CHAPTER 4

RESULTS

4.1 Mycelial Growth and Yield of Hot Dichloromethane Extracts of *Marasmius* species

Marasmius spp. grown on GYMP agar plates produced a confluent dense of mycelia spread. The yield of the freeze dried mycelia from the eight *Marasmius* spp in the present study are given in Table 4.1. From Table 4.1, *M. ruforotula* (KUM 20111) exhibited greatest growth density with mycelia weight of 23.40 g/L followed by *M. ruforotula* (KUM 20112) of 22.96 g/L and *M. selangorensis* (KUM 20181) (22.35g/L). Other *Marasmius* freeze dried mycelia yield ranged from 11.27 g/L – 18.14 g/L (Table 4.1).

The freeze dried mycelia from the eight species of *Marasmius* were extracted using hot dichloromethane solvent through the soxhlet extractor system. The dichloromethane extracts obtained was rotary evaporated under vacuum until dried and weighed. The percentage yields of dichloromethane crude extracts in the present study were calculated based on dry weight basis of mycelia and results are shown in Table 4.1.

The total yield percentages ranged from 1.12% - 3.87%. Based on the results shown in Table 4.1, *M. ruforotula* (KUM 20111) showed the highest total yield percentage of 3.87% followed by *M. kanchingnensis* (KUM 20160) with a yield percentage of 2.71% while the lowest is obtained by *M. guyanensis* (KUM 20044) with total yield percentage of 1.12% (Table 4.1). Referring to Table 4.1, the rest of the *Marasmius* crude dichloromethane extracts percentage yield ranged from 2.49% - 1.12%.

Table 4.1 Dry weight of mycelia biomass and their percentage yield based on dry weight basis of mycelia biomass for eight *Marasmius* spp.

Name and code of *Marasmius* spp.	Yield of freeze dried mycelia (g)/L	% yield of hot dichloromethane extract (dry weight basis, w/w)
M. guyanensis (KUM 20117)	13.12	2.00
M. guyanensis (KUM 20044)	15.25	1.12
M. guyanensis (KUM 20222)	11.27	1.42
M. kanchingnensis (KUM 20160)	12.55	2.71
Marasmius sp (KUM 20067)	18.14	2.27
M. ruforotula (KUM 20111)	23.40	3.87
M. ruforotula (KUM 20112)	22.96	2.49
M. selangorensis (KUM 20181)	22.35	1.25

4.2 Cytotoxic Activity of Hot Dichloromethane Extracts of *Marasmius* spp. against Cancer-Derived Cell Lines

In the present study, the dichloromethane extracts of *Maramius* were screened for their *in vitro* cytotoxic activities against six cancer-derived cell lines namely 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 a normal human lung cell line (MRC 5) using the neutral red cytotoxicity assay.

The cells were grown in their respective media and then incubated with dichloromethane extracts of *Marasmius* at a concentration of 20 µg/ml for 72 hours at 37°C. Negative controls consisted of cells not treated with the extracts. These controls exhibited normal proliferation rate and showed no signs of death after the incubation time of 72 hours. The killing percentage and standard deviations for the eight crude extracts are shown in Appendix B. The cytotoxicity data obtained were expressed as killing percentage relative to negative controls, shown in Table 4.2.

As indicated in the literature, usually natural products that produce toxicity activity at EC_{50} values between 20 to 100 µg /ml are used for clinical trial. They are considered to be toxic enough against the cancer cells with the lowest toxicity effect against normal cells. Referring to the results obtained in Table 4.2, the cytotoxic activity of crude dichloromethane extracts of *Maramius* demonstrated less than 50% inhibition rates at 20 µg/ml. Since the percentage inhibition at 20 µg/ml is less than 50%, these extracts are not considered actively cytotoxic hence no further testing was required. *Marasmius guyanensis* (KUM 20044) recorded the highest inhibition percentage of 33.7% on SKOV 3 cells at 20 µg/ml. This was followed by *M. ruforotula* (KUM 20111) with an inhibition percentage of 34.5%, whereas *M. ruforotula* (KUM 20112) and *M. guyanensis* (KUM 20022) both exhibited 33.4% and 33.8% inhibition rates,

respectively at 20 µg/ml. The killing percentage of the rest of the crude extracts ranged from 7.08% - 24.3% at 20 µg/ml. The least inhibition percentage was recorded with *Marasmius* sp. (KUM 20067) at 7.08% at the concentration of 20 µg/ml.

Marasmius ruforotula (KUM 20111) gave the highest inhibition rates at 20 µg/ml with 47.27% killing percentage of lysed KB cells, shown in Table 4.2, whereas *M. ruforotula* (KUM 20112) and *M. selangorensis* (KUM 20181) both resulted in the same killing percentage rates of 46.90%. The least inhibition percentage on KB cell was recorded with *M. guyanensis* (KUM 20044) at 8.95%.

