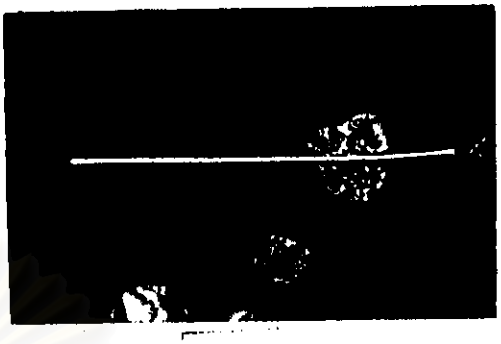
CHAPTER III

MATERIALS AND METHODS

1 Source of sample material

The marine sponge material, *Ircinia* sp., was collected by skin diving at the depth of 2-5 m from Palau Island which is located in south Pacific Ocean in July 1995. The sponge voucher specimen (code number PL-7-95-053) was preserved in alcohol and deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The *Ircinia* has siliceous spicules (Figure 5) with filamentous spongin threds filling the choanosome, which make the sponges extreme tough and difficult to tear; reticulated sand pattern on surface.

40 x



20 x

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Figure 5 Spicules of *Ircinia* sp.

Layer thickness : 250 μm.

Technique : One way ascending.

Distance : 10 × 20 cm.

Temperature : Ambient temperature (25 - 36 °C)

Detection : Both end sides of each plate were cut and used to locate the bands by the same detection methods as in 1.1.

2.1.3. Column chromatography.

2.1.3.1 Quick column chromatography.

This method is useful for the fractionation of large amount of complex mixtures especially those obtained from natural sources, because of speed and separating power. It can be used for routine work - up of reaction mixture. General requirements of the technique are as follows :

Adsorbent : Silica gel 60 (number 9385) particle size 0.040 - 0.063 mm (230 - 400 mesh ASTM) (E. Merck).

Packing : Suitable adsorbent was suspended in hexane solvent and filled into a sinterd glass filter as column (15 cm internal diameter). The adsorbent height is about 3-4 cm.

Sample loading : An appropriate solvent was used to dissolve the sample and mixed with kieselgulh (10 parts). The mixture was dried and pack onto the top of the adsorbent collumn.

Examination of the eluates : Compounds in each fraction were routinely checked up by a TLC technique. Locations of compounds on TLC plate were visualized under UV ligth, or by spraying with anisaldehyde reagent.

2.1.3.2 Flash column chromatography

Flash column chromatography is basically an air pressure drive, hybrid of medium pressure and short column chromatography, which has been optimized for particular rapid separation (Still, Kahn and Mitra, 1978). The

detailed of this technique are following :

Adsorbent : Silica gel 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM).

Packing : Adsorbent was wet - packed after being suspended in the eluent. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump, then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded onto the top of column.

Examination of eluates : Fraction were eaxamined in a similar manner described in section 1.3.1.

2.1.3.3 Gel filtration chromatography

This technique was used for a separation of the mixture containing compounds with different molecular weighs, the larger molecules move first and smaller later. The adsorbent is normally Sephadex LH - 20.

Packing : Adsorbent was suspended in the organic solvent system and left standing to swell prior to use for 24 hours. The suspension was poured in to the column and allowed to settle tightly (80-90 cm height)

Sample loading : The extract was dissolved in a small volumn of eluent and put on the top of the column.

Detection : Fraction were examined in the same manner as described in section 1.1.

2.1.3.4 Medium pressure liquid chromatography (BUCHI model 687)

The concept of Medium Pressure Liquid Chromatography (MPLC) was introduced in 1979 for the separation of diasteromeric oxazoline. The technique makes use of pressure of ca. 50-105 bar and can easily accommodate much larger sample load (100 mg - 100g) than are generally applied in Low Pressure Liquid Chromatography (LPLC) separation.

Adsorbent : Silica gel C_{18} reversed phase.

Column size : 2 cm diameter, 20 cm long.

Pressure : 8 bar.

Flow rate : 4 ml / min.

Detection : UV detector set at 230 nm.

Eluent : MeOH : H_2O, 8 : 2 (v/v)

Examination of fractions were followed to the section 1.3.1.

3 Crystallization technique.

The crystalization technique is followed to the "like dissolves like" theory as solid can be dissolved in solvents which have the same functional group, for example, polar solvent can dissolve polar compounds and nonpolar sovent can dissolve nonpolar compounds.

The compound were crystallized in a suitable solvents that rarely dissolved compound whereas extremely dissolved impure compound, and in a suitable condition (room or low temperature). The crystals was cleaned by appropriated solvent and evaporate that solvent by nitrogen gas blow.

