CHAPTER III

MATERIALS AND METHODS

Materials

1. Chemicals and reagents

1.1. Acenaphthene, standard (Sigma, 99%)

1.2. Acetone, AR grade (Mallinckrodt Chemical)

1.3. Dichloromethane (Lab Scan)

1.4. Disposable syringe filter, Nylon 13 mm diameter, 0.45 µm (Chrom Tech)

1.5. Ethyl acetate, AR grade (Lab Scan)

1.6. Ethylenediaminetetraacetic acid, Disodium salt dihydrate (Fluka, 98%)

1.7. Ficoll® 400 (Sigma-Aldrich)

1.8. Glass microfiber filter GF/C, 47 mm diameter (Whatman)

1.9. Methanol, AR and HPLC grades (Lab Scan)

1.10. Potassium cyanide (Merck, 97%)

1.11. Potassium dihydrogen phosphate (Fluka, 99%)

1.12. Renieramycin M, deangeloylrenieramycin M, renieramycin N, and renieramycin O were obtained from the blue sponge *Xestospongia* sp. (Suwanborirux *et al.*, 2003; Amnuoypol *et al.*, 2004).

1.13. Sodium hydroxide, pellets (J.T. Baker, 99%)

1.14. Sodium sulfate, anhydrous (Merck, 98.4%)

1.15. Sodium chloride (Merck, 99.5%)

1.16. Trypan blue 0.4%

2. Instruments

2.1. Hot air oven (CONTHERM)

2.2. Inverted microscope (OLYMPUS, model IX 51/IX 71 and CK 30)

2.3. Refrigerated centrifuge (Hettich Zentrifugen, model EBA 12R)

2.4. Centrifuge swinging bucket (BECKMAN, model Allegra X-12R)

2.5. Centrifuge tubes, 15- and 50-mL graduated, screw -cap

2.6. Hemocytometer (BOECO Germany)

2.7. Hand refractometer (N.O.W)

2.8. pH meter (EUTECH)

2.9. Thermometer

2.10. Muffle furnace (Gallenkamp)

2.11. Rotary evaporator (BUCHI)

2.12. Scanning electron microscope (JEOL, model JSM-5410LV)

2.13. Critical point dryer (Balzer, model CPD 020)

2.14. Sputter coater (Balzer, model SCD 040)

2.15. High-Performance Liquid Chromatography (Shimadzu)

2.15.1. Pump : Solvent delivery system module LC-10ADvp

2.15.2. Autosampler : SIL-10ADvp

2.15.3. Degasser : DGU-14A

2.15.4. Detector : Diode-array detector SPD-M10Avp

2.15.5. Controller : System controller SCL-10Avp

2.15.6. Software : Class-VP

3. Sources of the blue sponge *Xestospongia* sp.

The marine sponge *Xestospongia* sp. #2133 was identified by Dr. John N.A. Hooper and the voucher specimens have been deposited at Queensland Museum (serial No. QM G306998), Australia and the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. The sponge *Xestospongia* sp. (Class: Demospongiae, Order: Haplosclerida, Family: Petrosiidae) is a bluish sponge occurring commonly in the coral reef in the Gulf of Thailand.

For this study, the initial sponge colonies were firstly taken from Ran-Dok-Mai Island (13°09′07.77″N, 100°50′03.19″E), a small Island nearby Sichang Island at Chonburi Province, on February 12, 2005 by SCUBA diving at the depth range of 3-5 meters, and then the collected sponges were kept in a cage and left in the natural condition at Sichang Island (13°09′10.77″N, 100°48′41.93″E) at the depth range of 3-5 meters for sample collection (Figures 3.1 and 3.2).