As shown in Table 4.2, HCT 119 cells showed different cytotoxic profiles when treated with crude extracts of *M. ruforotula* (KUM 20111) and (KUM 20112) at 20 µg/ml. *Marasmius ruforotula* (KUM 20111) and (KUM 20112) exhibited similar killing percentage rates of 37.00% to 35.48%, respectively at 20 µg/ml. *Marasmius selangorensis* (KUM 20181) produced an inhibition percentage of 33.38% whereas *M. guyanensis* (KUM 20117) with 29.36%. The lowest percentage of inhibition of HCT 119 was obtained with *M. guyanensis* (KUM 20222) at 3.93%.

Cytotoxicity activity of the extracts at 20 µg/ml on HT 29 cells in this study was evaluated and the results are presented in Table 4.2. *Marasmius guyanensis* (KUM 20222) caused the highest inhibition percentage of 40.70% against the HT 29 cells at 20 µg/ml. *Marasmius ruforotula* (KUM 20111) and (KUM 20112) produced inhibition percentages of 27.08% and 25.40% respectively. However, the rest of the extracts only produced low inhibition percentages ranging from 4.24% - 15.64%. The lowest killing percentage of 4.24% on HT 29 cells was produced by *M. guyanensis* (KUM 20044) (Table 4.2).

With reference to MCF 7 cells, *M. guyanensis* (KUM 20222) and *M. kanchingnensis* (KUM 20160) produced inhibition rates of 23.21% and 21.33%, respectively at 20 µg/ml. The inhibition percentage produced by *M. ruforotula* (KUM

20111) and (KUM 20112) did not differ very much from each other. The inhibition percentages were 17.41% and 16.41%, respectively. *Marasmius selangorensis* (KUM 20181) produced less inhibition activity because only about 6.57% KB cells were killed at the concentration of 20 µg/ml. *Marasmius guyanensis* (KUM 20044) and *Marasmius* sp. (KUM 20067) inhibited KB cells at 7.74% and 7.84%, respectively at 20µg/ml.

The cytotoxic activities of crude dichloromethane extracts of *Maramius* on CaSki cells were evaluated and the results are presented in Table 4.2. *Marasmius* sp. (KUM 20067) inhibited CaSki cells by 32.03% at the concentration of 20 µg/ml. The least inhibition percentage 8.77% was obtained from *M. guyanensis* (KUM 20044). The crude dichloromethane extracts of *Marasmius* were also evaluated for cytotoxicity against normal cells (MRC 5). The crude dichloromethane extracts inhibited MRC 5 cells with percentage of inhibition less than 50% at 20 µg/ml (Table 4.2).

As shown in Table 4.2, the inhibition percentages produced by all crude dichloromethane *Marasmius* extracts ranged between 2.68% - 8.99%. The inhibition percentages of MRC 5 by these extracts were lower than 10% at the concentration of 20 µg/ml. This suggests that the extracts are less inhibitory to normal cell as compared to cancer cells. Since the percentage inhibition at 20 µg/ml is less than 50%, these extracts are not considered actively cytotoxic. No further testing was required. Based on the results shown in Table 4.2, *M. ruforotula* (KUM 20111) and (KUM 20112) produced percentage inhibition of only 7.77% and 7.60%, respectively. Meanwhile *M. kanchingnensis* (KUM 20160) showed a low inhibition percentage of 2.68% (Table 4.2).

Table 4.2: *In vitro* growth inhibition (%) of various human cancer cell lines by hot crude dichloromethane extracts of selected *Marasmius* spp. at concentration of 20 µg/ml

Crude dichloromethane extracts of *Marasmius* spp.	% inhibition						
	KB	**MRC 5**	**HT 29**	**MCF 7**	**HCT 119**	**SKOV 3**	**CaSki**
M. guyanensis KUM 20044	8.95 ± 2.41	4.40 ± 0.85	4.24 ± 0.44	7.74 ± 1.72	17.4 ± 0.61	37.7 ± 1.82	8.77 ± 1.21
M. guyanensis KUM 20222	12.4 ± 1.70	8.99 ± 0.65	40.7 ± 3.76	23.2 ± 1.30	3.93 ± 0.84	33.8 ± 3.35	15.4 ± 2.26
M. guyanensis KUM 20117	36.6 ± 2.59	6.53 ± 1.05	7.20 ± 2.25	9.37± 0.91	29.4 ± 1.46	17.4 ± 1.99	17.1 ± 0.82
M. kanchingnensis KUM 20161	16.6 ± 1.91	2.68 ± 0.31	15.6 ± 3.77	21.3 ± 2.88	6.92 ± 2.40	24.3 ± 1.71	15.0 ± 1.55
Marasmius sp. KUM 20067	25.2 ± 1.65	6.40 ± 0.70	13.7 ± 1.04	7.84 ± 1.35	14.6 ± 3.51	7.08 ± 0.66	32.0 ± 2.59
M. ruforotula KUM 20111	47.2 ± 2.04	7.77± 0.24	27.08 ± 2.18	17.4 ± 2.28	37.0 ± 3.72	34.6 ± 2.22	20.6 ± 1.14
M. ruforotula KUM 20112	46.9 ± 0.84	7.60 ± 0.66	25.40 ± 1.32	16.4 ± 1.97	35.5 ± 3.58	33.4 ± 2.10	22.9 ± 2.19
M. selangorensis KUM 20181	46.9 ± 2.20	7.91 ± 1.10	7.17 ± 1.18	6.57 ± 0.96	33.4 ± 3.67	18.3 ± 1.81	10.4 ± 2.09

