CHAPTER 3

MATERIALS AND METHODS

3.1 Maintenance of *Maramius* species and Preparation of Inoculum

Eight *Marasmius* cultures in the present study were previously identified and maintained on Malt Extract Agar (MEA) (Appendix A) slants deposited at the Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya (Yee Shin, 2007). The cultures were then inoculated on three 7 mm-diameter onto Glucose-Yeast-Malt-Peptone (GYMP) agar plates at three different points (Appendix A). Inoculated plates were incubated for 7-10 days at 25^{o}C. Table 3.1 shows the list of *Marasmius* spp. studied.

Table 3.1 List of *Marasmius* species studied

No	Species name	Sub-sect	Picture
1.	KUM 20044 *M. guyanensis* Mont.	Marasmius subsect Sicciformes	
2.	KUM 20222 *M. guyanensis* Mont.	Marasmius subsect Sicciformes	
3.	KUM 20117 *M. guyanensis* Mont.	Marasmius subsect Sicciformes	

4.	KUM 20160 *M. kanchingnensis*	Sicci subsect Siccine ser Leonini	Picture not available
5.	KUM 20181 *M. selangorensis* Y.S. Tan & Desjardin	Sicci, subsect Siccine, ser. Leonini	
6. 7.	KUM 20111 *M. ruforotula* Singer KUM 20112 *M. ruforotula* Singer	Marasmius subsect Sicciformes Marasmius subsect Sicciformes	
8.	KUM 20067 *Marasmius* sp.	Sicci	Picture not available

3.2 Liquid Fermentation for the Production of Mycelial Biomass

Production of mycelial biomass was carried out by inoculating ten 7 mm-diameter plugs of 7-10 days old *Marasmius* mycelia cut from the periphery of the

colony using a sterile cork borer into 100 ml of sterilized GYMP liquid media in 500 ml Erlenmeyer flasks. Liquid GYMP was prepared as described in Appendix A. Ten replicate flasks were prepared for each *Marasmius* species. Inoculated flasks were incubated for 14 days at 25^{o}C under static conditions to obtain mycelia biomass.

3.3 Preparation of Hot Dichloromethane Extract of *Marasmius* spp.

Mycelial biomass was harvested, freeze-dried and crushed into powder. Twenty grams of each dried mycelial biomass was extracted with dichloromethane (Mallinckrodt Chemicals, USA) boiled for 14 hours using the soxhlet extractor system. The resultant extract collected in the round bottom flask was subjected to evaporation using a rotary evaporator (Büchi Rotavapor R-114, Switzerland) to yield total solubles as crude extracts.

The percentage yield of hot dichloromethane extracts were calculated as below:

$$\text{Yield (\%)} = \frac{\text{weight of dried extract}}{\text{weight of freeze dried mycelia biomass}} \times 100$$

The dried extract was kept in a small vial covered with aluminium foil and stored at -20^{o}C until further analysis.

3.4 Determination of Cytotoxic Activity of *Marasmius* Extracts

Seven cell lines were used in this study. Human mouth epidermal carcinoma cell line (KB), human epidermal carcinoma of cervix cell line (CaSki), human colon cancer cell line (HT 29), human intestinal colon cancer cell line (HCT 119), human colorectal cancer cell line (SKOV 3), human breast cancer cell line (MCF 7) and human fibroblast

cell (normal cell) (MRC 5) were purchased from American Tissue Culture Collection (ATCC, USA).

3.4.1 Preparation of Cell Culture

The viability of the cells was checked before and after treatment by the tryphan blue exclusion dye method. Frozen cell stocks were stored in liquid nitrogen (-196^{o}C) prior to use.

The provial of cells was removed from liquid nitrogen and plunged into a beaker of ice. It was then, transferred to a 37^{o}C water bath (Grand Instruments) for quick thawing. The cells were transferred into 7 ml of 20% supplemented medium 199 or RPMI 1640 medium in a polypropylene tube and spun at 1000 rpm (Clements 2000) for 5 minutes. The supernatant was discarded. The pellet was subsequently resuspended in 5 ml of 20% supplemented medium 199 or RPMI 1640 medium and incubated in a 25 ml tissue culture flask (Falcon) at 37^{o}C in a 5% carbon dioxide (CO_2) incubator (Shel Lab).