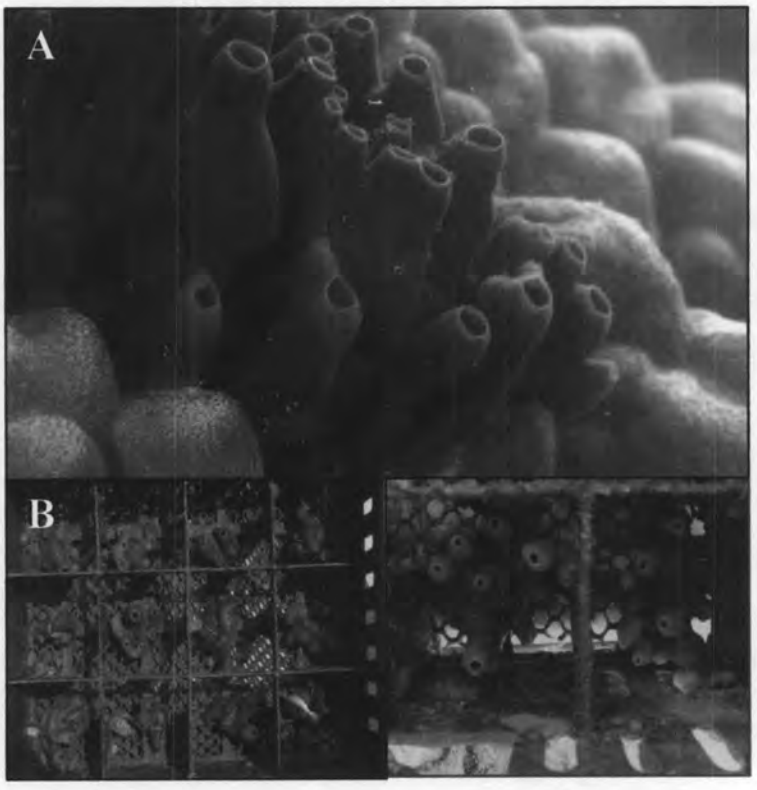


Figure 3.1. The blue sponge *Xestospongia* sp.: (A) on the coral in the Gulf of Thailand, and (B) in a cage at Sichang Island (photographs by Khanit Suwanborirux).