4.3 Antioxidant Activity of *Marasmius* spp.

The crude dichloromethane extracts of selected *Marasmius* spp. were tested for antioxidant activities. Antioxidant activity was monitored using three different assays:

i. Diphenyl picryl hydrazyl (DPPH) Radical Scavenging Assay.

ii. Reducing Power Assay.

iii. Metal Chelating Assay.

4.3.1 Diphenyl picryl hydrazyl (DPPH) Radical Scavenging Activity

Using this assay, antioxidant activity of mushroom extracts were assayed based on the scavenging activity against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals. Free radicals with odd electron, gave a strong absorption band at 515 nm with a deep violet colour. As these free radicals were scavenged and become paired off, DPPH will generate a yellow colour and the absorption vanishes. The decrease in absorbance is proportional to the number of DPPH molecules being scavenged. The change in absorbance produced in the reaction is quantitated to evaluate the antioxidant potential of extracts. Effectiveness of antioxidant properties were inversely correlated with their EC_{50} values. The antioxidant activity was measured based on the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (EC_{50}; unit = mg extract/ml methanol). With regards to EC_{50} value the lower the EC_{50}, the higher the antioxidant power. Steady state was considered to be achieved when further decrease in absorbance values were no longer significant, as determined statistically by one way analysis of variance (ANOVA).

Scavenging effects of each hot dichloromethane extract of *Marasmius* spp. on DPPH radicals was found to be concentration-dependent, with stronger inhibition occurring at higher concentrations. The reaction kinetics and graphs of different lengths of time (minutes) taken by the dichloromethane extracts of *Marasmius* spp. at various concentrations to react with DPPH radicals to a steady state was illustrated by the graphs shown in Appendix B. As illustrated in Appendix B, all extracts took approximately 60 minutes to reach a steady state and exhibited slow kinetic behaviour.

In this assay, L-ascorbic acid and BHA were the commercial standards used as positive controls in the present study. Ascorbic acid ($C_6H_8O_6$) with a molar mass of 176.16 g/mol was observed to react quickly with the DPPH radical in a dose dependent manner. Appendix B shows the scavenging activity (%) of DPPH radicals by ascorbic acid and BHA at concentrations of 0.01, 0.02, 0.05, 0.08, 0.10, 0.15 and 0.2 mg/ml. At 0.2 mg/ml the scavenging activities of ascorbic acid and BHA on DPPH radicals were very good at 91.4% ± 0.001 and 85.8% ± 0.004, respectively. The positive controls, ascorbic acid and BHA reacted rapidly with DPPH, indicating a rapid kinetic behaviour.

The data and graphs for the determination of time (minutes) required for the BHA and ascorbic acid to react with DPPH radicals to reach a steady state are illustrated in Appendix B. Dose-response graphs of scavenging activities of dichloromethane extracts from *Marasmius* spp. on DPPH radicals are shown in Appendix B. According to the dose-response graphs, DPPH radicals scavenging ability of *Marasmius* extracts were dose-dependant, with higher percentages of DPPH radical being scavenged at higher concentrations of the extracts. About 30% of DPPH radicals were scavenged by *M. ruforotula* (KUM 20112) at concentration of approximately 55 mg/ml. However, higher concentration of about 150 mg/ml was needed by *Marasmius* sp. (KUM 20067) to scavenge about 49.74 % DPPH radicals in Appendix B.

For crude extracts of *M. ruforotula* (KUM 20111) and (KUM 20112), *M. guyanensis* (KUM 20117, KUM 20222) and (KUM 20044), *M. kanchingnensis* (KUM 20160), *M. selangorensis* (KUM 20181) and *Marasmius* sp. (KUM 20067) the scavenging activity on DPPH radicals ranges between 25.03 %, 21.81%, 19.48%, 25.79%, 15.64%, 29.63%, 20.10% and 13.26%, respectively when tested at 40 mg/ml. Extracts from the eight *Marasmius* spp. showed different degrees of scavenging ability against DPPH radicals. Among them, the most potent DPPH radical scavenging ability was exhibited by *M. kanchingnensis* (KUM 20160).

