CHAPTER 3
MATERIALS AND METHOD

3.1 Materials

The reagents used for the isolation and purification of active compounds were of analytical grade whereas high performance liquid chromatography (HPLC) grade solvents were used for HPLC analysis. Chemicals and solvents were purchased from Merck KGaA and Fisher Scientific. Molecular biology grade dimethyl sulphoxide (DMSO) (Sigma-Aldrich) was used for dissolving drug samples for 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) bioassay. The promyelocytic leukemia cells, HL60 was purchased from the American Type Culture Collection (ATCC®). The Roswell Park Memorial Institute (RPMI) 1640 with phenol red media and Foetal Bovine Serum (FBS) used for cell culture were purchased from Gibco while RPMI 1640 without phenol red media used for MTT bioassay was from Sigma-Aldrich. Pheophorbide-*a* standard used in the bioassay was purchased from Frontier Scientific.

3.2 Instrumentation

All thin layer chromatography (TLC) spots were observed under Spectroline® Longlife™ filter UV lamp at 254 and 366 nm. Separation of extracts and fractions were carried out with a Shimadzu analytical HPLC with SPD-M10AVP PDA detector and LC-10ATVP pumps, as well as a Shimadzu HPLC with SPD-20A UV-Visible detector and LC-8A pumps. Mass spectra of the compounds were acquired using Agilent 1200 Series Liquid Chromatography with auto-sample injector attached to a 6400 Series Triple Quadruple MS System. Nuclear Magnetic Resonance (NMR) spectra for 1D (^{1}H and ^{13}C) were measured using JEOL ECA 400 MHz NMR (^{1}H at 395.88 MHz and ^{13}C at 99.55 MHz) and 500 MHz Bruker NMR spectrometer (^{1}H at 500.18 MHz). Thermo Labsystems Opsys MR

Spectrometer was used to measure the optical density (OD) at 570 nm for the MTT bioassay.

3.3 Sample Collection and Processing

Fourteen seaweed species were collected from Port Dickson during low tides, as shown in Figure 3.1. The specimens were collected based on their abundances at the collection sites.

Site of collection

Figure 3.1: The sites of seaweeds collection
GPS values for the locations of collection:
1. Cape Rachado - N02° 24.829' E101° 51.624'
2. Teluk Kemang - N02°26.772' E101°51.381'
3. Pantai Dickson - N02° 25.080' E101° 53.622'
(Adapted from Google Map)

The samples consisted of seven Pheophyta (brown seaweeds), five Chlorophyta (green seaweeds) and two Rhodophyta (red seaweeds). Details of the seaweeds collected from the different sites, namely Pantai Dickson, Cape Rachado and Teluk Kemang are shown in Table 3.1.

Table 3.1: Details of the seaweeds collected from Port Dickson

Seaweed Species	Type of Seaweeds	Site of Collection	Date of Collection
Sargassum baccularia	Pheophyta	Cape Rachado	18 April 2006
Sargassum binderi	Pheophyta	Cape Rachado	18 April 2006
Sargassum siliquosum	Pheophyta	Cape Rachado	12 July 2006
Sargassum polycystum	Pheophyta	Teluk Kemang	12 July 2006
Dictyota dichotoma	Pheophyta	Cape Rachado	12 July 2006
Turbinaria conoides	Pheophyta	Cape Rachado	18 April 2006 Recollection was done on July 2007
Padina australis	Pheophyta	Cape Rachado	18 April 2006
Chaetomorpha linum	Chlorophyta	Pantai Dickson	11 July 2006
Cladophora patentiramea	Chlorophyta	Pantai Dickson	11 July 2006 Recollection was done on July 2007
Caulerpa racemosa	Chlorophyta	Cape Rachado	12 July 2006
Caulerpa lentilifera	Chlorophyta	Cape Rachado	9 August 2006
Caulerpa sp.	Chlorophyta	Cape Rachado	12 July 2006
Gracilaria edulis	Rhodophyta	Pantai Dickson	18 April 2006
Gracilaria salicornia	Rhodophyta	Cape Rachado	18 April 2006

The samples collected were transported back to the laboratory for cleaning immediately after the trip. All samples were kept in seawater and stored with ice during transportation. Samples were rinsed with tap water and separated into respective species before storing them at -20 ^{o}C in freezers. The *Caulerpa* samples were processed differently from the other seaweeds. They were cleaned at the collection site and dried in a sealed plastic bag filled with silica desiccant as the seaweed disintegrates easily.

