CHAPTER 3

Materials and Methods

3.1 Materials

Table 3.1 Chemical and reagents

Chemical and reagents	Company
3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)	Bio Basic Inc., Canada
AccuGel (29:1)	National diagnostics, USA
Acetone	Lobal Chemic, India
Acrylamide	Bio Basic Inc., Canada
Agarose	Merck, Germany
Ammonium persulfate (APS)	Bio Basic Inc., Canada
Bovine calf serum	JR Scientific Inc., USA
Bovine serum albumin (BSA)	Sigma, USA
Bromophenol blue	BDH, UK
Cocktail protease inhibitor set III, animal free	Calbiochem®, USA
Coomassie Brilliant Blue G-250	Bio Basic Inc., Canada
Coomassie Brilliant Blue R-250	Bio Basic Inc., Canada
Crystal violet	Merck, Germany
Dichloromethane	Lobal Chemic, India
Dithiothreitol (DTT)	Bio Basic Inc., Canada
Dimethyl sulfoxide (DMSO)	Sigma, USA
(3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT)	Sigma, USA
Dry strip cover fluid	GE Healthcare, UK
Ethanol	Merck, Germany
Ethylacetate	Lobal Chemic, India

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Table 3.1 (continued)

Chemical and reagents	Company
Ethylenediamine (*N, N, N′, N′*) tetraacetic acid (EDTA)	Scharlau, Spain
Fetal bovine serum	Hyclone®, USA
Glacial acetic acid	QreC^{tm}, New Zealand
Glutaraldehyde	Sigma, USA
Glycine	Research organics, USA
Glycerol (87%)	QreC^{tm}, New Zealand
IPG buffer, pH 3-10 NL	GE Healthcare, UK
IPG buffer, pH 4-7	GE Healthcare, UK
Iodoacetamide (IAA)	GE Healthcare, UK
Low molecular weight protein marker	GE Healthcare, UK
Methanol	Merck, Germany
Minimum essential medium (MEM)	Hyclone®, USA
85% phosphoric acid	Scharlan, Spain
N, N′-methylene bisacrylamide	Bio Basic Inc., Canada
N, N, N′, N′-tetramethylethylenediamine (TEMED)	Bio Basic Inc., Canada
Penicillin / Streptomycin	Gibco®, USA
Phenylmethylsulfonyl fluoride (PMSF)	Bio Basic Inc., Canada
Silica gel type 100 (70-230 mesh ASTM)	Merck, Germany
Silica gel 60 GF254	Merck, Germany
Sodium acetate	Carlo Erba, France
Sodium bicarbonate	Merck, Germany
Sodium chloride	LobaChemi, India
Sodium dodecyl sulfate (SDS)	GE Healthcare, UK
Sodium thiosulfate	Carlo Erba, France
Shaker (platform)	Major Science, Taiwan
Thiourea	Carlo Erba, France
TLC Aluminium sheet (TLC siliga gel / 60F 254)	Merck, Germany
Trypsin-EDTA (0.5%)	Gibco®, USA

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Table 3.2 Instruments

Instrument	Company
Urea	AppliChem GmbH, Germany
Autoclave	Sanyo, Japan
Biosafety cabinets class II	ESCO®, USA
Cell culture flask, 25 cm^2	CORNING®, USA
Cell culture flask, 75 cm^2	Cellstar®, Germany
Cell culture plate 24 wells	NUNC^{tm}, Denmark
Centrifuge	Beckman Coulter, USA
Refrigerated centrifuge	Hettich, USA
Centrifuge tube	Vivantis, UK
CO_2 incubator	Shellab, USA
Compound microscope	Olympus, USA
Electrophoresis apparatus	GE Healthcare, UK
Ettan IPG Strip holder 13 cm	GE Healthcare, UK
Freezer -20°C	Whirlpool, USA
Freezer -80°C	New Brunswick Scientific, UK
Immobilized (IPG) strip pH 4-7 linear	GE Healthcare, UK
Immobilized (IPG) strip pH 3-10 nonlinear (NL)	GE Healthcare, UK
Lyophylizer	FTS system, USA
Microcentrifuge	Eppendrof, Germany
Microcentrifuge tube	Lab connection, Thailand
Micropipette	PZ HTL S.A., Poland
Microplate reader	Biotek, UK
Microscope	Olimpus optical, Japan
pH meter	Precisa, Switzerland
Pipette tips	Lab Connection, Thailand
Power supply	Bio Rad, UK
Rotary evaporator	BUCHI, Japan
Spectrophotometer	Thermo Fisher Scientific, USA
Vortex mixer	Scientific Industries Inc., Thailand

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3.1.1 Cells and Viruses

Vero cells (African monkey kidney cell) were supported from Division of Microbiology, Department of Medical Technology, Faculty of Associated Medical Science, Chiang Mai University. The standard herpes simplex virus type 1 (strain 1F) and herpes simplex virus type 2 (strain 2G) was obtained from Division of Microbiology, Department of Medical Technology, Faculty of Associated Medical Science, Chiang Mai University.

3.2 Methods

3.2.1 Extraction of *Spirogyra* spp.

Dry powders of algae (250 grams) which were collected from the natural pond at Na Ku Ha Village, Suankhun district, Amphoe Muang, Phrae province, were macerated with 4 liter of 95% ethanol for 3 days at room temperature or soaked with distilled water at 45ºC in water bath for 3 hours. Then, the suspension of algal extracts was filtered through filter paper Whatman No.1 (Whatman, USA). The filtrate was concentrated by rotary evaporator to remove the solvent and dried by lyophilizer to obtain crude extracts. Next, the dried extract was reconstituted by dimethylsulfoxide (DMSO, Labscan, Ireland) and stored in amber glass bottle at 4°C before investigation of anti-HSV activity.