4 Spectroscopy.

4.1 Infrared (IR) absorbtion spectra.

The IR spectra were obtained on a Fourier Transform - Infrared spectroscopy (FT- IR, Spectrometer Spectrum 2000, Perkin Elmer) (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

4.2 Mass spectra (MS).

The mass spectra of all compounds were obtained on a VG Platform II, Fisons Instrument mass spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). EIMS and CIMS was performed under 70 eV and 30 eV with methane as reagent gas.

4.3 Ultraviolet spectroscopy (UV)

The UV spectra were obtained on a Spectronic 3000 array, Milton Roy (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). The Methanol was used as solvent.

4.4. Nuclear Magnetic Resonance spectroscopy (NMR).

The NMR spectra of ^{13}C-NMR (P43), HMBC (P43 and KP9) and HSQC (KP9) were obtained on a JEOL JMN α series (500 MHz for ^{1}H- NMR and 125 MHz for ^{13}C- NMR) (The Scientific and Technological Research Equipment Center, Chulalongkorn University). The NMR spectra of P1, P44 and P45 were obtained on a Avance DPX 300 NMR Spectrometer Bruker Spectrospin (300 MHz for ^{1}H- NMR and 75 MHz for ^{13}C- NMR) (Faculty of Pharmaceutical sciences, Chulalongkorn University). Deuterated Chloroform was used as a solvent and as an internal reference.

5 Bioactivity determination.

5.1 Brine shrimp lethality activity test.

The bioassay was applied from the microwell cytotoxity assay method (Solis *et al.*, 1993) and as shown in Scheme1 . This method is recognized as a simple bioassay for natural products research with the advantage of being rapid, inexpensive and simple (no aseptic technique are required). Activities of broad range of known active compound are manifestes as toxicity to the brine shrimp, a tiny crustacean. Brine shrimp have been utilized in various bioassay system, for example analysis of pesticide residue, mycotoxin, stream pollutant, anaesthetics dinoflagellate toxin, Toxicity of oil dispersants, cocarcinogenicity of phorbol ester and toxicity in marine environment (Mayer *et al.*, 1982).

Brine shimp.

The egg of brine shimp (*Artemia salina* & LEACH) (Aquarium product®, USA) were hatched in a shallow rectangular box fill with artificial sea water. The box was divided into two compartments by a septum which had a few 2-mm holes. One compartment containing the brine shimp's eggs was darken, while another one was illuminated by a tangsten lamp. After 24 hrs, the nauplii of brine shimp would hatch and move directly to the bright side.