As seen with the EC_{50} values shown in Table 4.3, it is clear that the eight dichloromethane of *Marasmius* extracts possessed varying degrees of free radical scavenging capabilities. The scavenging activities of all the dichloromethane *Marasmius* extracts differ significantly from each other (ANOVA, $P < 0.05$). Among the eight fractions, dichloromethane extract of *M. kanchingnensis* (KUM 20160) showed the highest scavenging activity with an EC_{50} value of 67.49 mg/ml followed by *M. guyanensis* (KUM 20222) with an EC_{50} value of 77.56 mg/ml. *Marasmius* sp. (KUM 20067) with an EC_{50} value of 150.78 mg/ml, exhibited the weakest ability to scavenge DPPH. *Marasmius ruforotula* (KUM 20111) and (KUM 20112), *M. selangorensis* (KUM 20181) had EC_{50} values of 79.90 mg/ml, 91.70 mg/ml, and 99.50 mg/ml, respectively (Table 4.3). The EC_{50} value of ascorbic acid obtained was much lower, 0.08 mg/ml while that of BHA was 0.107 mg/ml.

Table 4.3: EC_{50} values of dichloromethane extracts of *Marasmius* spp. BHA and ascorbic acid in DPPH scavenging assay.

Species code	#Time (min) reach steady state	*EC_{50} values (mg/ml)
Control: BHA	0	0.11 ± 0.012^{b}
Control: Ascorbic acid	0	0.08 ± 0.005^{a}
M. guyanensis KUM 20044	60	141.80 ± 0.007^{bc}
M.guyanensis KUM 20117	60	102.65 ± 0.037^{a}
M.guyanensis KUM 20222	60	77.56 ± 0.004^{c}
M. kanchingnensis KUM 20160	60	67.49 ± 0.004^{d}
Marasmius sp. KUM 20067	60	150.78 ± 0.015^{a}
M. ruforotula KUM 20111	60	79.90 ± 0.004^{c}
M. ruforotula KUM 20112	60	91.70 ± 0.013^{a}
M. selangorensis KUM 20181	60	99.50 ± 0.022^{ab}

#Raw data for the determination of time (minutes) required for the hot dichloromethane extracts and DPPH radicals to react and reach a steady state are stated in Appendix B. *Each EC_{50} value is expressed as mean standard deviation (n=3). Means with different alphabet letters within a column are significantly different (ANOVA, $p < 0.05$). Raw data for ANOVA and multiple range tests are stated in Appendix B.

4.3.2 Reducing Power Activity

The presence of reductants (i.e. antioxidants) in the extracts leads to the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. The reducing capacity of an extract may serve as an indicator of its potential antioxidant activity.

Figure 4.1 shows the reducing powers (as indicated by the absorbance at 700 nm) of eight dichloromethane extracts from *Maramius* spp. compared with BHA and ascorbic acid as positive standards. As illustrated in Figure 4.1, ascorbic acid and BHA showed excellent reducing powers of 1.881 nm and 2.539 nm, respectively at 10 mg/ml. Based on the results shown in Figure 4.1, *M. selangorensis* KUM 20181 showed good reducing power ability in relative to BHA and ascorbic acid at all concentrations of extracts (5, 10, 15 and 20 mg/ml). The reducing power of *M. selangorensis* (KUM 20181) is the highest reaching peak absorbance of 1.57 nm corresponding at 10 mg/ml. At higher concentration the change in absorbance was insignificant (Figure 4.1). *Marasmius kachingnensis* (KUM 20160), *Maramius sp.* (KUM 20067) and *M. guyanensis* (KUM 20044) exerted moderate reducing powers abilities whereas; the lowest reducing power was exhibited by *M. guyanensis* (KUM 20222) with 0.116 nm at 10 mg/ml. The reducing powers of *M. ruforotula* (KUM 20111), *M. guyanensis* (KUM 20044), *Marasmius* sp. (KUM 20067), *M. kanchingnensis* (KUM 20160), *M. guyanensis* (KUM 20117) and *M. guyanensis* (KUM 20222) were 0.871 nm, 1.144 nm, 1.387 nm, 1.519 nm, 0.546 nm and at 0.157 nm at 20 mg/ml respectively.