3.2.2 Anti-herpes simplex virus assay

3.2.2.1 Cultivation of Vero cells

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Vero cells were used throughout this study for propagation of herpes simplex virus and investigation of anti-HSV activity. The cells were obtained from division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Vero cell were cultured as monolayer in Eagle's minimum essential medium; MEM (Gibco, UK) and supplemented with penicillin/streptomycin (PAA, Austria). Heat inactivated fetal bovine serum, 10% (Hyclone, UK) was added before use. The cells were cultured until confluence at 37ºC in humidified 5% CO_2 atmosphere incubator. Vero cells were subcultured every 2-4 days in Biosafety Cabinet Class II. The cells were washed twice

with PBS (1X) and detached with trypsin-ethylenediamine tetra-acetic acid (Trypsin-EDTA, 0.05%, PAA, Austria). Trypsin-EDTA was removed and growth medium was added. The cells were transferred into new flask with spilt ratio of 1:3 and further incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days.

3.2.2.2 Propagation of herpes simplex viruses

Standard strains of herpes simplex virus (HSV) type 1 strain F and type 2 strain G (HSV-1F and HSV-2G) were used throughout this study. HSV-1F and HSV-2G were obtained from Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University. Clinical ACV-resistant HSV-1 isolates 1A, 1B, 11, 12 and 22 were also used. The virus at multiplicity of infection (MOI) of 1.0 was propagated on monolayer of Vero Cells in 25 cm^2 flask after adsorption at room temperature for 1 hour. After adsorption period, the inoculum was removed by washing twice with PBS (1X). Then, maintenance medium supplemented with 2% heat inactivated fetal bovine serum was added. The infected cells were cultured at 37ºC in humidified 5% CO_2 atmosphere incubator until 80-90% of cytopathic effect (CPE) of the cells was observed. The infected cells were collected and virus stock was prepared by freezing and thawing of infected cells twice.

3.2.2.3 Plaque titration assay

HSV viral stock was serially ten-fold diluted in MEM and each dilution of HSV was added into the confluence of Vero cells in 24-well tissue culture plate. After viral adsorption for 1 hour, the overlay media containing growth medium and 1.5% sodium carboxymethyl cellulose were added to each well. After incubation at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes for observation of toxicity on Vero cell. Then, plaques were counted and viral titers were expressed as plaque forming unit/ml (PFU/ml).

3.2.2.4 Cell cytotoxicity

Cytotoxicity of *Spirogyra* spp. extracts was investigated on Vero cells. The extracts were serially two-fold diluted with MEM and each concentration of the

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extracts was added to quadruplet wells onto 96-well tissue culture plate. Then, Vero cells at concentration of 1×10^5 cells/ml were added to each well and incubated for 24 hours at 37ºC in humidified 5% CO_2 atmosphere incubator. After that, the algal extracts at different concentration were added to each well and incubation was carried out at 37°C for 48 hours. Then, 15 µl of MTT (5 mg/ml) was added to each well and incubated at 37°C in 5% CO_2 incubator for 4 hours. Finally, DMSO was added in each well to form MTT-formazan product. Determination of cell viability was performed using ELISA reader by measuring the absorbance at 540 nm with reference wavelength at 630 nm.

3.2.2.5 Plaque reduction assay

Vero cells were grown as monolayer in 24-well tissue culture plates. Each well was infected with 50-100 PFU of HSV and incubated on rocking platform for 1 hour at room temperature for viral adsorption to the cell. Then, *Spirogyra* spp. extracts or fractions at non-toxic concentrations and ACV at 50% effective dose (ED_{50}) were added to the cells and overlaid with overlay medium. After incubation for 3-4 days at 37ºC in humidified 5% CO_2 atmosphere incubator, the extracts were removed and washed once by PBS (1X). Then, the cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. The number of plaques was counted comparing with untreated virus control and expressed as plaque forming unit per ml (PFU/ml). 50% effective dose (ED_{50}) was expressed as 50% inhibition of plaque formation and calculated by dose-response curves.

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3.2.2.6 Acyclovir

Acyclovir (ACV, Vilerm), 0.10 g was dissolved in 1 ml of sterile deionized water and stored in -20°C before use. ACV was diluted in MEM and tested against both types of HSV. ACV at 50% effective dose (ED_{50}), which was the dose that inhibited HSV infection by 50% was determined and selected as positive control when investigation of anti-HSV activity.

3.2.2.7 Mechanism of *Spirogyra* spp. extracts on HSV inhibition

(1) Inactivation kinetic

Inactivation kinetic of the *Spirogyra* spp. extracts on HSV particles was investigated. HSV was treated with the highest non-toxic concentration of algal extracts. The mixture of *Spirogyra* spp. extracts was incubated at room temperature for 60, 120, 180 and 240 minutes.

The mixture was collected at the end of each incubation period and kept at -80ºC. Titers of residual virus after treatment with *Spirogyra* spp. extracts were determined by plaque titration assay and the percentage of viral particles inhibition by algal extracts was calculated and compared to untreated virus control.