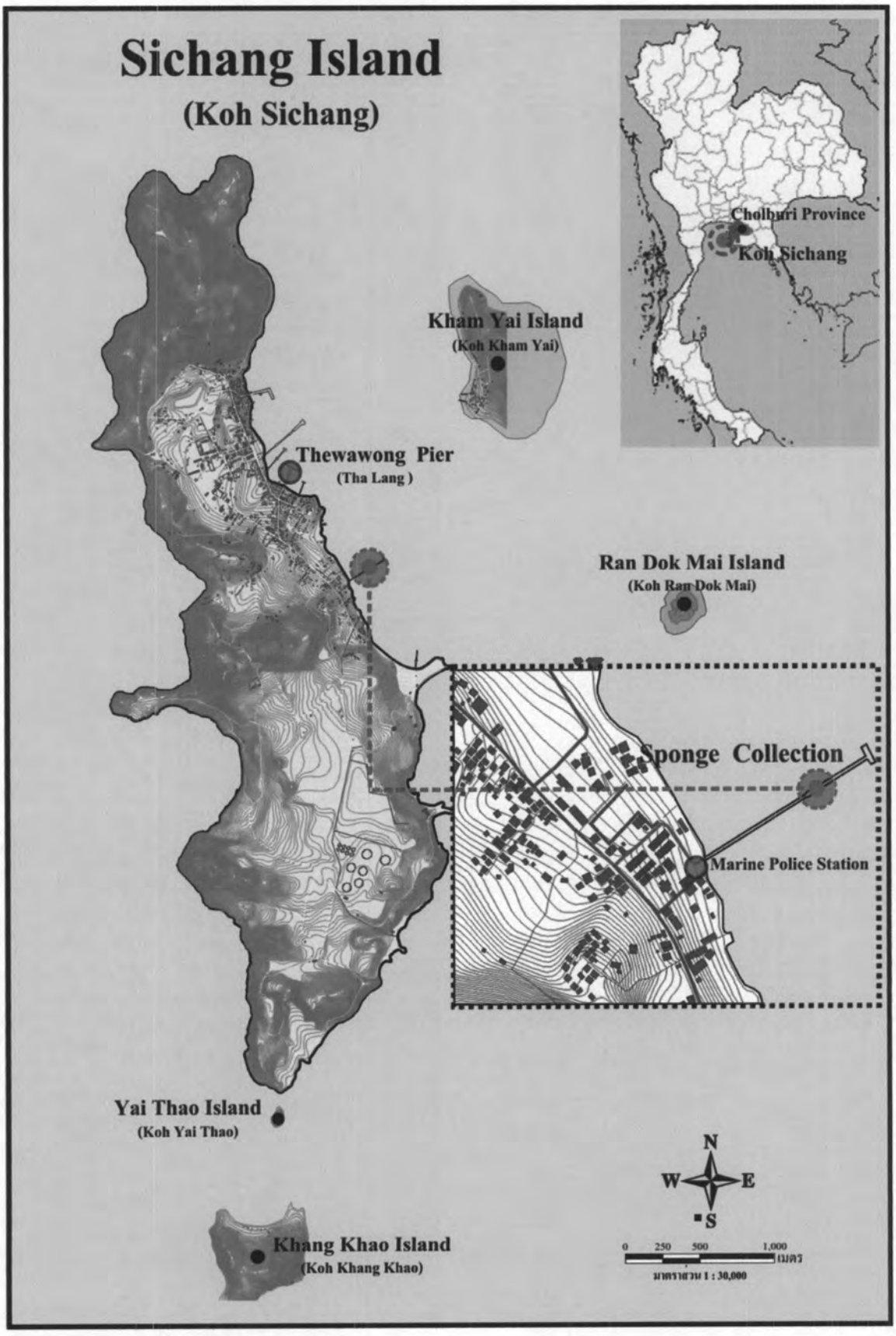


Figure 3.2. The initial sponge *Xestospongia* sp. from Ran-Dok-Mai Island and in-sea cultured sponge collection sites at Sichang Island at Cholburi Province.

Methods

1. Quantitative determination of the blue sponge *Xestospongia* sp.

1.1. Collection of the sponge *Xestospongia* sp.

The sponge *Xestospongia* sp. samples in the cage were monthly collected for 12 months (from August 2005 to July 2006). In each month, three colonies were randomly collected. The samples were cut into small pieces about one centimeter size and then the seawater in the sponge body was immediately removed by a tissue paper for one minute. The semi-dried samples in the separate collecting bags were kept in an ice cooler during transportation. Then, the samples were stored at -20 °C at our laboratory until extraction.