On the following day, the samples were thawed after removing from the freezer and brushed thoroughly with a toothbrush and forceps to remove the epiphytes and coralline algae growing on the Seaweeds. This process was done in diluted seawater to prevent leaching of compounds into water. Subsequently, they were rinsed repeatedly with distilled water to remove the salt and sand before drying in the open air for at least two days. The

samples were further dried in an oven at 40 ^{o}C and kept in a sealed plastic bag for storage. The dry weight of all samples was recorded.

3.3.1 Preparation of herbarium specimens

All samples were made into herbarium specimens as permanent records except for *Gracilaria edulis* due to limited material supply. The samples were cleaned thoroughly, patted dry and arranged neatly on herbarium papers. They were then covered with a layer of gauze and pressed with layers of newspapers and cardboards. After drying in an oven at 40 ^{o}C for a few days, dried samples were sewed neatly onto a clean herbarium paper and labeled accordingly.

3.3.2 Preparation of extracts

Dried samples were ground into fine powder or cut into smaller pieces and weighed prior to extraction. Samples were soaked in adequate amount of methanol and the extract was repeatedly concentrated in *vacuo*. The sample was repeatedly soaked in fresh solvents until the color of the extract became almost clear. The dried crude extracts were combined and weighed prior to storage at 4 ^{o}C .

3.4 Bioassay

Isolation and purification of pure compounds from the extracts was guided by MTT bioassay that was carried out at every stage of fractionation to determine which samples or fractions to be further fractionated until pure compounds were yielded.

3.4.1 Cell culture

Promyelocytic leukemia cell (HL 60) was routinely cultured in RPMI with phenol red media and 10% FBS at 37 ^{o}C in a humidified 5% CO_2 incubator.

3.4.2 MTT bioassay for photo-cytotoxicity

Photo-cytotoxicity of the samples were evaluated using MTT-based short term survival assay adapted with slight modification from Mosmann (1983). The number of HL 60 cells was counted with a hemocytometer and diluted to approximately 15 000 cells/mL using RPMI without phenol red media in 5% FBS. Meanwhile, the samples to be screened were prepared at 10 mg/mL stock concentration and further diluted to the desired screening concentrations using the media.

Two sets of 96-well assay plates were then seeded with 50 µl of the cells (~15 000 cells/well) followed by 50 µl of sample solutions prepared earlier in four replicates. A known photosensitiser, pheophorbide-*a* was used as a positive control at 0.01 µg/mL while 8 wells of seeded cells were added with 50 µl blank media which served as the negative control. One of the plates was irradiated with a light dose of 9.6 J/cm^2 for 10 minutes after two hours' incubation whereas the other plate was kept in the dark. After 24 hours incubation, 15 µl of 5 mg/mL MTT solution was added into both plates and left to incubate for another four hours. Subsequently, 70 µl of the medium was carefully pipetted out from each well without disturbing the deposition of formazan crystal in the wells. DMSO (100 µl) was added to dissolve the crystals after removing the supernatant before measuring the OD at 570 nm. The percentages of cell viability of the dark-control and light-treated samples

were determined. All samples were screened with at least two sets of assay to ensure reproducibility of the observed activity.