(2) Effect of *Spirogyra* spp. extracts on HSV before viral attachment

Effect of *Spirogyra* spp. extracts on viral attachment to cellular receptor was evaluated. Various non-toxic concentrations of *Spirogyra* spp. extracts were added into a confluence of cell monolayer in 24-well tissue culture plate and incubated at room temperature for 1 hour. After that, the extracts were removed and the cells were washed with PBS (1X) once. Then, 50-100 PFU of virus was added and overlaid with overlay media. The inoculum was incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days. After incubation, the overlay media were discarded and plaques were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. Percentage of HSV inhibition was determined by calculation of plaque number after treatment with algal extracts comparing with antiviral agent, ACV and virus control. ED_{50} values of algal extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

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(3) Effect of *Spirogyra* spp. extracts on HSV during viral attachment

Effect of *Spirogyra* spp. extracts on viral attachment to cell culture was investigated. The *Spirogyra* spp. extracts at various non-toxic concentrations and 100 plaques/0.1 ml of HSV were added into monolayer of Vero cell in 24-well tissue culture plates. Then, the virus was adsorbed at room temperature for 1 hour on rocking platform. After that, the inoculum was removed before overlaid with overlay medium and incubated at 37ºC in humidified 5% CO_2 atmosphere incubator for 3-4 days. After incubation period, the numbers of plaques were counted after stained with 0.1% crystal

violet in 1% ethanol for 20 minutes and percentage of inhibition was determined by calculation of plaque number compared to untreated viral infected cells control. ED_{50} values of algal extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

(4) Effect of *Spirogyra* spp. extracts on HSV after viral attachment

Vero cells were cultured as monolayer at 37ºC in humidified 5% CO_2 atmosphere incubator in 24-well tissue culture plates. Then, 100 plaques/0.1 ml of HSV were added for 1 hour at room temperature on rocking platform. After that the cells were treated with various concentrations of *Spirogyra* spp. extracts and overlaid with overlay medium before incubation for 3-4 days at 37ºC in humidified 5% CO_2 atmosphere incubator. Then, media was discarded and infected cells were stained with 0.1% crystal violet in 1% ethanol for 20 minutes. Percentage of inhibition was determined by reduction of plaque number comparing to untreated virus control. ED_{50} values of *Spirogyra* spp. extracts on HSV inhibition were also calculated by dose-response curves and expressed as concentration that plaque formation was inhibited by 50%.

(5) Effect of *Spirogyra* spp. extracts on HSV multiplication

Effect of *Spirogyra* spp. extracts on viral multiplication was determined. Vero cells were grown as monolayer in 6 well tissue culture plates. The cells were infected with $1x10^6$ PFU/ml of HSV for 1 hour at room temperature on rocking platform. After that, the inoculum was removed and unattached virus was washed twice by PBS (1X). Then, the highest nontoxic concentrations of algal extracts were added to each well and incubated at 37ºC in 5% humidified CO_2 atmosphere incubator. ACV at ED_{50} concentration and MEM were used as positive and negative control, respectively. Infected cells were collected at 0, 6, 24, 30 and 36 hours after viral infection. Moreover, *Spirogyra* spp. extracts that showed the strong inhibition of HSV multiplication were selected and used at lower concentration to compare inhibitory activity against HSV multiplication cycle. After that the cells were frozen and thawed

twice and virus in supernatant was kept at -80ºC before determination of virus titers by plaque titration assay.

3.2.3 Separation and partial purification of *Spirogyra* spp. extracts

Spirogyra spp. extracts that showed the highest anti-HSV activity were selected and separated by partition technique and subsequently further purified using column chromatography to identify the major secondary metabolites. The procedure of separation was as follows.

3.2.3.1 Separation of *Spirogyra* spp. extracts by partition technique

Crude extracts of *Spirogyra* spp. extracts, which showed the highest anti HSV activity were separated by partition technique. Different solvent based on polarity was used. Distilled water (56 ml) was initially added to 25.0 g of dried powder of extract before it was partitioned with 50 ml of hexane in separatory funnel four times to give the fraction. The residual of extracts was further partitioned by gradually enrichment with 50 ml of ethyl acetate four times to give the EtOAc fraction. Then, the supernatant were selected and further partitioned with dichloromethane fraction. Finally, each partition fractions were dried in high vacuum to obtain dry yield of four fractions and the fractions of extracts were further dissolved in DMSO before testing for their anti-HSV activity by plaque reduction assay.

3.2.3.2 Isolation of *Spirogyra* spp. extracts

The ethanolic and methanolic extract of *Spirogyra* spp. (100 g) was fractionated by column chromatography using silica gel as stationary phase. The silica gel was mixed with each *Spirogyra* spp. extract and packed in column and elution was started with eluted solvent; dichloromethane, EtOAc, 95% EtOH and methanol consecutively. Each 20- 25 ml fraction was collected and combined after visualization with TLC using ultraviolet light (254 and 366 nm). The isolation of *Spirogyra* spp. was performed.

After that, each fraction was dried in high vacuum and dissolved in DMSO before study the efficacy against HSV activity by plaque reduction assay.

