CHAPTER 5

Conclusion

In this study, the highest percentage yield of this algal extract was obtained from methanolic extract (ME) of *Spirogyra* spp., which was 23.52%. The algal extracts were also determined for their cytotoxicity on Vero cells. The result revealed that the aqueous extract (AE) of *Spirogyra* spp. showed the lowest cytotoxicity with CD_{50} value of 4,363.30 µg/ml, while the ME extract of *Spirogyra* spp. revealed the highest cytotoxicity in Vero cells with CD_{50} value of 250.80 µg/ml. Furthermore, HSV-1F, HSV-2G and ACV-resistant HSV-1 isolates were treated with the highest non-toxic concentration of *Spirogyra* spp. extracts to determine the potential anti-HSV activity. From plaque reduction assay, the result exhibited that the AE extract of *Spirogyra* spp. could inhibit plaque formation of HSV-1F or HSV-2G more than 40% when treated before viral attachment to Vero cell. On the other hand, the ME extract of *Spirogyra* spp. had the highest inhibitory effect on ACV-resistant HSV-1 by 66.50%. From our results, the high anti-HSV activity of the ME and ethanolic (EE) extract of *Spirogyra* spp. during viral attachment to Vero cell were 96.25 and 94.52 % inhibition, respectively. The high inhibitory effect of ME extract on ACV-resistant HSV-1 of 52.68 - 75.94% was also observed. In addition, the highest inhibitory effect on HSV infection was shown by the EE extract of *Spirogyra* spp. by 52.24% when treatment after viral attachment to cell culture. The EE extract of *Spirogyra* spp. could also inhibit plaque formation of ACV-resistant HSV-1 isolates more than 50%. Thus, the *Spirogyra* spp. extracts which showed strong effective inhibition of HSV infection were further evaluated for anti-HSV infection on various stages of HSV multiplication cycles.

The result revealed that the AE extract of *Spirogyra* spp. extract exerted the highest inhibitory effect against the standard HSV-2G and HSV-1F infection with ED_{50} values of 271.40±0.80 and TI values of 16.08±0.65 when treatment before viral attachment. On the other hand, the ME extract of *Spirogyra* spp. showed the highest anti-viral activity against ACV-resistant HSV-1 infection with ED_{50} values of 85.14±0.50 µg/ml and TI values of 2.95±0.61 when treatment before viral attachment.

Moreover the ethanolic extract of *Spirogyra* spp. had the highest efficacy to inhibit HSV-1F infection with ED_{50} values of 164.20±1.26 µg/ml and TI values of 2.17±0.85 when treatment during viral attachment on Vero cell. While as, HSV-2G infection were potential inhibited by ME extract of *Spirogyra* spp. with ED_{50} values of 75.03±1.12 µg/ml and TI values of 3.34±0.90 when treatment during viral attachment on cell culture. The ME extract of *Spirogyra* spp. also showed the high inhibitory effect on ACV-resistant HSV-1 infection with ED_{50} values of 70.45±0.80 µg/ml and TI values of 3.56±0.72 when treatment during viral attachment on Vero cell. The highest efficacy of EE extract on HSV-1F and ACV-resistant HSV-1 infection was exhibited that ED_{50} values of 166.40±0.70 and 128.18±1.00 µg/ml when treatment after viral attachment. Nevertheless, the AE extract showed the highest efficacy to inhibit standard HSV-2G infection with ED_{50} values of 621.58±0.80 µg/ml and TI values of 7.02±0.71 when treatment after viral attachment. In summary, HSV-1F and ACV-resistant HSV-1 isolates were inhibited by the EE extract better than HSV-2G when treatment during and after viral attachment. However, the AE extract had high inhibitory effect on HSV-1F and HSV-2G when treatment before viral attachment.

Furthermore, direct inactivation of HSV-1F, HSV-2G and ACV-resistant HSV-1 particles by algal extracts was performed. The result showed that the ability of EE and ME extract on inactivation of HSV-1F and HSV-2G particle was better than AE extract. HSV-2G was the highest inactivated 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. The potential ability of extract on direct inactivation of ACV-resistant HSV-1 strains was significantly increased by the time. The results showed that the HSV-2G, HSV-1F and ACV-resistant HSV-1 isolate No.1A, 1B, 11, 12 and 22 titer were observed at 4 hours after treatment with ME extract at concentration of 500 µg/ml with significantly reduction by 7.62±0.11, 5.97±0.03, 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.