The screening for photo-cytotoxicity was conducted using 20 µg/mL of extracts. This concentration was used following the recommendation of the US NCI screening program on natural compounds where a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value in KB carcinoma cells is less than 20 µg/mL, after incubation between 48 and 72 hours (Boik, 2001). The concentration of the extracts screened was reduced by half after each fractionation. For example, the concentrations of the fractionated fractions were tested at 10 µg/mL while its extract was screened at 20 µg/mL. Fractions collected from the subsequent stage of isolation were then screened at the concentration of 5 µg/mL and further reduced accordingly until the pure compound was obtained.

3.4.3 Photo-cytotoxicity

Photo-cytotoxicity of the sample is determined by the comparison between two sets of MTT assay plates. A sample is concluded as active when the viability percentage of dark-control cells is measured to be ~100%, while the light-treated cells is to at least 50% or less cell viability percentage, as shown in the scenario of Sample 1 in the bar graph (See Figure 3.2). However, some samples may possess some cytotoxic properties in the dark condition, as observed in sample 2-4's circumstances. The light-treated assay plate should have at least two fold or less cell viability percentage as compared to its respective dark-control set, as in the case of sample 2 but not sample 3. In the scenario where samples showed strong cytotoxic properties (less than 20% of cell viability) in both conditions (sample 4), the

samples were also chosen to be followed up to identify if there was any hidden photo-cytotoxicity.

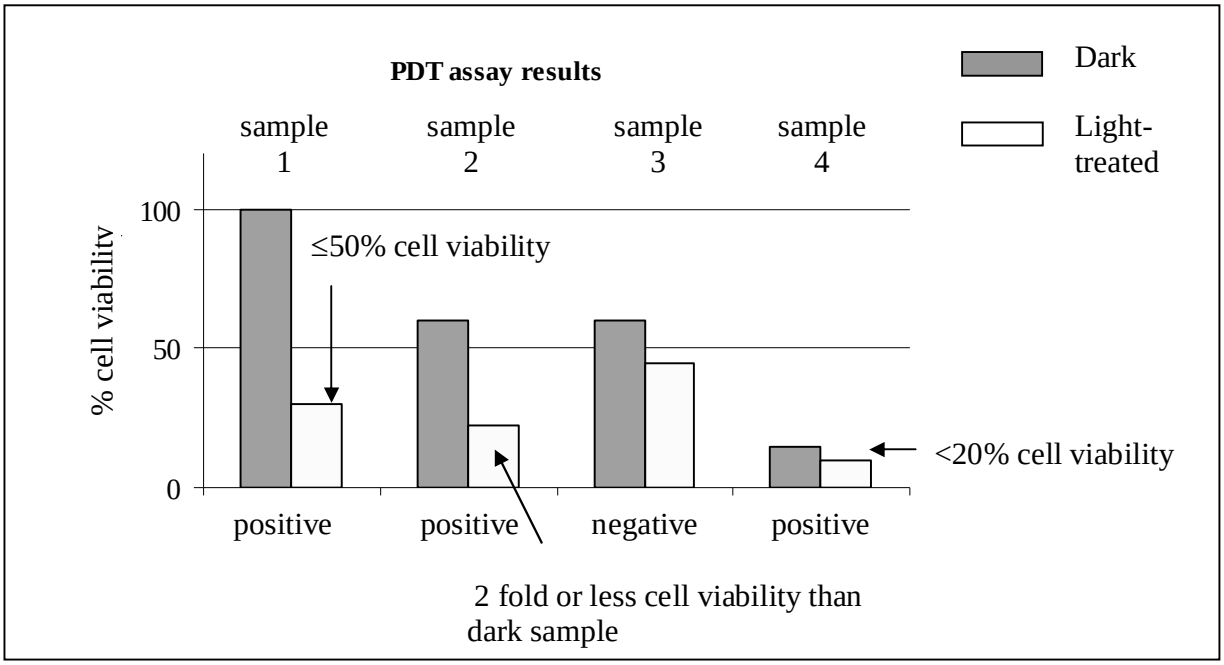

Figure 3.2: Example of typical PDT assay results observed in MTT screening

3.5 Isolation and purification of chemical compounds

In general, various chromatographic methods may be used to yield pure chemical compounds from a crude extract. Early fractionation of the crude extract into semi-pure fractions may involve liquid-liquid partition and/or column chromatography while further purification of smaller quantities into pure compounds may employ preparative thin layer chromatography (pTLC) and/or HPLC techniques. The choice of chromatographic method to be applied for fractionation or purification is dependent on several criteria including the abundance, polarity and complexity of the samples.

