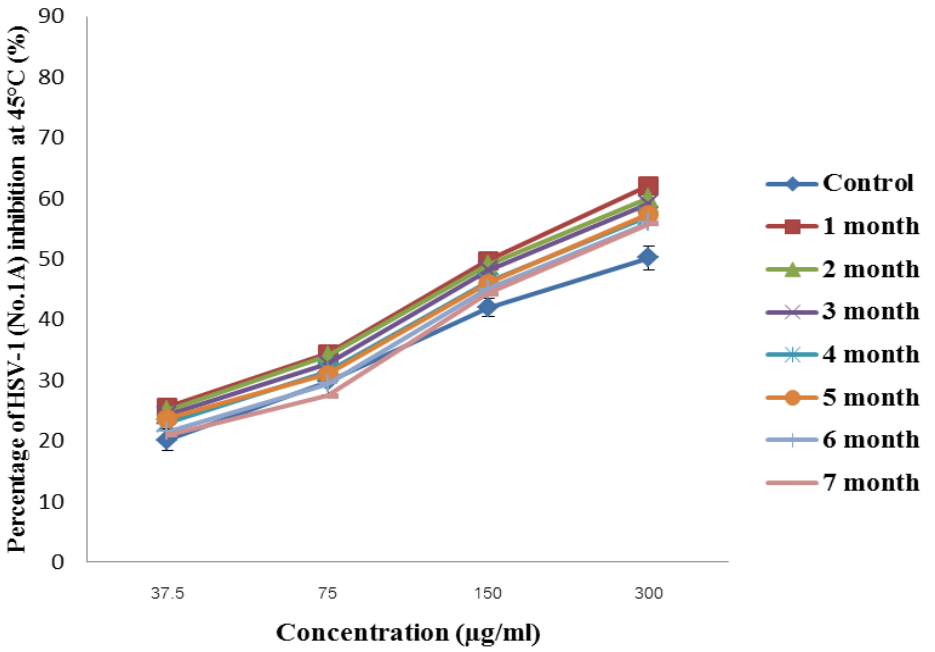


Figure 4.36 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1A) after storage at 45ºC for 7 months

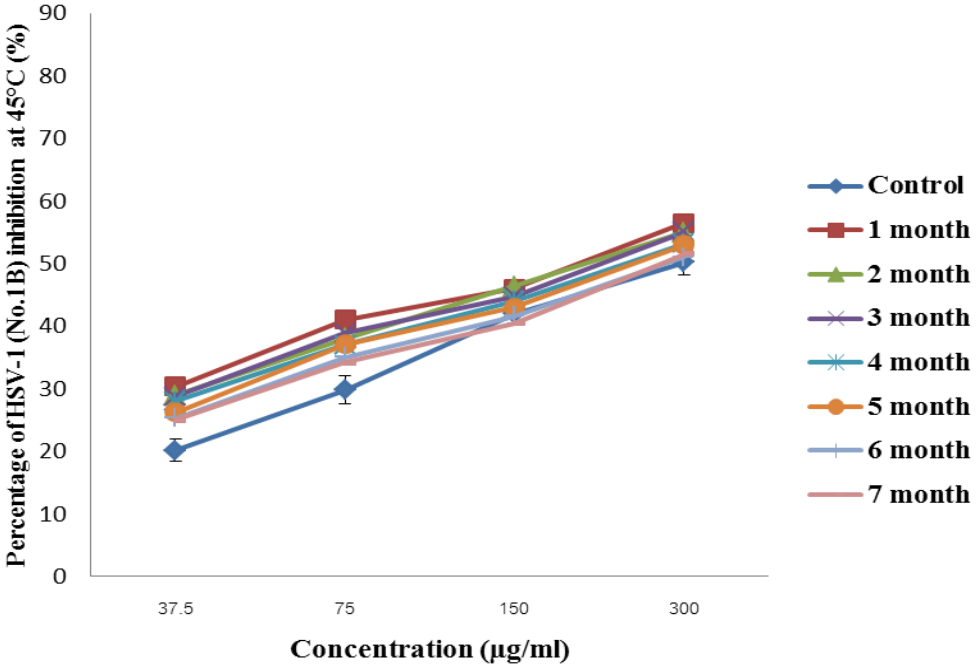


Figure 4.37 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.1B) after storage at 45ºC for 7 months

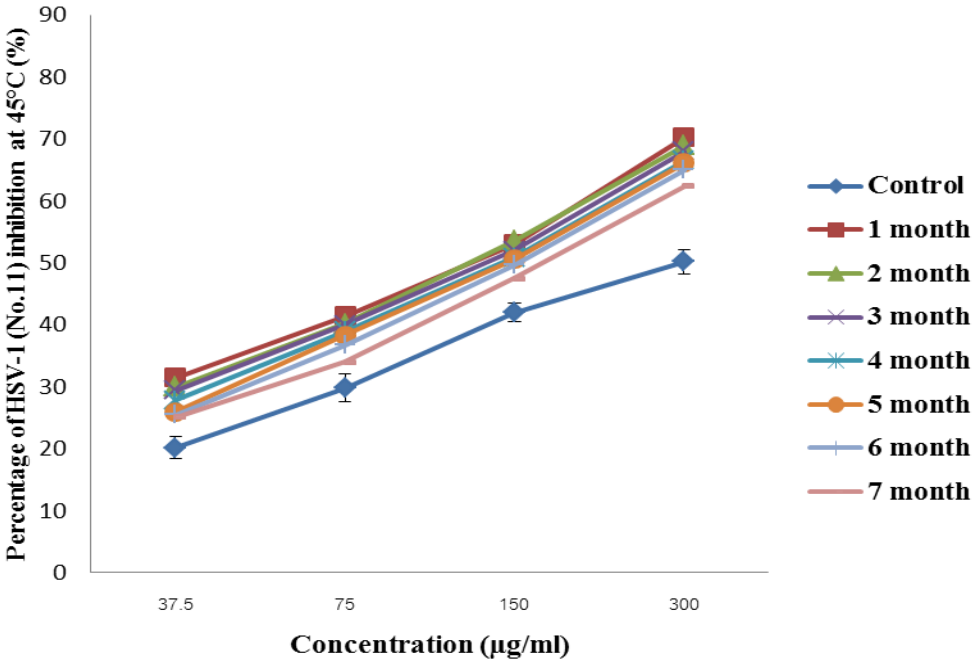


Figure 4.38 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.11) after storage at 45ºC for 7 months