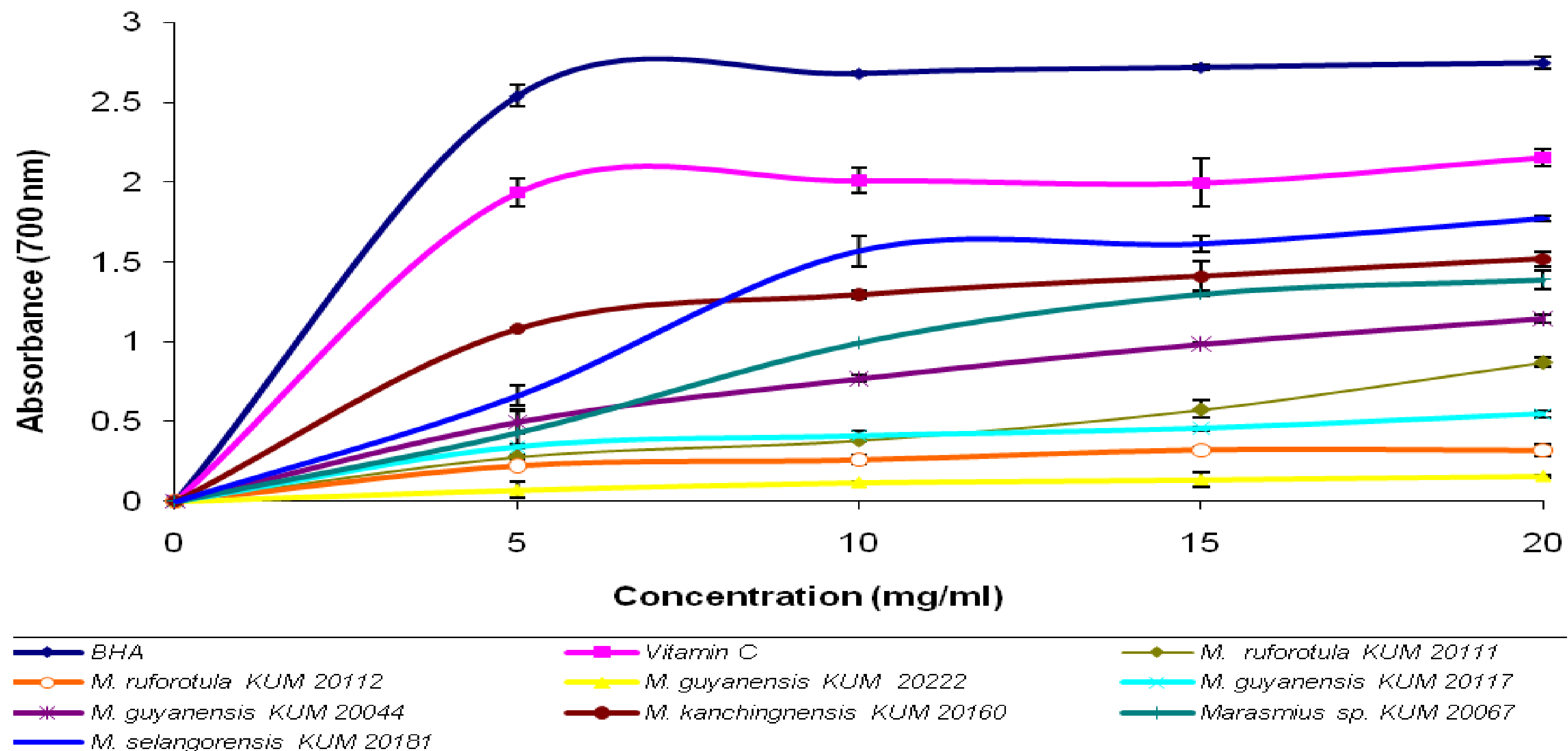

Figure 4.1 Reducing power of dichloromethane extracts from *Marasmius* spp. compared with BHA and ascorbic acid as standard. Each value is expressed as mean±standard deviation of triplicate measurements (n = 3).

4.3.3 Metal Chelating Activity

Metal chelating assay measures the reduction of Fe^{3+}/ferricyanide complex to the ferrous form occurs due to the presence of reductants in the test sample. Absorbance of Fe^{2+} can be measured at 700 nm. The metal chelating efficiency of samples was determined by comparing with the chelating activity of EDTA.

As illustrated in Figure 4.2, chelating effects of the eight dichloromethane extracts of *Marasmius* spp. on ferrous ions increased slowly with increasing concentrations (1, 2, 3, 4 and 5 mg/ml). EDTA exhibited the strongest and excellent chelating ability of 95.7% chelating effect on ferrous ions at a concentration as low as 1 mg/ml, which is significantly higher than all of the eight extracts.

Figure 4.2 show that when compared with EDTA, all extracts possess low chelating ability. Chelating effects of *M. selangorensis* (KUM 20181) on ferrous ions in relative to EDTA showed an increase of 25.85% at 1 mg/ml to 29.88% at 2 mg/ml and maintained a plateau of 33.87% - 35.95% at 3 - 5 mg/ml (Figure 4.13). *Marasmius selangorensis* (KUM 20181) exhibited a better chelating effect when compared to the other extracts.

The chelating abilities of the rest of the extracts are considered weak. The chelating abilities of the eight extracts were 25.99%, 21.90%, 32.05%, 29.82%, 23.77%, 25.86% and 34.01% at 5 mg/ml for *M. ruforotula* (KUM 20111), (KUM 20112), *M. guyanensis* (KUM 20222), (KUM 20044) and (KUM 20117), *Marasmius* sp. (KUM 20067) and *M. kanchingnensis* (KUM 20160) respectively. However among these extracts, chelating activities of *M. selangorensis* KUM 20181 and *M. kanchingnensis* KUM 20160 were slightly better than those of the other extracts.