Sometimes crude extracts are subjected to methylation prior to fractionation in an attempt to esterify all free acids and thereby to improve the recovery yield of the fractions during

silica-based chromatography, which is one of the most common stationary phases used in chromatography. This is because poor recovery yield of fractions is often observed during the isolation of acidic or polar chemical constituents within the fractions, from silica chromatography and methylation of the crude extracts will convert any free acids that are present in the crude extracts to their methyl esters which will prevent the compounds from being retained by silica during chromatographic separations. The general flow of the isolation and purification process of a crude extract to yield pure compounds was illustrated in Figure 1.1.

The following sub-sections describe the various methods employed in the isolation and purification of compounds in this study.

3.5.1 Methylation

Approximately 2 g of extract was dissolved in about 100 mL of methanol in a round-bottomed flask. The solution was subjected to the methylation reaction by adding 5% of concentrated sulphuric acid in droplets to the mixture kept in an ice-water bath and stirred continuously at room temperature in the dark overnight. TLC was used to monitor the reaction by comparing the differences of separation profiles throughout the methylation process. The reaction was complete when there was no change in the observed separation TLC profiles. Upon completion of the reaction, the mixture was neutralized slowly with saturated sodium bicarbonate solution and partitioned with 50 ml of dichloromethane for at least 3 times. The organic layer was dried *in vacuo* and the yield was determined.

3.5.2 Liquid-liquid partition

Crude extracts were subjected to liquid-liquid partition to further remove salt and precipitate that may be present. Crude extract was dissolved in minimal chloroform and washed with equal amount of distilled water in a separating funnel. The organic layer was then collected and washed again with at least two volumes of distilled water. The organic layer was later concentrated with rotary evaporator while the aqueous layer was freeze-dried using a freeze dryer. The final weights of the yields were recorded.

3.5.3 Open column silica gel chromatography

Open column silica gel chromatography was applied in big scale sample fractionation. The extract was dissolved in methanol and mixed with 230-400 mesh silica gel 60 (Merck KGaA) at a ratio of 1:5. The mixture was subsequently dried *in vacuo* to obtain dried silica powder coated with the extract. The powder was then introduced into a glass column packed with 230-400 mesh silica gel 60 for fractionation. Extracts were fractionated using solvents of increasing polarity, consisting of hexane, ethyl acetate and methanol.

3.5.4 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is generally used to visualise the separation profile of a sample prior to select its appropriate method for further isolation. Samples were dissolved in acetone and spotted on a pre-coated silica gel 60 F_{254} aluminium-backed TLC sheet (Merck KGaA) using capillary tubes. It was further developed with appropriate solvent systems in a glass chamber. Developed TLC was subsequently observed with visible eyes for colored spots. Meanwhile, it was also observed under UV light (254 and 366 nm). In addition, TLC sheet was sprayed with vanillin reagent 1% (w/v) in sulphuric acid followed by heating at 110^{o}C for 5 minutes to visualise the presence of terpene compounds.

3.5.5 Preparative thin layer chromatography (PTLC)

Preparative thin layer chromatography (PTLC) is used to purify samples that are in minute amount (usually <20 mg) and have a reasonably good separation profile based on the TLC. The sample was spotted repeatedly onto a pre-coated 20 x 20 cm silica 60 F_{254}, 2 mm thickness, glass-backed plate (Merck KGaA) and developed multiple times with pre-determined solvent system in a glass chamber to ensure good separation. The bands of interest were colored and thus, could be observed with visible eyes. In addition, UV light (254 and 366 mm) was used to visualize the presence of colorless but UV-active bands. Silica-containing bands were then identified and scrapped out from the plate into vials and soaked with acetone. They were then filtered, washed with acetone and the filtrate was dried *in vacuo* and weighed.