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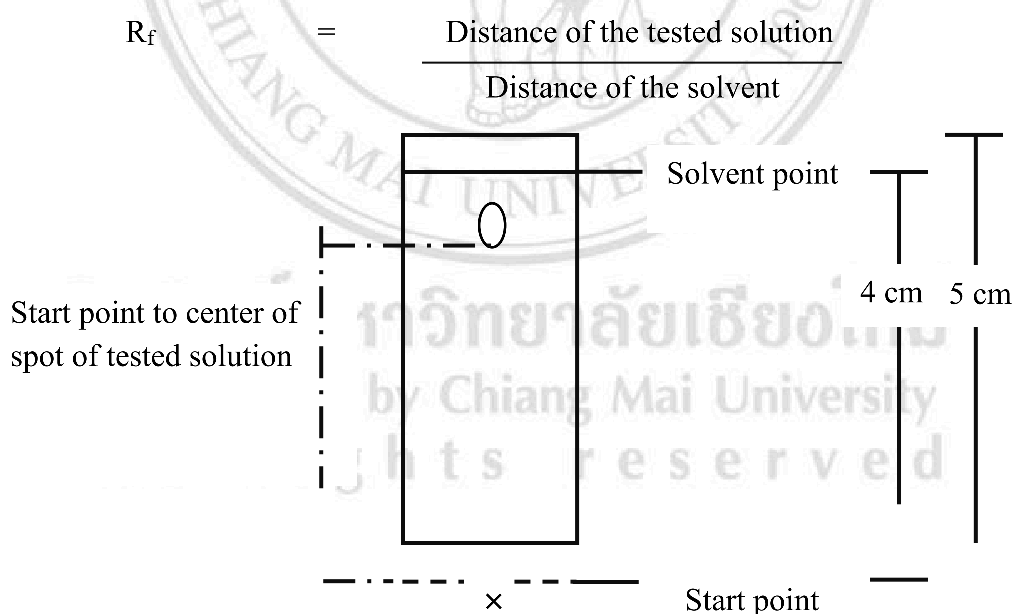
3.2.3.3 Thin layer chromatography (TLC)

Identification of phytochemical constituents was further carried out by TLC using pre-coated silica gel 60F 264 (Merck, Germany) as an adsorbent with layer thickness of 0.2 mm and 5 cm distance. First, a small spot of each sample solution was applied to a silica gel plate at starting point about 1 cm from the base and the plate was placed in to saturated solvent chamber system. Different compounds in the sample mixture could move at different rates due to differences in solubility in the solvent and stationary phase. Then, TLC plate was removed after the solvent moved to the solvent point and air-dried at room temperature. After that, the numbers of spots on the developed plates were observed under UV light at 365 nm. Spots were visualized by spraying with 2% anisaldehyde-sulfuric acid reagents to determine the different compounds following by heating in oven at 110ºC for 5-10 minutes. Colorful spots of positive control after spraying this reagent were varied depending on the compound observed. In addition, Rf values of each spot were calculated and measured as follows.

$$R_f = \frac{\text{Distance of the tested solution}}{\text{Distance of the solvent}}$$



3.2.3.4 Determination of phytochemical compounds in *Spirogyra* spp. extracts

Phytochemical compound in crude extracts and fractions of the algal were investigated in this study. The major compounds of the algal extracts were identified for secondary metabolite constituents such as alkaloids, antraquinones, lactone, essential oil, terpenoid, steroid, flavonoid, phenolics and tannins using reference phytochemical test.

(1) Detection of alkaloids by Dragendorff's reagent

Alkaloids were determined by dissolving 0.3 g of the powdered extracts in 15 ml of 2N hydrochloric acid (HCl) and incubated in water bath for 10 minutes. Then, the mixture was cooled and filtered using Whatman No. 1 filter paper. After that, 2 ml of filtrate was divided into 4 tubes and each individual tube was treated carefully with various alkaloid reagents.

A few drops of Mayer's reagent (Potassium Mercuric Iodine) were added to the filtrate of the first test tube. A turbid or white color of precipitate indicated the presence of alkaloids in the filtrate. An orange or reddish color of precipitate indicated the presence of alkaloids, was observe after adding a few drops of Dragendorff's reagent (Solution of Potassium Bismuth Iodide) to the filtrate of the second test tube. A few drops of Wagner's reagent (Iodine in Potassium Iodide) were added to the filtrate of the test tube. A brown or reddish color of precipitate indicated the presence of alkaloids. A few drops of Hager's reagent (Saturated picric acid solution) were added to the filtrate of the fourth test tube. A yellow color of precipitate indicated the presence of alkaloid

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(2) Detection of antraquinone by potassium hydroxide

Potassium hydroxide's reagent was used to identify antraquinone. Briefly, 0.2-0.5 g of the extract was mixed with 20 ml of KOH in the test tube and incubated for a few minutes on water bath for 5 minutes. Then, the layer was collected and 1N NaOH was added to the layer. Formation of rose-pink or red of alkaline layer indicated the presence of antraquinone.

(3) Detection of lactone by Kedde's test

3,5-Dinitrobenzoic acid was used to identify lactone. Briefly, 0.2-0.5 g of the extract was mixed with 10 ml of 3,5-Dinitrobenzoic acid in the test tube. Then, the layer was collected and 1N NaOH was added to the layer. Formation of rose-pink or violet of alkaline layer indicated the presence of lactone.

(4) Detection of essential oil by phosphomolydic acid

Phosphomolydic acid in 95% EtOH was used to identify essential oil. Briefly, 0.2-0.5 g of the extract was mixed with 10 ml of phosphomolydic acid in the test tube. Then, the layer was sprayed on the layer. Finally, the layers were incubated in 105°C before presence the formation of deep blue or black of alkaline layer indicated the positive of essential oil.

(5) Detection of terpenoid by sulfuric acid

Sulfuric acid in 5% EtOH was used to identify terpenoid. Briefly, 0.2-0.5 g of the extract was mixed with 10 ml of sulfuric acid solution in the test tube. Then, the layer was sprayed on the layer. Finally, the layers were incubated in 105°C before presence the formation of brown or red-violet of alkaline layer indicated the positive of terpenoid.

(6) Detection of flavonoid and tannins by ferric chloride

Ferric chloride solution was used to identify flavonoid and tannins. Briefly, 0.2-0.5 g of the extract was mixed with 10 ml of ferric chloride solution in the test tube and incubated for a few minutes on water bath for 5 minutes. The formation of blue-gray, gray and blak of alkaline layer indicated the positive of flavonoid and tannins.