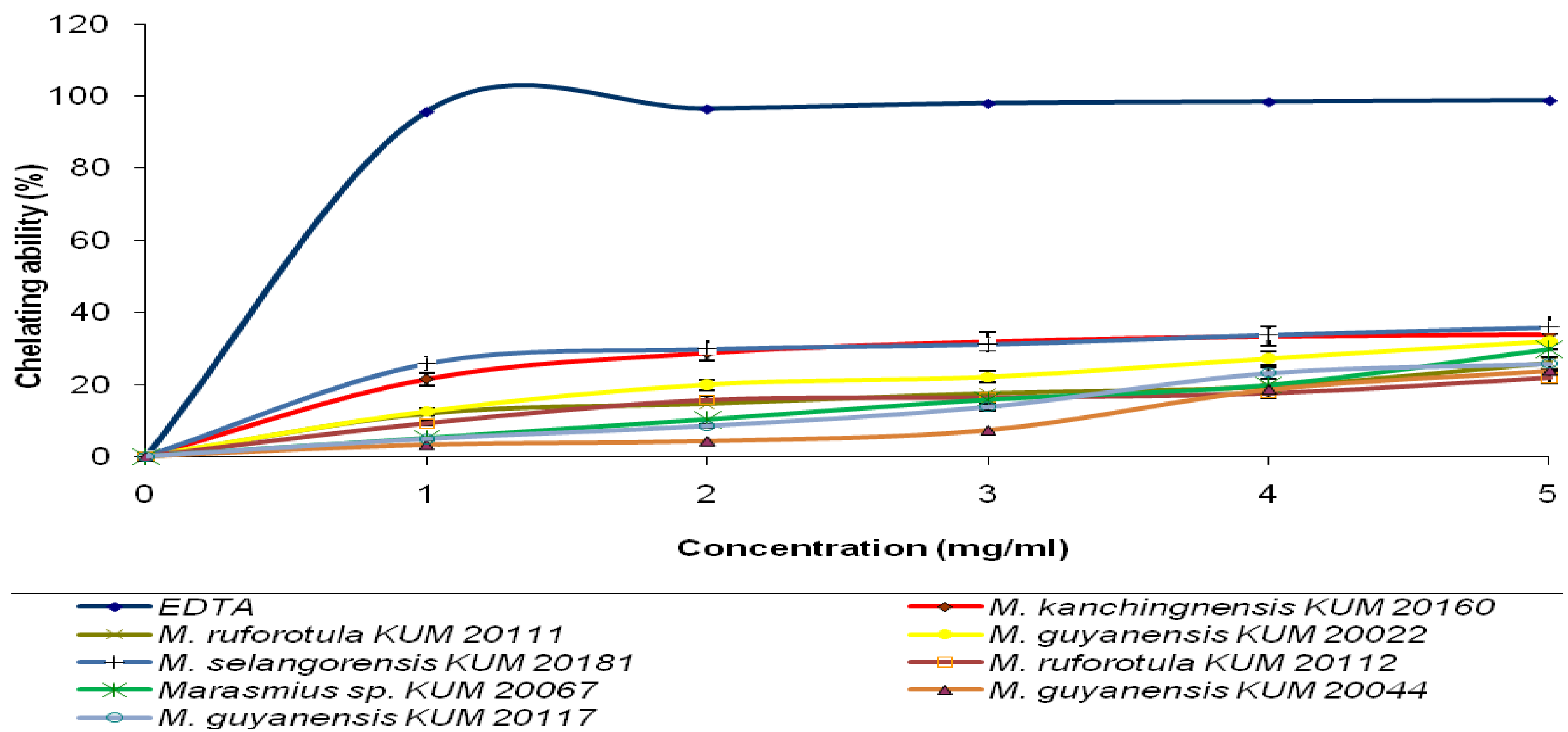

Figure 4.2 Chelating effects of dichloromethane extracts from *Marasmius* spp. compared with EDTA as standard on ferrous ions. Each value is expressed as mean±standard deviations of triplicate measurements (n = 3).

CHAPTER 5

DISCUSSION

5.1 Yield of *Marasmius* Crude Extracts

In this study, eight species of *Marasmius* were cultured and subsequently extracted using dichloromethane. They were grown by submerged fermentation in liquid GYMP for 14 days. The rates of growth of each species tested were different as shown by the weight of dried mycelia obtained, which varies from 11.27 g/L - 23.40 g/L with *M. ruforotula* (KUM 20111 and KUM 20112) exhibited highest growth density. The growth development of fungi is dependant on factors such as temperature, pH, light and oxygen (Ingold & Hudson, 1995). Some strains of wild mushrooms may develop poorly in an artificial environment and this might have influenced the ability of the mushrooms to produce active substances exhibiting potential medicinal properties. In other words, different species may exhibit different optimum mode of growth against the environmental factors.