CaSki, HT 29 and MCF 7 cells were maintained in RPMI 1640, KB cells in 199 medium, SKOV 3 cells in DMEM, HCT 116 cells in McCOYs and MRC 5 cells in MEM in 25 ml tissue culture flask. The culture was incubated in a 5% CO_2 incubator kept at 37^{o}C in a humidified atmosphere. The culture was subcultured every 2 or 3 days and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination.

Adherent cells attached and formed a single layer in a culture flask. Confluent cells were washed twice using Phosphate Buffered Saline (PBS). The cells were detached from the flask by incubating in a 1 ml accutase and 3 ml PBS solution for 5 minutes at 37^{o}C, and then sharply tapped to detach the cells from the flask. The floating cells were transferred into a centrifuge tube and centrifuged for 5 minutes at 1000 rpm.

The supernatant was removed and 2 ml 10% supplemented medium was added to the pellet. The cells were split and transferred into different flasks each containing 7 ml culture media. The flask was then further incubated.

Cells were detached from the flask with 0.25% solution of accutase DPBS in 0.5 mM EDTA (Innovative Cell Technology Inc) and washed in culture medium. The cell pellet was obtained by centrifugation at 1000 rpm for 5 minutes and the density of the viable cells was counted by 0.4% of tryphan blue exclusion in a haemocytometer. For cell enumeration, 100 µl of the stock cell suspension was transferred into a provial with 900 µl of 0.4% tryphan blue and mixed well. The haemocytometer (Scherf) was covered by a glass cover slip and 20 µl of the suspension with dye was loaded at the two edges of the cover slip so that it could flow into the chambers by capillary action. The haemocytometer was then examined under an inverted microscope and the unstained viable cells were counted. The cell suspension at a concentration of 3×10^4 cells/ml (concentration of 5×10^4 cells/ml was used for HT-29 cells) was prepared according to the formula below:

$$P_1 \times 10^5 \times V_1 = P_2 \times V_2$$

P_1 : average number of viable cells counted from the haemocytometer

10^5 : Counting chamber conversion factor & dilution factor with dye

V_1 : Volume of stock cell suspension needed

P_2 : The desired cell concentration in the cell suspension

V_2 : Volume of 10% supplemented RPMI 1640 medium used for seeding

Cells were then plated in a 96-well microtiter plate (Nunc), at a concentration of 30 000 cells/ml for KB cells and at concentration of 50 000 cells/ml for CaSki, MCF 7 and HT 29, in a total volume of 190 µl. The plate was incubated in a CO_2 incubator at 37^oC for 24 hours to allow the cells to adhere and achieve 60 -70% confluence before

addition of the test agents. After 24 hours, the crude extracts were then added to the wells for prescreening at 20 µg/ml of each crude extract. The cells were incubated with the extracts for 72 hours. The untreated cells with just growth medium (without addition of any extract) served as negative controls.

3.4.2 Preparation of Stock Dichloromethane Extracts

The dried fractions of the crude dichloromethane extracts were weighed and dissolved in dimethysulphoxide (DMSO) to form stock solutions at 20 mg/ml for use in the Neutral Red Cytotoxicity Assay.

3.4.3 *In Vitro* Neutral Red Cytotoxicity Assay

The neutral red cytotoxicity assay was based on the initial protocol described by Borenfreund and Puerner, (1984) and it quantitates the amount of the neutral red dye accumulated in the lysosomes of viable and uninjured cells. After the 72 hour incubation period with the extracts, the media were replaced with a medium containing 50 µg/ml Neutral Red.

The plates were incubated for another 3 hours to allow for uptake of the vital dye into the lysosomes of viable and injured cells. After the incubation period, the media were removed and cells were washed with the Neutral Red washing solution. The dye was eluted from the cells by adding 200 µl of Neutral Red resorb solution and incubated for 30 minutes at room temperature with rapid agitation on a microtiter plate shaker (LT BioMax 500). The optical density (OD) of the dye absorbance was measured at 540 nm using ELISA reader (Titertek Multiskan MCC/340). Three replicates were used to determine the cytotoxicity activity of the extract.

The average data from the triplicates for each dichloromethane extracts concentration was expressed in terms of killing percentage relative to negative controls.