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3.2.4 Effect of *Spirogyra* spp. extracts on HSV viral DNA

Viral DNA was extracted from infected cells after treatment with and without algal extracts. These cells were harvested and lysed in lysing solution containing 5M NaCl, 10 mg/ml proteinase K and 10 mg/ml RNAse and incubated at 37°C. After 1 hour, the suspension was centrifugated and proteins were separated by phenol-chloroform extraction method. Then, the supernatant was dissolved in distilled water. Isolated HSV DNA were determined by real time PCR comparing to control.

3.2.5 Sample collection of HSV infected cell for proteomics analysis

The culture medium was removed to 15 ml centrifuge tube, then 5 ml of ice cold 1X PBS was added. After that the cell monolayer was scraped with cell scraper. The mixture was poured in the 15 mL centrifuge tube and keep in ice box, 5 mL of ice cold 1X PBS was added. Next, cell suspension was centrifuged at 4°C, 1,200 rpm for 5 minutes and the supernatant was removed. The cell pellet was mixed with 1 mL of ice cold 1X PBS, then centrifuged at 4°C, 3,000 rpm for 10 minutes. Finally, the supernatant was removed, the cell pellet and culture medium were stored at -80°C until analysis.

3.2.5.1 HSV protein extraction for proteomics

The protein sample and cell culture were mixed with rehydration buffer (8 M urea, 2 M thiourea, 48 mM DTT, 4% (w/v) CHAPS, 10% isopropanol, and 5% isopropanol) for 50-200 µL. Then, the protein cocktail inhibitor (Calbiochem®, USA) was added and mixed gently before further analysis.

3.2.5.2 Determination of total protein concentration

Total protein concentration were measured following the method as described by Bradford assay. The protein samples was bound with Coomassie Brilliant Blue G250 dye (Bio Basic Inc., Canada). Under acidic condition, the dye is expressed in protonated red cationic form. After the proteins bind to the dye, they are converted to a stable unprotonated blue anionic form. The absorbance of the solution was measured at 595 nm. For a standard curve, BSA was used as a standard solution.

3.2.5.3 Analysis of protein pattern by SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique which is used for mornitor the protein pattern after total protein concentration was examined. 12.5% SDS was used and the apparatus were cleaned with 70% (v/v) ethanol. The polyacrylamide gels were prepared, which consisted of resolving gel solution and stacking gel solution.

(1) 12.5% SDS-PAGE preparation

The glass cassette were assembled. The resolving gel was prepared following the detail that illustrated in Table 3.3. The casting frame was placed into the casting stand. Then, the glass plate was marked at 1.5 cm below the comb teeth. Gel mixture were prepared by APS and TEMED were added. Then, the gel was

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prepared into the gel casting and surface of resolving gel was covered with cold 70% ethanol. The gel was allowed to polymerize for 30 minutes. Next, 70% ethanol was removed and stacking gel was prepared. The air bubbles should be avoid during gel preparation. The comb was put into the 4% stacking gel layer and stacking gel was allowed to polymerize at least 30 minutes. Finally, the casting stand was moved into 1X electrophoresis buffer and the comb was removed.

Table 3.3 The solution mixture for preparation of 12.5% SDS-PAGE

Type of gel solution	Chemicals	Volume (ml)
12.5% resolving gel solution	30.0% 29:1 (acrylamide:bisacrylamide)	4.17
	1.5 M Tris-HCl, pH 8.8	2.50
	Steriled DI water	3.12
	10.0% (w/v) SDS	0.10
	10.0% (w/v) APS	0.10
	TEMED	0.01
4.0% stacking gel solution	30.0% 29:1 (acrylamide:bisacrylamide)	1.33
	1.0 M Tris-HCl, pH 6.8	2.50
	Steriled DI water	6.01
	10.0% (w/v) SDS	0.10
	10.0% (w/v) APS	0.05
	TEMED	0.01

(2) Protein sample preparation and electrophoresis

Protein sample was prepared before separation in 12% SDS-PAGE, 15 μg of protein sample was mixed with 3X loading sample buffer in the volume of 1/3 protein sample in 1.5 mL of steriled microcentrifuge tube. Then, the sample was mixed gently and incubated at 95°C for 5 minutes and the sample was immediately stored in the ice. Next, the mixture was centrifuged at 13,000 rpm for 15 seconds to eliminate the steam on the lid of microcentrifuge tube. After that, 3 μL of low molecular weight protein marker and protein samples were added into each well of 12.5% SDS-PAGE gel. The power supply voltage was set at 80 volts for SDS-PAGE running, and stopped when the dye front reached the bottom of gel. Finally, the glass cassette was diassembled and gels were stained with Coomassie Brilliant Blue R-250.

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(3) Gel staining

The gel was stained with Coomassie Brilliant Blue R-250 until the band of proteins appeared within the clear background. Next, the gel was stored in DI water overnight and dried in cellophane membranes.

3.2.5.4 Two-dimensional gel electrophoresis (2-DE) analysis

The effect of *Spirogyra* spp. extract on the global protein of HSV infection cells was studied in this experiment. Protein samples were separated by IEF and used IPG strip (pH 3-10, nonlinear and pH 4-7) before running 12.5% SDS-PAGE.