1.2. Preparation of 10 mM potassium cyanide (KCN) in 0.05 M phosphate buffer solution, pH 7

1.2.1. 0.05 M phosphate buffer solution, pH 7 (USP XXIII)

Solution A: 0.2 M potassium dihydrogenphosphate (0.2 M KH_2PO_4) solution

Accurately weighed 2.72 g to a 100-mL volumetric flask and adjusted to volume with water.

Solution B: 0.2 M sodium hydroxide (0.2 M NaOH) solution

Accurately weighed 0.80 g to a 100-mL volumetric flask and adjusted to volume with water.

To make pH 7.0, mixed 50 mL of **solution A** and 29.1 mL of **solution B** in a 200-mL volumetric flask and adjusted to volume with water.

1.2.2. 10 mM KCN in 0.05 M phosphate buffer solution, pH 7 (10 mL)

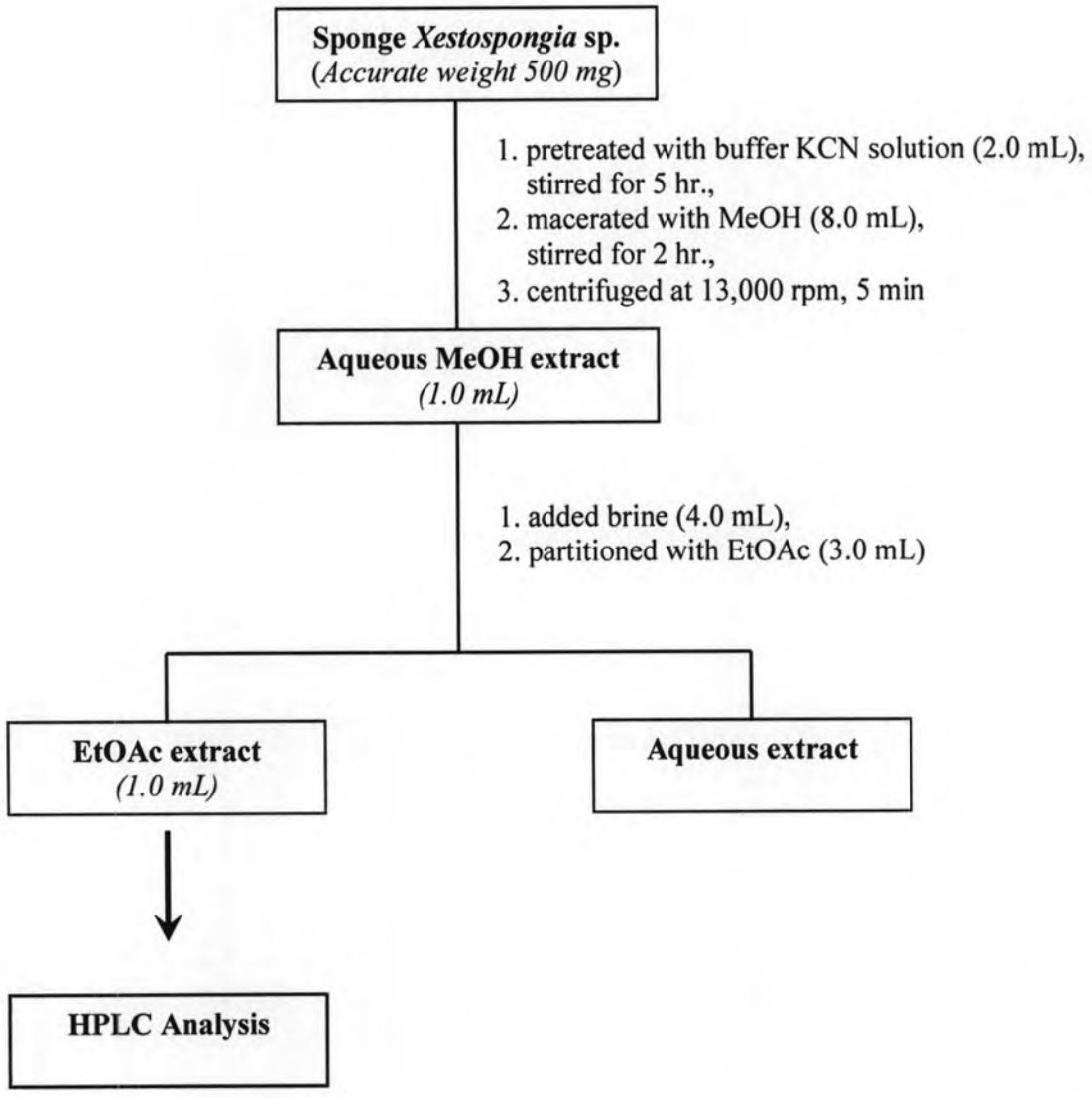
Accurately weighed 65.12 mg of potassium cyanide (KCN) in 10 mL of 0.05 M phosphate buffer solution and adjusted to pH 7.0.