The percentage of inhibition of each of the test samples was calculated according to the following formula as shown below:

$$\% \text{ of inhibition} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100\%$$

The ED_{50} is the concentration of extract that causes 50% inhibition or cell death (Chiang *et al.,* 2003). ED_{50} for each extract was extrapolated from the graphs plotted using the % of inhibition values obtained. Lower ED_{50} values indicate higher effectiveness in antioxidant properties of the studied extracts. The absorbance decrease is connected with the radical scavenging ability by the antioxidants contained in the studied extracts. The faster the absorption decreases, the stronger the antioxidant, possessing higher ability of hydrogen donation (Yen and Duh, 1994). The range of extract concentrations and measurement frequencies were established experimentally.

3.5 Determination of Antioxidant Capacity of *Marasmius* Extracts

3.5.1 Scavenging Effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radicals

This method which determines the scavenging effect of the extracts on 2,2-diphenyl-1-picrylhydrazyl radicals was applied according to (Brand-Williams *et al.,* 1995). One mM DPPH solution was prepared and diluted to 0.06 mM with methanol. Stock solutions of each extracts were prepared by dissolving 0.05 g of crude extract in 1 ml methanol. This produced stock solutions with the concentration of 50 mg/ml. An aliquot of 3.9 ml methanolic solution containing 0.06 mM DPPH was added to 0.1 ml of methanol diluted crude extracts to obtain final concentrations of 2.5 - 500 mg/ml.

The solution was mixed vigorously and absorbance was then measured at 515 nm using a spectrophotometer (UVmini-1240, Shimadzu Corporation Japan) with

methanol as the blank. The decrease in absorbance was recorded at 0, 1, 2 minutes and every 15 minutes until the reaction reached a plateau. The time taken to reach steady state was determined by one-way analysis of variance (ANOVA) which compare the decrease in absorbance values. Disposable plastic cuvettes with the dimension 1 cm x 1 cm x 4.5 cm from Kartell, Italy were used for visible absorbance measurements. All determinations were performed in triplicates. The scavenging activity on DPPH was expressed as percentage radical scavenging calculated as follows:

$$\% \text{ radical scavenging effect} = \frac{[A_o - A_s]}{A_o} \times 100\%$$

A_o refers to the absorbance of 0.06 mM DPPH methanolic solution only whereas A_s is the absorbance of the reaction mixture.

EC_{50} value (mg /ml) is the effective concentration of extract at which DPPH· radicals were scavenged by 50%, EC_{50} values were obtained by interpolation from linear regression analysis. Antioxidant activities of the extracts were compared with those of L-ascorbic acid, BHA as positive standards.

3.5.2 Reducing Power Assay

The reducing power of the prepared extracts was determined according to the method of Oyaizu (1986). Briefly, each extract in varying amounts of 5 mg, 10 mg and 20 mg was dissolved in 1 ml of methanol to which was added 2.5 ml of 0.4 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) solution of potassium ferricyanide (Sigma).

The mixture was incubated in a water bath at 50^{o}C for 20 minutes. Following this, 2.5 ml of 10% (w/v) trichloroacetic acid (Sigma) solution was added and the

mixture was then centrifuged at 650 rpm for 10 minutes. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a 0.1% (w/v) solution of ferric chloride (Sigma). Absorbance of reaction mixture was read spectrophotometrically (UV-VIS Hitachi U-2000) at 700 nm. Increased absorbance of the reaction mixture indicates greater reducing power. Mean values from the three independent samples were calculated for each extract. L-ascorbic acid and BHA were used as a positive reference.

3.5.3 Metal Chelating Method

The chelation of ferrous ions by the extracts and standard was estimated by the method of Dinis, Madeira and Almeida (1994). Extracts were added to a solution of 1 mM $FeCl_2$ (0.05 ml) (50 µl). The reaction was initiated by the addition of 1 mM ferrozine (0.1 m) (Sigma) after which the mixture was shaken vigorously and left standing at room temperature for 10 minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm.

All tests and analyses were done in triplicates and results averaged. The control contains $FeCl_2$ and ferrozine complex formation molecules. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below:

$$\% \text{ inhibition} = \frac{[A_o - A_1]}{A_o} \times 100\%$$

Where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of crude extracts and standards. EDTA (Sigma) acts as a positive control.

3.6 Statistical Analysis

Data were recorded as means $\pm$ standard deviations and analysed by STATGRAPHICS Plus version 3. One-way analysis of variance (ANOVA) was carried out to test for any significant differences between the means; the mean values of antioxidant activities between two extracts or two treatments were analysed by independent-samples t-test. P-values less than 0.05 were considered statistically significant. All analyses were performed in triplicates.