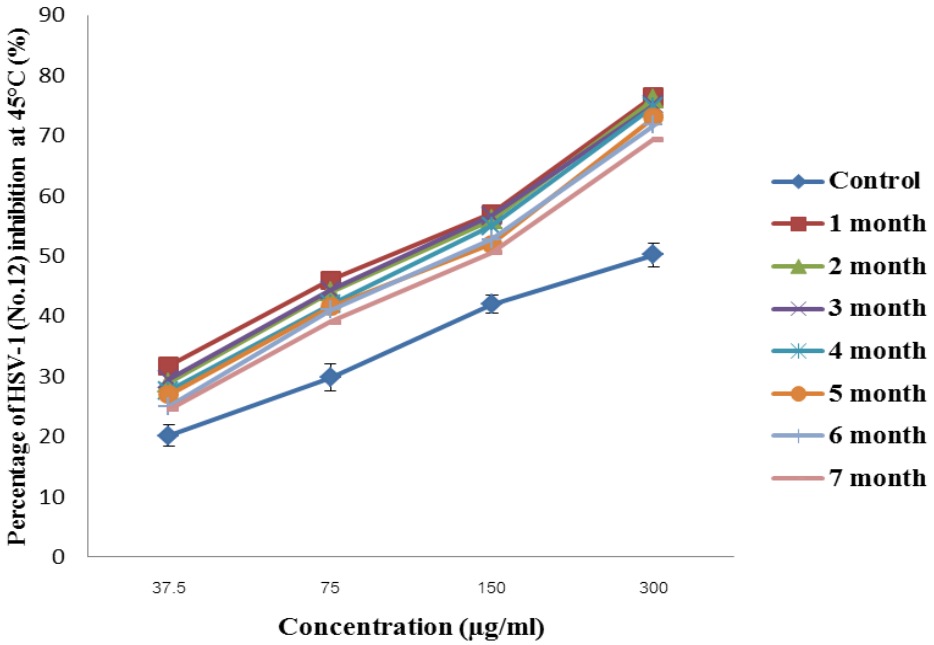


Figure 4.39 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.12) after storage at 45ºC for 7 months

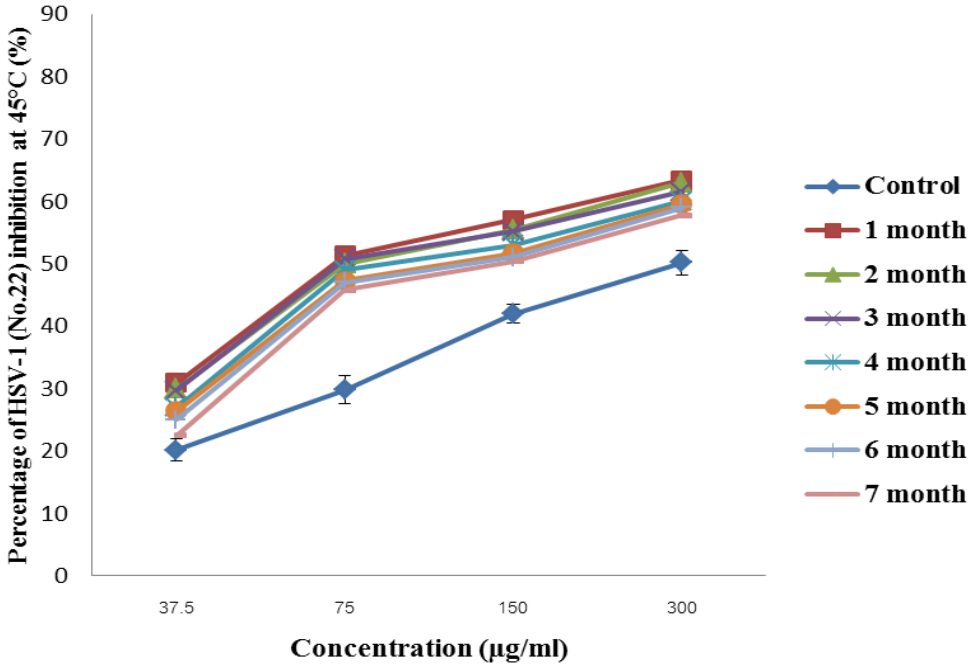


Figure 4.40 Inhibitory effect of algal gel containing ethanolic extract of *Spirogyra* spp. against HSV-1 (No.22) after storage at 45ºC for 7 months

Plaque reduction assay was used to confirm the efficacy of algal gel against HSV infection. After the algal gel was kept at 45ºC for 7 months, it was found that the algal gel also retained their inhibitory efficacy on HSV infection, which was similar to the antiviral activity when storage at 4 and 25ºC. Although, the physical properties of algal gel product was changed when kept at various condition. However, it still retained their antiviral effect against both types of HSV and ACV-resistant HSV-1 infection. Hence, this preliminary study on algal gel product contained ethanolic extract of *Spirogyra* spp. should be further developing the stability of this algal gel.