3.5.6 Analytical high performance liquid chromatography (HPLC)

Analytical HPLC has the similar applications as TLC, where it is also used to visualize the separation profile of samples, however with the attachment of different detectors. In addition, the resolution of separation profiles can be improved by adjusting several parameters such as the solvent system, the flow rate and the types of HPLC column, prior to further isolation.

The sample was dissolved in an appropriate solvent and pre-filtered with Sartorius Minisart SRP 4, PTFE-membrane, single-use syringe filter prior to analysis. Chromolith® Performance RP-18 encapped column 100-4.6 mm (Merck KGaA) was used for HPLC separations. It was eluted with gradient of acetonitrile and water (Table 3.2) at a flow rate of 1 ml/min. Meanwhile, non-polar compounds were profiled via normal-phase HPLC

using Onyx® monolithic Si column 100-4.6 mm (Phenomenex), eluted with linear gradient of hexane and ethyl acetate in 30 minutes at a flow rate of 1 ml/min .

Table 3.2: Gradient system of acetonitrile and water for reversed-phase HPLC profiling

Time (min)	% water	% acetonitrile
0	50	50
5	40	60
10	30	70
15	20	80
20	10	90
25	0	100

Analytical HPLC was also used for the isolation and purification of fractions with amount less than 100 mg. Mid-polar to polar samples were separated with gradients of acetonitrile, methanol and water using Gemini 5u C6-phenyl column 110A 150 x 4.6 mm 5 µm (Phenomenex) and Chromolith® Performance RP-18 column encapped 100-4.6 mm (Merck KGaA). Meanwhile, non-polar samples were purified with gradient of hexane and ethyl acetate using Licrocart® Lichrospher® Si 60 column (5 µm, 125-4 mm).

All analytical HPLC profiles were detected at three wavelengths, namely 254 nm, 370 or 400 nm and 650 nm simultaneously to examine the presence of compounds with absorption at specific wavelengths.

3.5.7 Semi-preparative HPLC

Semi-preparative HPLC works in a similar manner as analytical HPLC and is carried out on extracts and fractions with more than 100 mg. The samples were developed using optimal gradient solvent system with appropriate analytical HPLC column and scaled-up accordingly. Lichrocart® Lichrospher® Si 60 5µm 250-10 mm (Merck KGaA) column was

used for the normal-phase semi-preparative collection while Onyx® monolithic semi-PREP C18 100-10 mm (Phenomenex) and Gemini 5u C6-phenyl 110A 250 x 10 mm 5 µm (Phenomenex) were used for reversed-phase semi-preparative collection. The peaks from the semi-preparative HPLC runs were detected at 400 nm and 650 nm simultaneously and the fractions were collected accordingly and weighed after being dried *in vacuo.*

3.6 Criteria of the selection of samples

Three main criteria were taken into consideration in the selection of samples to be followed up during the isolation process. The criteria are the extent of photo-cytotoxicity based on MTT assays, as well as the abundance and the complexity of the contents of the samples.

Apart from being photo-cytotoxic (See Section 3.4.3), the abundance and complexity of the samples are important in justifying whether further investigations are worthwhile as equipment such as 1D-NMR may require at least 1.0 mg for the analysis. Many fractionated samples were excluded from further isolation due to the fraction's complexity and low amount of material.

3.7 Naming samples

All samples were named according to their sequence of collection separated by their fractionation and purification stages. The stage of fractionation and purification is indicated by hyphens in the naming process. For example, Tur-1 indicates the first fraction collected from Tur extract via first fractionation while Cla-4-3 indicates the third fraction that was purified from the forth fraction collected in the first fractionation.