1.3. Extraction of the sponge *Xestospongia* sp.

The semi-dried *Xestospongia* sample was cut into fine pieces and accurately weighed for 500 mg. A solution of 10 mM potassium cyanide (KCN) in 0.05 M phosphate buffer solution, 2.0 mL pH 7.0, was added and the sample was stirred for 5 hr. Then, the suspension was macerated with 8.0 mL of methanol and stirred for additional 2 hr. After centrifugation at 13,000 rpm speed for 5 min, the aqueous methanolic

solution (1.0 mL) was transferred to 4.0 mL of brine and further partitioned with ethyl acetate (3.0 mL).

The ethyl acetate layer (1.0 mL) was further dried under nitrogen gas and *in vacuo*, respectively, to give a crude ethyl acetate extract. The crude extract was stored at -20 °C prior to HPLC analysis (see Scheme 3.1).



Scheme 3.1. Preparation of the crude ethyl acetate extract from the sponge *Xestospongia* sp.

2. Cell separation of the blue sponge *Xestospongia* sp.

2.1. Preparation of the sponge *Xestospongia* sp. for cell dissociation and fractionation

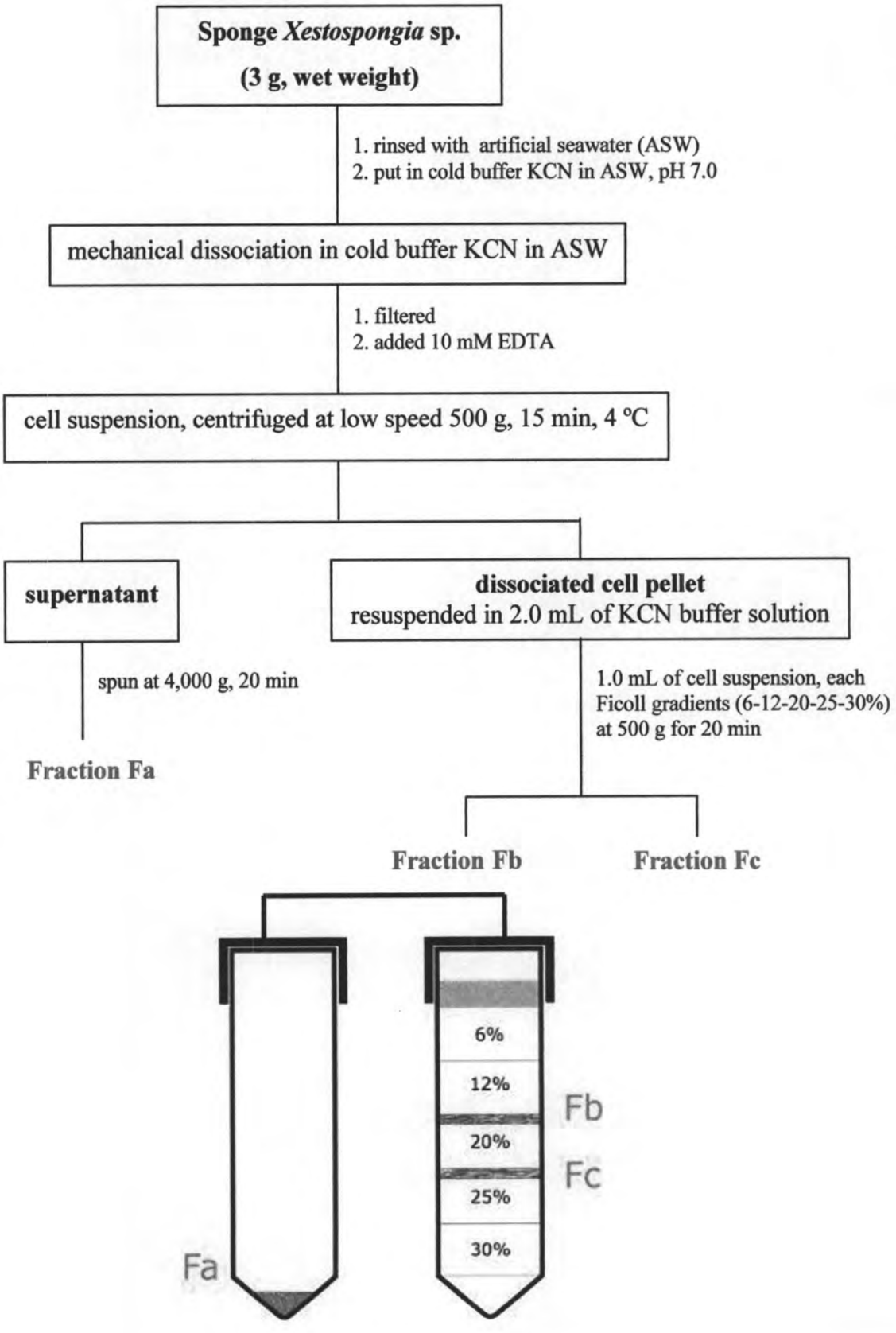
The fresh sponge *Xestospongia* sp. samples were cleaned and washed with artificial seawater (ASW) to remove associated organisms and dirt. The cleaned samples were then kept in the collecting tubes with 10 mM potassium cyanide in artificial seawater, pH 7.0, in an ice cooler. The sponge samples were stored at 4°C prior to use for cell dissociation and scanning electron microscopy (SEM).

The wet sponge *Xestospongia* sp. was accurately weighed for 3 g and then roughly chopped by using a scalpel blade, and gently crushed with mortar and pestle with 10.0 mL of 10 mM potassium cyanide buffer solution containing 10 mM ethylenediaminetetraacetic acid (EDTA, 1.0 mL). The sponge tissue suspension was then filtered through a clean fine cloth (80 μm mesh) to remove spicules and cell debris to give a cell suspension. The filtered cell suspension was spun at 500 g for 15 min at 4 °C to give a **supernatant** and a **dissociated cell pellet**. The **supernatant** was further centrifuged at higher speed (4,000 g for 20 min) to give a cell pellet, designated as **Fa**, which was resuspended in 1.0 mL of 10 mM potassium cyanide buffer solution for continuing extraction (see in Section 2.2). The **dissociated cell pellet** was resuspended in 2.0 mL of potassium cyanide buffer. Then, 1.0 mL of the suspension was used for cell fractionation by centrifugation (at 500 g for 20 min) across discontinuous Ficoll gradients (6%, 12%, 20%, 25%, and 30% in artificial seawater) using a centrifuge swinging bucket. Ficoll gradient centrifugation gave two sharp bands of cells, fraction **Fb** in the low-density fraction (at the 12/20% interface) and fraction **Fc** in the high-density fraction (at 20/25% interface). The bands of cells (fractions **Fb** and **Fc**) were collected by a Pasteur pipette and washed twice with KCN buffer solution to remove Ficoll (at 500g for 10 min). Each fraction was resuspended in 1.0 mL of potassium cyanide buffer solution and a small suspension (20 μL) of fractions **Fb** and **Fc**, each, was observed for cell counting by hemocytometer under inverted microscope and by SEM. Each remaining suspension (fractions **Fb** and **Fc**) was used for extraction (see Section 2.2) and further detected for the quantity of renieramycins by HPLC method (see Scheme 3.2).