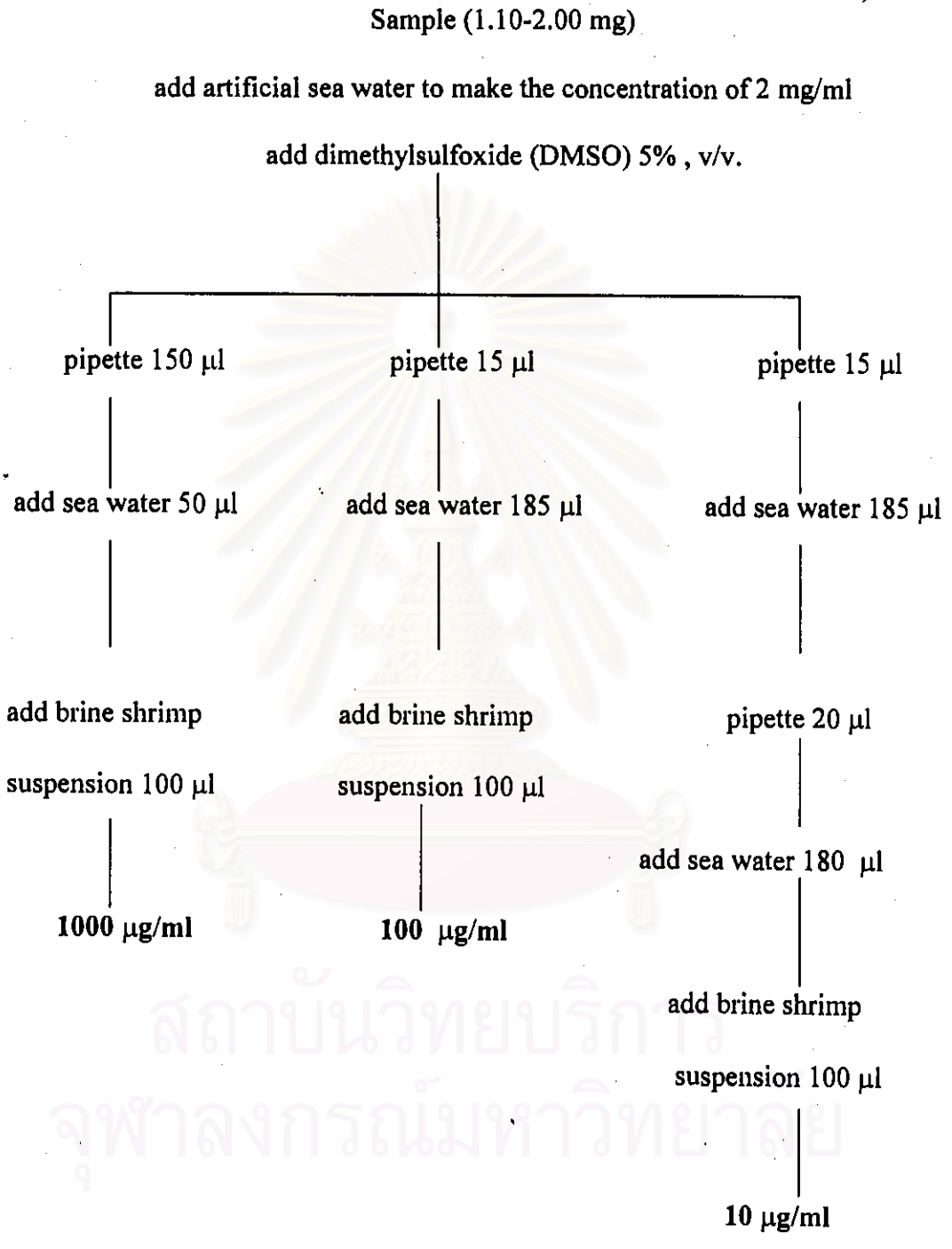
Artificial sea water.

Salt (Instant Ocean, Aquarium system., 38g) was dissolved in 1 litter of purified water and the solution was filtered through a filter paper (Whatman® Filter paper No. 1).

Bioassay.

About ten brine shimp (in 100 µl of brine suspension) were transfer to each wells with a micropipette. The microwell were maintained under illumination. After 24 hrs, dead and survivors were count under stereomicroscope.

The number of dead brine shrimps in the test well of each concentration were subtracted from those in the control well, then calculated in term of LD_{50} by using Finney equation program. The LD_{50} value and 95% confident intervals were determined from the 24- hours counts the probit analysis method describe by Finney program. Incase where data were insufficient for this technique, the dose response data were transformed into a straight line by mean of alogic transformation and LD_{50} was derived from the best fit line obtain by linear regression analysis.



Scheme 1. Brine shrimp lethality test at the concentration of 1000, 100 and 10 µl/ml.

5.2 Anti-herpes simplex virus assay (anti-HSV assay)

For testing anti HSV activity, the modified procedure which has been described previously (Skehan *et al.*, 1990) was used. In brif, cell were counted and diluted to 1×10^5 cell/ml with fresh medium and gently mixed. The suspension was added to 96 well microtiter plate (190 µl/well) were incubated for 30 min at 37 °C in a CO_2 incubator. For testing compounds, compound solution (dissolved in 10 µg of 100 % DMSO) and virus (30 PFU/cell) 60 µl were add to well 30 min before adding 130 µl of cold 50 % aquest trichroroacetic acid (TCA). The plates were incubated for 3 days at 37 °C in a CO_2 incubator. After incubation period, cell were fixed by addition of 100 µl of cold 50 % aqueous trichloroacetic acid (TCA). The plates were incubated at °C for 30 min, washed 4-5 timeswith tape water and air-dried at room temperature. The TCA-fixed cell were stained with 100 µl sulforhodamine B (SRB) (0.4% W/v SRB in aqueous acetic acid) for 30 min. FreeSRB solution was then removed by rinsing with 1 % acetic acid (4 times). The plates werethen air-dried at room temperature and the bound dry solution with 200 µl of 10 mM Tris base(pH 10). The plate were placed on shaker for 5 min, and the absorption was determined at 515 nm using an ELISA plate reader. Finally the absorption valued obtained with each of the treatment procedures were average, and the average value obtained with the zero day control was subtracted. These values were then expressed as a percentage, relative to solvent-treated control incubations, and ED_{50} values were calculated using non linear regression analysis (percent survival versus concentration). In every exeriment, aclycovir was used as positive control.