The efficacy of algal extracts on viral multiplication cycle was also demonstrated. The results found that the titers of HSV-1F and ACV-resistant HSV-1 isolate No.1A, 1B, 11, 12 and 22 titer were 5.36±0.20, 4.00±0.60, 4.40±0.50, 4.78±0.20, 4.91±0.20 and 5.01±0.50 log PFU/ml, respectively, compared to viral control after treatment with EE extract of *Spirogyra* spp. at 36 hours. While, yields of HSV-2G were

the highest reduction after treatment with AE extract to 2.63±0.10 log PFU/ml at 36 hours.

From the results of multiplication cycle of HSV infection, the EE and AE extracts showed the strongest anti-HSV-1 and anti-HSV-2 activity, respectively. Then, EE and AE extracts were selected to investigate their algal bioactive compounds that affected HSV infection. The results found that HSV-1F, HSV-2G and ACV-resistant HSV-1 isolates were sensitive to EE01 and EE02 fractions isolated from EE extract wih percentage of inhibition ranged from 42.51- 98.03%. While, AE01, AE02 and AE03 showed low effect on HSV-1F, HSV-2G and ACV-resistant HSV-1 Infection, which inhibited by 30.60- 49.65%.

Crude ethanolic extract of *Spirogyra* spp. were selected to further elucidate the major phytochemical constituents profile by phytochemical analysis. The EE01 and EE02 fractions were composed of alkaloid, essential oil and terpenoid.

In this present study, the inhibition of viral DNA synthesis was also evaluated. The results found that EE extract of *Spirogyra* spp. showed the potential inhibitory effect on HSV-1F and ACV-resistant HSV-1 isolate No. 1A, 1B, 11, 12 and 22 which observed by the lowest percentage of DNA remaining by 81.24±1.49, 75.16±2.49, 70.95±1.64, 62.13±3.88, 69.81±3.43 and 58.14±2.26, respectively. The effect of AE extract of *Spirogyra* spp. on HSV-2G DNA synthesis showed the highest percentage of DNA remaining by 84.36±2.85.

In addition, the results showed that the natural active substances of *Spirogyra spp.*extract showed high efficiency to inhibit DNA of HSV-1F, HSV-2G and ACV-resistant HSV-1 isolates. 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 EE extract by 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 AE extract was 18.86±3.79. In summary, the mechanism of action of HSV DNA polymerase after treatment with the algal extracts was decreased when compare with HSV DNA control.

In this study, the validation of three extraction buffers for the protein extraction of HSV infection on Vero cell was evaluated. The result of 1-DE and 2-DE

examination showed that the CHAP-Urea-Thiourea buffer was appropriated to extract global proteins with high reproducibility and reliability. Furthermore, the efficacy on inhibition of HSV protein synthesis was examined in the presence or absence of *Spirogyra* spp. extract. The result suggested that this algal extracts could inhibit various viral proteins. Especially, the EE extract of *Spirogyra* spp. had potential to inhibit HSV-1F protein synthesis. Additionally, AE extract of *Spirogyra* spp. had efficacy to inhibit HSV-2G protein synthesis.

After 2-DE analysis, the reduction of HSV proteins was observed after treatment of infected cells with *Spirogyra* spp. extracts. The protein spots decreased in *Spirogyra* spp. extract treated cells, which was revealed in a range of 545-1638 protein spots. It was found that the marked areas of separated proteins spots in 2-DE gels were preferably in the acidic pI, pH 4-5 and molecular weight of 30-97 kDa. Moreover, the identification and expression of HSV-1F and HSV-2G proteins were evaluated. The results of the HSV-1F and HSV-1F infected cell after treatment with EE extract of *Spirogyra* spp. revealed nineteen differentially expressed protein spots. There were thirteen spots that indicated the down-regulation of protein expression in treated condition. While, six spots of proteins were highly up-regulated. However, the comparison of protein expression of HSV-2G infected cell and HSV-2G treated cell after treatment with AE extract of *Spirogyra* spp., reported twenty-six differentially expressed protein spots. There were fifteen spots that revealed down-regulation in treated condition and eleven spots were highly up-regulated expressed proteins. Then, all of the forty-five protein spots were chosen for protein identification by ESI-QUAD-ToF MS analysis.