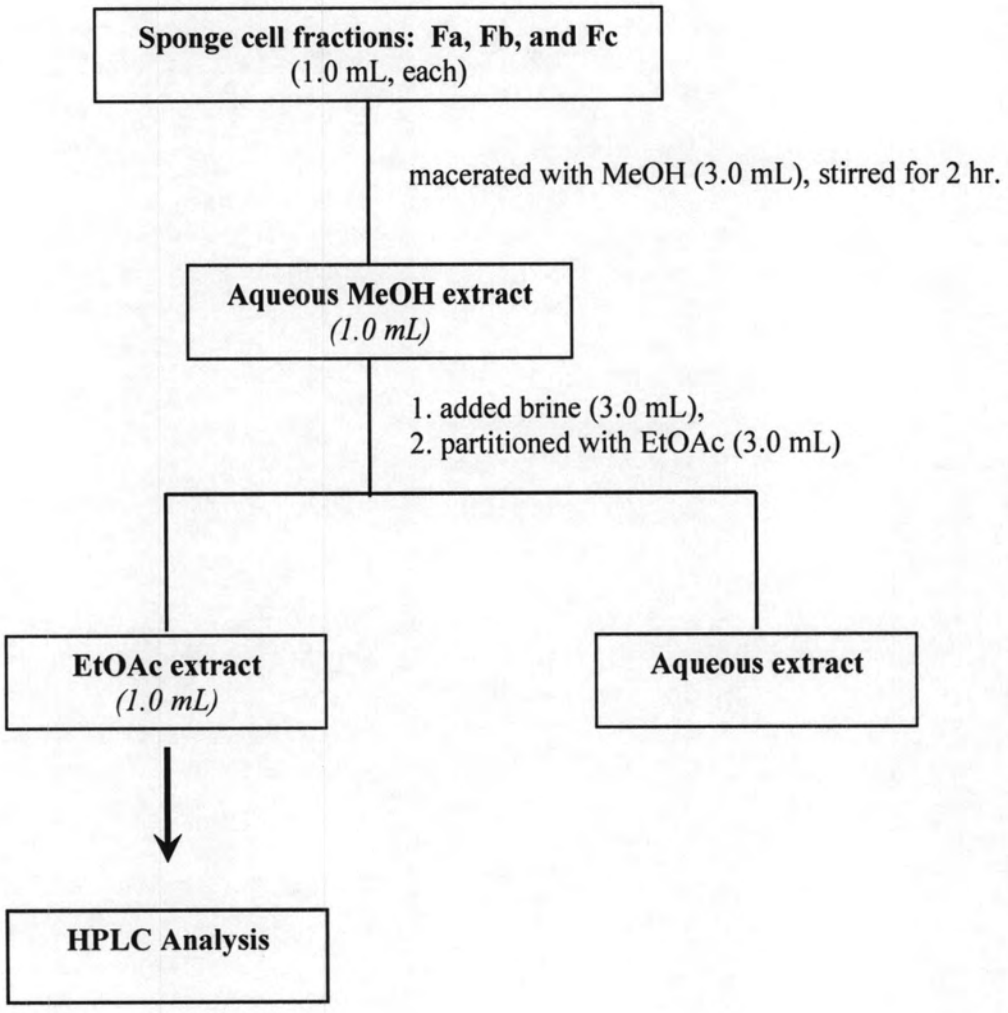
2.2. Extraction of the sponge *Xestospongia* sp. cell fractions

Each sample suspension in Section 2.1 (fractions, **Fa**, **Fb**, and **Fc**) was macerated with 3.0 mL of methanol and stirred for 2 hr. The aqueous methanolic solution (1.0 mL) was transferred to 3.0 mL of brine and partitioned with ethyl acetate (3.0 mL).

The ethyl acetate extract (1.0 mL) was further dried under nitrogen gas and *in vacuo*, respectively, to give a crude ethyl acetate extract. The extract was stored at -20 °C prior to HPLC analysis (see Scheme 3.3).



Scheme 3.2. Scheme of sponge cell fractionation.



Scheme 3.3. Preparation of the ethyl acetate extracts of cell fractions, **Fa**, **Fb**, and **Fc** for HPLC analysis.

2.3. Scanning electron microscopic (SEM) study

Specimens:

1. The sponge *Xestospongia* sp. tissues
2. The sponge *Xestospongia* sp. cell fractions, **Fa**, **Fb**, and **Fc**

Specimen preparation for SEM:

2.3.1. Fixed specimens overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer in artificial seawater, pH 7.2.

2.3.2. Filtered pass through membrane filter, pore size 0.45 μm (only the sponge cell fractions specimens).

2.3.3. Rinsed specimens twice in phosphate buffer for 10 min/each and once in distilled water for 10 min.

2.3.4. Dehydrated specimens with a graded series of ethanol, 30%, 50%, 70%, 90% for 10 min/each and absolute ethanol 3 times (10 min/time).