(1) First dimension : Isoelectric focusing (IEF)

Isoelectric focusing (IEF) is the method for separate protein according their isoelectric point (pI). This study used 13 cm IPG strip, pH 3-10 nonlinear for secretomome analysis and pH 4-7 for intracellular protein analysis. The 250 µg protein sample was mixed with 2.0% IPG buffer and rehydration buffer. Next, the plastic cover of IPG strip was removed and IPG strip was embedded into the strip holder then the protein sample was loaded. After that, the surface of IPG strip was cover with Immobilined DryStrip Cover solution at the volume of 400 µL. Finally, the lid of strip holder was slided onto strip holder and the strip holder was put into Ettan IPGPhor II Manifold. The strip was allowed to rehydration step for 12 h until the end process by following program.

Prot#8 13 cm pH 3-10 NL / 13 cm pH 4-7

Rehydration 12:00 Hr @20°C

IEF @20°C 50µA/strip

2 strips 5 steps

S1	Step and hold	120V	200Vhr
S2	Step and hold	500V	500Vhr
S3	Gradient	1000V	800Vhr
S4	Gradient	8000V	11300Vhr
S5	Step and hold	8000V	4400Vhr

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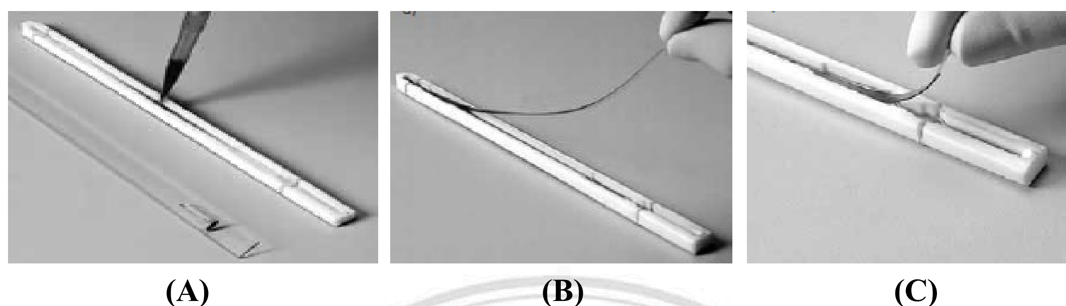
At the end of program running, IPG strip was removed from Ettan IPG Phor II and rinsed IPG strip with steriled DI water before run second dimension.



(A) **(B)** **(C)**

Figure 3.1 Sample loading and IPG strip preparation for IEF (A) Load sample into strip holder. (B) Place IPG strip onto strip holder and (C) Positioning the IPG strip, avoid air bubbles from cathode end to anode end.

(2) Second dimension: SDS-PAGE

The second dimension for protein sample separation was performed after IEF step by SE 600 Ruby (GE Healthcare). The glass cassette were assembled. The resolving gel was prepared following the detail that illustrated in Table 2.4. The casting frame was placed into the casting stand. Then, the glass plate was marked 5 mm below the top of glasses. After the gel mixture were added, APS and TEMED were added for degassed. Then, the surface of resolving gel was covered with 70% cold ethanol and allowed to gel polymerize for 30 minutes. Next, 70% ethanol was removed and the gel was stored in sterile water overnight at 4°C before use.

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(2.1) IPG strip equilibration

IPG strip was equilibrated before SDS-PAGE running. SDS equilibrium buffer was performed. Then, the equilibration buffer was divided to 2 tubes. Each tube, DTT and IAA were added into the equilibration buffer and adjusted the total volume to 5 mL at the final concentration 10 mg/mL and 25 mg/mL, respectively. Next, the equilibration buffer mixture with DTT was aliquoted to 2.5 mL in each tube. IPG strip was placed into the falcon tube with the equilibration buffer with DTT, sealed the falcon tube with paraflim and embedded the falcon tube to the rotating platform at 50 rpm for 15 minutes. After that, the IPG strip was transferred to the equilibration buffer with IAA, sealed the tube with parafilm and rotated the tube on the rotating platform at

50 rpm for 15 minutes. Finally, IPG strip was rinsed with steriled DI water before separation by SDS-PAGE.

(2.2) Electrophoresis

IPG strip was placed on the top surface of SDS-PAGE gel and avoided air bubble. After that, the 3x3 mm^2 of sterlied filter paper with 3 μL of low molecular weight protein marker was added at the space of anode end. Then, the top of SDS-PAGE was sealed with 1% (w/v) agarose sealing solution which containing bromophenol blue. Next, the SDS-PAGE running apparatus was moved into the SE 600 Ruby™ tank with the SDS-PAGE 1X running buffer and connected to the power supply. The electrophoresis program was set as described below.

Step	**Currents (mA/gel)**	**Approximate running duration (h : min)**
1	15	0 : 15
2	40	2 : 00 – 5 : 00

This step was run until the dye front appeared at the bottom of SDS-PAGE gel. Then, the gel was removed from the glasses and the gel was stained with Coomassie Brilliant Blue G-250.

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(2.3) Detection of the protein spots using Coomassie Brilliant Blue G-250

After SDS-PAGE electrophoresis, the proteins were separated and fixed in the fixation solution for 1 h and then the fixation solution was removed. Next, the gel was stained with the working solution of Coomassie Brilliant Blue G-250 for overnight. After that, the gel was destained with steriled DI water until the protein spot appeared on the clear background. Finally, the gel was scanned using Lab Scan 5 program.

3.2.5.5 Image gel analysis

(1) Work space creation

The ImageMaster 2D Platinum programe was operated and the window of ImageMaster was opened. Next, the workspace was created. After the work space was created, the project name and other details were set up. Next, the class or group of gel images was filled in the folder and then saved the data (Figure 3.2).