The percentage yield of hot dichloromethane extracts of the eight selected *Marasmius* spp. ranged from 1.12% - 3.87% as shown in Table 4.1. Eight selected *Marasmius* spp. were extracted using a soxhlet extractor system. The soxhlet extraction was widely used for the extraction of plant materials using heat and a selected solvent. In this study, the dried mycelia biomass from each of the *Marasmius* spp. was extracted with dichloromethane solvent. Application of heat during extraction may help to increase yield of extraction (Houghton & Raman, 1998). Though widely used, there are limitations to the soxhlet method; the soxhlet extraction system is restricted to usage with pure extraction solvent, as mixture of solvents will produce vapour with a different composition to the liquid solvent in the lower flask. The advantage of this system is that

instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled therefore the amount of solvent used will be significantly reduced.

The extraction process is considered complete when the colour of extract laden solvent emitted from the soxhlet becomes clearer. This is an indication that all the extract from the biomass has leached out. To avoid further loss of extract weight after evaporation, empty bottles where final evaporation take place were weighed beforehand so that the final yield of extract can be calculated easily without having to transfer the extract into another bottle after evaporation.

All eight *Marasmius* spp. in this study were grown on the same GYMP agar formula for cultivation. However, the rate of growth and production of bioactive compound can be further optimized by culturing the *Marasmius* spp. on different media composition because different species vary greatly in their preferences to certain agar nutrient. *Marasmius* spp. with slow growing mycelium on GYMP agar might grow better in different types of agar nutrient as the use of different types of media may help the mycelium to better suit for survival.

5.2 Cytotoxic Activity of *Marasmius* spp. against Cancer Cell Lines

Cytotoxic assays are widely employed in *in vitro* toxicology studies. Cytotoxicity is one of the chemotherapeutic targets of anti-tumour activity (Suffness & Pezzuto, 1991). The most commonly employed cytotoxicity assays for the detection of cell viability following exposure to toxic substances are the lactate dehydrogenase leakage (LDH) assay, a protein assay, the methyl tetrazolium (MTT) assay and the neutral red (NR) assay. Different cyctotoxicity assays can give different results depending on the test agent used and the duration of exposure (Weyermann *et al*.,

2005). Previous studies revealed that the NR and the MTT assay are the most sensitive cytotoxicity assays.

MTT assay gives satisfactory responses using cell membrane damaging agents like triton X-100, but can be misleading if the toxic agent influences intracellular activities e.g. sodium azide which inhibits the respiratory chain. The MTT assay, which is dependent on enzymatic reactions, might be influenced by enzyme inhibition like chloroquine. According to Fotakis and Timbrell (2006), the MTT assay is greatly influenced by the availability and metabolism of glucose by the bioindicator cell and thus the MTT specific activity will vary among cell lines.

Although both assays yield comparable ranking of cytotoxicity data, the optical density absorbance with the NR assay is about twice that obtained with the MTT assay, and thus require fewer cells for analysis (Borenfreund & Puerner, 1985). Therefore, for these reasons, the NR was used to evaluate the cytotoxicity potentials of the eight *Marasmius* extracts against human cancer-derived cell lines; CaSki, KB, HT 29, HCT 119, SKOV 3, MCF 7 and also on normal human fibroblasts cell MRC 5. The NR assay is based on the initial protocol described by Borenfreund and Puerner (1984). It determines the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells. Damage to the cell surface or sensitive lysosomal membranes decreases the uptake and binding of the NR (Lullmann-Rauch, 1979), making it possible to differentiate between viable intact cells and dead/damaged cells. This assay has been considered the gold standard for determining viability of cells *in vitro* because it is rapid, highly stringent and inexpensive method to measure cell death (Weyermann *et al.*, 2005).

The NR assay was performed after 72 hours of treatment of the *Marasmius* extracts on the CaSki, KB, HT 29, HCT 119, SKOV 3, MCF 7, MRC 5 cell lines. Research done by Riddell *et al.*, (1986) suggested that the 72 hours treatment period is

more suitable for NR method as compared to the 24 hours treatment. This is because the latter may not be sufficient and therefore result in an unacceptable number of false negative indications of cytotoxicity. Certain bioactive compounds may need longer time to exert their cytotoxic effects particularly those that inhibit cell division or affect cell viability through other long-term effects.

From time to time the culture was observed under microscope to ensure that the cells achieve 60 - 70% confluence in growth. It is best to have 60 - 70% confluency at the time of testing so that the cells are fully exposed to the testing agents (Borenfreund & Puerner, 1986). Borenfreund and Puerner (1985) also reported that it was advantageous to pre-incubate NR medium overnight at 37°C to remove fine precipitate and dye crystals which might appear in the mixture. The deposition of NR crystals onto the cell cultures need to be eliminated prior to the incubation period as it will interfere with the assay. In the present study, NR medium was firstly pre-incubated overnight at 37°C and then centrifuged for 10 minutes at 1,000 rpm twice right before use to facilitate the removal of the dye crystals. After incubation with the NR, the cells were rapidly rinsed with washing solution to remove extracellular NR, as well as to prevent detachment of cells during the subsequent extraction procedure (Borenfreund & Puerner, 1986).