2.3.5. Critical point dry (a critical point dryer, Balzer model CPD 020), mounted and coated with gold (a sputter coater, Balzer model SCD 040).

2.3.6. Observed under a SEM (JEOL, model JSM-5410LV)

3. High Performance Liquid Chromatography (HPLC)

3.1. Preparation of internal standard acenaphthene solution

Accurately weighed 15.0 mg of acenaphthene and adjusted with methanol into a 100-mL volumetric flask (150 μg/mL, concentration) as the acenaphthene stock solution. Pepetted 5.0 mL of the stock solution into a 50-mL volumetric flask and adjusted to volume with methanol as the internal standard working solution (**solution A**, 15 μg/mL or 300 ng/20 μL, concentration).

3.2. Preparation of renieramycin M reference solution

Accurately weighed 1.0 mg of renieramycin M and adjusted with **solution A** in a 10-mL volumetric flask as the renieramycin M stock solution (**solution B**, 100 μg/mL, concentration). Pipetted 0.10, 0.20, 0.30, 0.40, and 0.50 mL of **solution B** into separate 2-mL volumetric flasks and adjusted to volume with **solution A** as the renieramycin M working solutions to give five serial concentrations 5, 10, 15, 20, and 25 μg/mL,

respectively, and each serial concentration contains 15 μg/mL of the internal standard acenaphthene. Each concentration was performed in triplicate.

A volume of 20 μL of each renieramycin M working solution was injected into the HPLC instrument for analysis. Each peak area ratio between renieramycin M and internal standard acenaphthene monitored at 270 nm were plotted against individual renieramycin M concentrations to construct the calibration curve.

3.3. Sample preparation

Each of the crude ethyl acetate extracts from Section 2.2 was dissolved in 1.0 mL of **solution A** and then the sample solution was filtered through a filter paper, 0.45 μm before HPLC injection.

3.4. HPLC condition

The qualitative and quantitative analyses of renieramycin M in the sponge preparation was achieved by HPLC. The renieramycin M content was detected under the UV detection at 270 nm and compared with the retention time and the UV spectrum of the renieramycin M reference. The amounts of renieramycin M were computed by the internal standard quantification method. Each of sample and renieramycin M reference was performed in triplicate.

Column:	LiChrospher® C-18e, 125 x 4 mm, 5 μm
Mobile phase:	Methanol-water (7:3)
Flow rate:	1.0 mL/min
Detector:	Photo-diode array (PDA) at 270 nm
Injection volume:	20 μL

Calibration curve: 5 different concentrations on renieramycin M reference solutions (5, 10, 15, 20, and 25 μg/mL) in the internal standard acenaphthene solution (15 μg/mL, concentration) were analyzed.

4. Validation of the HPLC method

4.1. Linearity

Each of renieramycin M reference solutions (5, 10, 20, 30, and 35 μg/mL) in the internal standard acenaphthene solution (15 μg/mL, concentration) was injected into the HPLC system. The calibration curve was plotted between concentrations (ng/20 μL) of renieramycin M vs. peak area ratios between renieramycin M and the internal standard acenaphthene. The relationship between the variables was calculated and expressed in terms of the **coefficient of determination** or **correlation coefficient** ($\mathbf{R^2}$). Each concentration was performed in triplicate.

4.2. Accuracy

Three known amounts of renieramycin M (RM_A; 50, 150, and 300 ng) were separately spiked into the ethyl acetate extracts of the sponge *Xestospongia* sp. Three replications of the unspiked ethyl acetate extract (RM_U) and three spiked samples (RM_S) were injected into the HPLC system. The accuracy of the HPLC method was evaluated by determination in terms of the **percentage of recovery** (**%R**) from the true value.