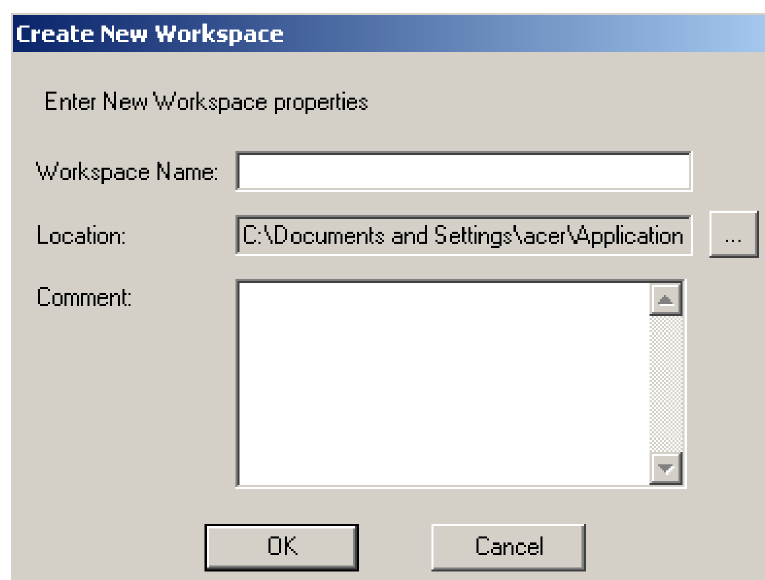

Figure 3.2 The new project creation.

(2) Contrast adjusting

The project was set to the active project and the gel images on the right table were opened. In addition, the gel images were adjusted the contrast by using adjust contrast mode.

(3) Spot detection

Before spot detection process, the detection parameters were set. The value of smooth, min area and saliency were set as 2, 5 and 1.0, respectively (Figure 3.3).

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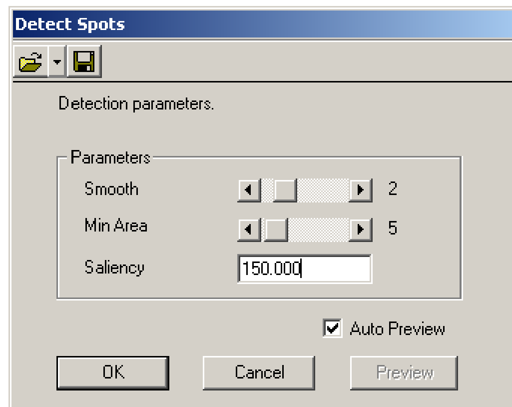

Figure 3.3 The spot detection parameter setting.

(4) Spot editing

After the process of auto spot detection was performed, the spots were edited such as split, grow, reduce and merge the protein spots (Figure 3.4).

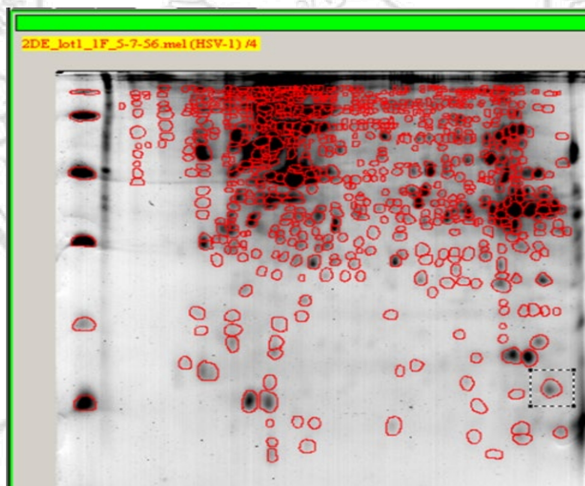

Figure 3.4 The spot editing parameter

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(5) Gel matching

In this step, the landmark was created and the reference gel was selected. Then, all of the gel images were matched using “Auto-Match gels” function in the menu bar. After that, the gel images were paired and the vecters was checked. If the mismatch pair of the protein spots were appeared, the mismatch point was edited by choosing the “Add” or “Delete” at the menu bar (Figure 3.5).

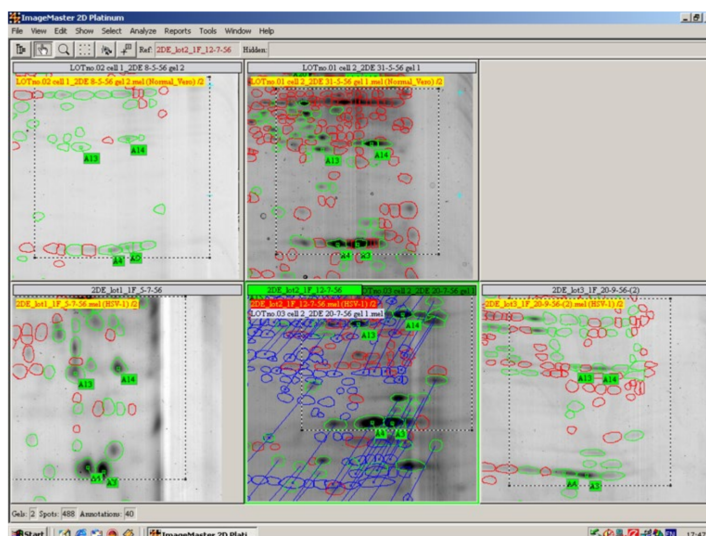

Figure 3.5 The landmark of protein spot setting and vector direction of the protein spots.

(6) Data analysis

In the process of data analysis, choosing " Analyze > Class report…" at the menu bar. Then, the window of class report was opened and choosing "Ratio" on the up-right of the window. After that, the data was analyzed by statistic analysis with student T test. Next, the spot ID was labeled and added color. The histogram of the protein spot was shown and 3D view of protein spots were displayed by click "3D view" (Figure 3.6).