Interestingly, the identification of differentially expressed HSV-1F and HSV-2G protein spots were analyzed by Mascot database programe. The results of highly up-regulated expressed proteins of HSV-1F infected cell after treatment with the EE extract of *Spirogyra* spp. extract were reported. The up-regulated nesprin-1 protein, Interleukin-25, GATA-type zinc finger protein 1, Protein X and protein FAM162B were identified by ESI-QUAD-ToF MS analysis. In addition, the results from Mascot analyzed the down-regulation proteins of HSV-1F infected cell after treatment with the EE extract, which included the RPGR-interacting protein 1-like protein, FAM162B

protein, Interleukin-25, F-box domain, neogenin-like partial protein and fusion protein. Nevertheless, the results of up-regulated expression of HSV-2G infected cell after treatment with AE extract of *Spirogyra* spp. were revealed the nesprin-1 protein, regulatory protein E1, protein VP3, major tail sheath protein, tegument protein UL47 homolog, nucleoprotein and protein FAM162B. While, the down-regulation of proteins in HSV-2G infected cell after treatment with AE extract were identified. The regulatory protein E1, nesprin-1 protein, Interleukin-25, DNA packaging tegument protein UL25, gene 9 protein, FAM162B protein, neogenin-like protein, nuclear transcription factor subunit gamma, large delta antigen, virion-packaging protein UL17, apoptosis-stimulating of p53 protein, polyprotein, mitoferrin-2, major tail sheath protein and spike protein were shown. Then, all of the identified proteins were selected for analysis of protein-protein interactions (PPIs) by String database.

This present study analyzed the protein-protein interactions of two conditions included the HSV-1F infected cells and HSV-1F infected cell after treatment with EE extract. HSV-2G infected cells and HSV-2G infected cell after treatment with AE extract of *Spirogyra* spp. The String network database reported the association of functional proteins and analysis of protein-protein interaction. The results of protein interaction between HSV-1F and host cell proteins after treatment with EE extract were represented the mechanism of Nesprin-1 protein, which up-regulated expression, but the integrin beta-2 and E3 ubiquitin were down-regulated expression. The results of protein-protein interactions (PPIs) suggested that the functional mechanism of global proteins were involved of the nuclear organization and structural integrity of HSV-1F protein changed after treatment with EE extract. Therefore, the connection to the cytoskeleton and F-actin which located in cytoplasm was interacted with the nuclear envelope. The bioactive compounds of EE extract inhibited HSV-1F protein expression via the protein pathway of host cell. Then, HSV-1F could not replicate and assembly to new virion. Additionally, the results of PPIs between HSV-2G and host cell protein after treatment with AE extract were analyzed. The Nesprin-1 protein was up-regulated, but the nuclear transcription factor subunit gamma, regulatory protein E1 and E3 ubiquitin were down-regulated. The network database of PPIs revealed the functional mechanism of global proteins including the regulation of cell cycle and intracellular

non-membrane bounded organelle of HSV-2G infected cell after treated with AE extract. The PPIs showed the necessary expression of activating and regulatory enzyme subunit for cell cycle progression. Thus, HSV-2G replication and protein expression was inhibited by the AE extract of *Spirogyra* spp.

Therefore, this present study suggested that HSV-1F and HSV-2G infectivity on Vero cells was inhibited when treatment with algal extracts during and after viral attachment on host cell by interfering or blocking the viral adsorption and viral entry across the cell membrane. HSV DNA replication and viral protein synthesis also inhibited after treatment with *Spirogyra* spp. extracts.

Moreover, the EE extract of *Spirogyra* spp. showed the highest efficacy against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection. Thus, gel contained crude ethanolic extract of *Spirogyra* spp. was developed. Hence, stability test of algal gel product was also investigated in this study. The result revealed that, the physical properties of algal gel was changed when storage at 4, 25 and 45ºC for 7 months. However, the algal gel product also retained high efficacy of anti-HSV activity. Moreover, the algal gel also showed strong inhibitory effect on HSV infection after heating-cooling at 4ºC for 48 hours and changed to 45ºC for 6 cycles. Furthermore, satisfaction surveys and skin irritation were examined on human volunteer. The results presented the report from 30 surveys that received from eighteen patients and twelve volunteer, which showed mostly satisfied with the physical, silky texture of algal gel without staining on skin. The result also confirmed that algal gel did not generate any irritation on skin.

This algal gel product containing EE extract of *Spirogyra* spp. demonstrated high antiviral activity against HSV-1F, HSV-2G and ACV-resistant HSV-1 infection and it also did not irritate to skin. Furthermore, this result suggested the potential efficacy of this algal gel for development as an alternative therapeutic anti-HSV agent. However, anti-viral activity should be confirmed *in vivo* in further study.

In conclusion, these report supported various usefulness and application of freshwater green macroalgae from Northern of Thailand as a valuable source for development of potential, inexpensive and safe therapeutic antiviral agent herpes simplex virus.