Recovery calculations for an analysis are determined using the following formula:

$$\%\text{Recovery} = \frac{[RM_S - RM_U] \times 100}{RM_A}$$

Where;

RM_S = measured amount of renieramycin M in the spiked ethyl acetate extract

RM_U = measured amount of renieramycin M in the unspiked ethyl acetate extract

RM_A = amount of added renieramycin M

4.3. Precision

Six replications of three renieramycin M reference solutions (10, 25, and 35 μg/mL, concentration) were injected into the HPLC system in the same day. The precision of the HPLC method was presented in terms of **the percentage relative standard deviation** (**%RSD**) or **percent of coefficient of variation** (**%CV**).

$$\%\text{RSD} = [SD / \bar{X}] \times 100$$

Where;

SD = standard deviation of peak area ratio or retention time

$\bar{X}$ = mean of peak area ratio or retention time

5. Determination of environmental parameters (Bennet and Bogorad, 1972; Parson *et al.*, 1989; Eaton *et al.*, 2005)

5.1. Chlorophyll-a

5.1.1. Dried filter papers (Whatman GF/C, pore size 0.45 μm) at 450 °C in a muffle furnace for 6 hr. Stored and cooled in a desiccator to a constant weight until needed.

5.1.2. Dropped a saturated magnesium carbonate solution about 3-4 drops on the filter paper before filtering 1,000 mL of seawater sample to prevent chlorophylls degradation. Each seawater sample was performed in triplicate.

5.1.3. Placed the filter paper in the opaque screwed test tube by wrapped with aluminium foil to protect light and extracted chlorophylls with 10.0 mL of 90% acetone solution and stored at -20 °C for 12 hr.

5.1.4. Measured the optical densities (OD) or absorbance of the acetone extract at wavelengths 750, 664, 645 and 630 nm by a UV spectrophotometer. Calculated the concentration of chlorophylls-a using the following equation:

a) Concentration of chlorophyll-a (mg/L)

$$Ca = 11.85 \times (OD_{664} - OD_{750}) - 1.54 \times (OD_{645} - OD_{750}) - 0.08 \times (OD_{630} - OD_{750})$$

Where;

OD = corrected optical density (with a 1-cm light path) at the wavelengths (750, 664, 645 and 630 nm)

After determining the concentration of chlorophylls in the acetone extract, the amount of chlorophylls per unit volume was calculated as follows:

$$\text{Chlorophyll-a, mg/m}^3 = \frac{\text{Ca} \times \text{extract volume, L}}{\text{volume of seawater sample, m}^3}$$

Where;

Ca = concentration of chlorophyll-a which is substituted in the above equation, mg/L

5.2. Total suspended solids (TSS) and total suspended organic matters (TSO)

5.2.1. Dried filter paper (Whatman GF/C, pore size 0.45 μm) at 450 °C in a muffle furnace for 6 hr. Stored and cooled in a desiccator to a constant weight and accurately weighed the filter paper (**A**).

5.2.2. Filtered 1,000 mL of seawater sample through the filter paper.

5.2.3. Dried the filter paper in a hot air oven at 100 °C for 24 hr. Stored and cooled in a desiccator and accurately weighed (**B**). Placed the filter paper in muffle furnace at 450 °C for 6 hr. Stored and cooled in a desiccator and accurately weighed (**C**).

5.2.4. Calculated TSS and TSO using the following equations:

Total suspended solids, TSS (mg/L)

= [(**B** - **A**) × 1000] / the volume of water sample, mL

Total suspended organic matters, TSO (mg/L)

= [(**B** - **C**) × 1000] / the volume of water sample, mL

5.3. Total dissolved solids, TDS and total dissolved organic matters, TDO

5.3.1. Placed the porcelain at 450 °C in a muffle furnace for 6 hr. Stored and cooled in a desiccator to a constant weight and accurately weighed (**D**).

5.3.2. Filtered 1,000 mL of seawater samples through the filter paper and put 50 mL of the filtrate in the porcelain

5.3.3. Dried the porcelain in a hot air oven at 100 °C for 24 hr. Stored and cooled in a desiccator and accurately weighed (**E**) and then placed the porcelain in a muffle furnace at 450 °C for 6 hr. Stored and cooled in a desiccator and accurately weighed (**F**).

5.3.4. Calculated TDS and TDO using the following equations:

Total dissolved solids, TDS (mg/L)

= [(**E** - **D**) × 1000] / the volume of water sample, mL

Total dissolved organic matters, TDO (mg/L)

= [(**E** - **F**) × 1000] / the volume of water sample, mL

6. Statistical Analysis

All data were expressed in terms of mean ± standard deviation and/or range. In addition, data concerning the amounts of renieramycin M contents in the blue sponge *Xestospongia* sp. were analyzed by one-way ANOVA for identification of significant statistical differences along the studied period. The Pearson correlation coefficient (r) was also computed for the relationship between the environmental parameters (water temperature, salinity, pH, TDS, TSS, and chlorophyll-a) and renieramycin M contents in the blue sponge *Xestospongia* sp. during August 2005 to July 2006.