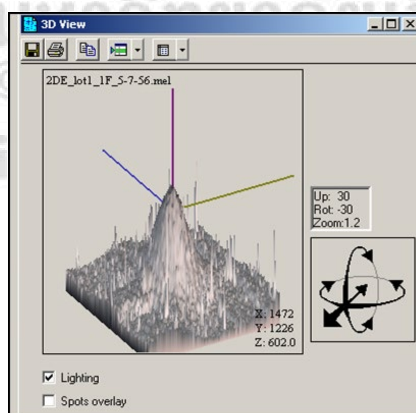

Figure 3.6 The 3D view of the protein spots and protein spots creation.

(7) pI and MW setting on gel

The pI and MW of protein spot was created by choosing “View > Grid Lines > Show…” at the menu bar. The pH and MW of protein spots were marked at the corner of the gel image. The information of pI and MW of protein spots were showed after click “Window > Information” and moved the curser to the protein spot (Figure 3.7).

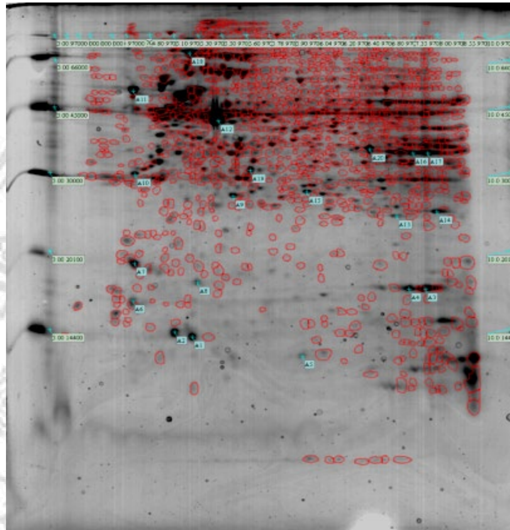

Figure 3.7 Setting of pI and MW.

3.2.5.6 MS analysis

Data analysis step of protein spot in 2-DE gel, the selected protein spots were picked up from 2-DE gels and storaged in steriled microcentrifuge tube with steriled water. The protein spot samples were analyzed with LC-MS/MS at Proteomics Research Laboratory, National Center for Genetic Engineering and Biotechnology (Biotec), Thailand. The protein samples were digested with trypsin and the peptide products were separated by high-performance liquid chromatography (HPLC). After that, the fractions of mixture were manipulated through mass spectrometer using electrospray ionization (ESI) technique and the ions were separated by hybrid analyzer or quadrupole-time-of-flight (Quad-ToF) analyzer. Finally, the ion was detected resulting in MS/MS data.

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3.2.5.7 Protein identification using peptide mass fingerprint data

The PMF or MS/MS Ion Search (STITCH) data, which was obtained from individual protein spot, was searched for its peptide sequence using Mascot program (available at http://www.matrixscience.com/). The databases from SwissProt and NCBInr were used for peptide sequence searching. The taxonomy using in this study were "*Primate*", "*Mammals*" and "*Viruses*". The fixed and variable modifications were set as "Carbamidomethyl (C)" and "Oxidation (M)", respectively. The mass values and peptide mass tolerance were "Monoisotopic" and "±1.2 Da". The results were reported in the scores of peptide match to database.

3.2.5.8 Protein interaction and associated identification

After data analysis was employed, the name of the interest proteins were manipulated through STRING database version 9.1 (http://string-db.org/) to search for protein interaction and association.

3.2.6 Development of algal gel product for treatment of HSV infection

Spirogyra spp. extract, which showed the highest anti-HSV activity was selected and used to prepare anti-viral gel product. The algal extracts at highest non-toxic concentration was added to gel base (Sangtongdee *et al*., 2006), which contained carbopol, methyl paraben, propylene glycol, tween 80, triethanolamine and distilled water. The contents were mixed thoroughly by homogenizer. After that, the algal gel was tested against standard HSV-1F and HSV-2G using plaque reducing assay.

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(1) Stability test of algal gel product (modified from Boonmachai, 2010)

The algal gel containing *Spirogyra* spp. extract was produced. Then, the algal gel was stored at different temperatures which were 4, 25 and 45ºC for 210 days. Moreover, the stability of algal gel and efficacy against HSV infection were determined every month for 7 months.

(1.1) Physical properties determination

The stability of gel and physical properties were observed such as the separation of layer, homogenization, color, smell, pH and intensity.

(1.2) Toxicity of herbal gel product

The toxicity of the algal gel product was performed on Vero cell.

(1.3) The efficiency of the algal gel product against HSV-1F and HSV-2G infection was investigated by plaque reduction assay.

(1.4) Stability of the algal gel product

Heating-cooling cycle of algal gel was performed. The algal gel was kept at 4ºC for 48 hours and then kept at 45ºC for 48 hours for 6 cycles and anti-HSV activity were tested by plaque reduction assay. Physical properties were also determined such as pH, viscosity, color and odor.

(1.5) The irritation test and satifaction of algal gel product on volunteers skin

Irritation test of algal gel product to the skin was tested in 30 healthy volunteers. The algal gel was applied on a surface area approximately was 1×1 cm^2 of lateral arms compared with deionized water. The gel was further washed to remove any remaining gel residue after 24 hours of the test. The tested skin was observed for the irritation and satification record.

3.2.7 Statistical analysis

All values were given as mean ± standard deviation (SD) from triplicate samples of three independent experiments for antiviral assay. Data values of cell viability assay was expressed as means ± SD from triplicate experiment. Different alphabets were considered statistically significant when $p < 0.05$.

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