Anti-tumour substances have been identified in many mushroom species. Basidiomycetes mushrooms were reported to produce various classes of primary and secondary metabolites. These mushroom metabolites are usually used as adaptogen and immunostimulants and they are now considered to be one of the most useful anti-tumour agents for clinical uses (Jose & Janardhanan, 2000). Maitake-d-fraction, an over-the-counter immunostimulator compound derived from Maitake's β-1,3-d-glucans and β-1,6-dglucans, made news in 1998 when the Food and Drug Administration (FDA) approved the application for Investigational New Drug (IND) from the company

that produces it, Maitake Products, Inc. of New Jersey, to conduct a phase-2 clinical trial using Maitake d-fraction as an oral agent for advanced breast and prostate cancer patients. This company also developed a new medicinal mushroom preparation from Maitake (SF-Fraction-Glycoprotein), which helps maintain healthy cardiovascular functions and a healthy circulatory system (Elinoar, 2009). In Japan, an immunomodulator compound derived from Maitake β-glucans called Grifolan, a branched β-1,3-d-glucan extracted from *Grifola frondosa* was found to promote tumor regression and necrosis, and was approved to be used in the treatment of cancer (Mao *et al*., 2007).

The search for new anti-tumour substances from mushrooms continues. In the present study, the screening of cytotoxic activity in *Marasmius* was carried out at a concentration of 20 µg/ml. Extracts having an ED_{50} values less than 50% inhibition at 20 µg/ml is considered not cytotoxically active hence no further testing is required (Geran *et al.,* 1972). The results collected from this study revealed that all of the extracts were not actively cytotoxic against all the cancer cell line tested including normal human fibroblast, with percentage of killing less than 50% at 20 µg/ml. Therefore, no ED_{50} was registered at the concentration evaluated and no further testing was carried out on them.

Though not considered actively cytotoxic, the *Marasmius* extracts were compared based on the inhibition percentage that they produce. *Marasmius ruforotula* (KUM 20111) with the percentage inhibition of 47.2% followed by *M. ruforotula* (KUM 20112) and *M. selangorensis* (KUM 20181) with 46.9% respectively, are the four *Marasmius* extracts that produced relatively higher inhibition percentages against KB cell line at 20 µg/ml. Overall results also showed that KB, SKOV 3 and HCT 119 cell line were more susceptible to *M. ruforotula* (KUM 20111) extracts as compared to HT 29, CaSki and MCF 7 cells. *M. guyanensis* (KUM 20044) produced highest

percentage of inhibition towards SKOV 3 cell line as compared to other cell lines evaluated in this study. HT 29 cell line seemed to be more susceptible to *M. guyanensis* (KUM 20222) with the percentage inhibition of 40.75% as compared to the other cell lines where the percentages of inhibition were found less than 40%. As for CaSki cell line, *Marasmius* sp. (KUM 20067) demonstrated a percentage of inhibition of 32.0% at 20 μg/ml.

The overall results however did not totally indicate that they do not contain anti-tumour properties. They might exert their anti-tumour action through other mechanisms. It can be further investigated for other anti-tumour mechanisms, which will be useful for mechanistic studies in the future. Clinical studies on the active metabolites isolated should be carried out to elucidate and understand the toxicity level and mechanism(s) of action of the *Marasmius* extracts at the molecular and biochemical level. Extracts that did not show potent cytotoxic activity against a particular cancer cell can be tested against other cancer cell lines.

Based on the present study, all the *Marasmius* extracts though not considered cytotoxically active, they demonstrated varying degrees of inhibition against the cancer cells tested. Different types of cancer cell line showed different sensitivities to different *Marasmius* extracts. For *M. ruforotula* (KUM 20111 and KUM 20112), the percentage inhibition was high against KB cells however the percentage inhibition was low for MCF 7. In previous study of other mushroom; *Pleurotus ostreatus* exerts its anti-proliferative activity towards HT29 cancer cells and is significantly less effective towards normal human fibroblast (Lavi *et al*., 2006). Similar to this study, *Marasmius* dichloromethane extracts also possess ultimately less inhibition percentage towards MRC 5 normal cells. Therefore it is necessary to evaluate the cytotoxic effect of these *Marasmius* extracts against other types of human cancer in order to confirm its selectivity.

Previous researchers found that mushrooms polysaccharides or polysaccharide-protein complexes are the anti-tumour active fractions (Hobbs, 1995). Polysaccharides are mainly present as glucans with different types of glycosidic linkages such as (1→3), (1→6)-beta-glucans and (1→3)-alpha-glucans, and as true heteroglycans, while others mostly bind to protein residues as polysaccharide-protein complexes (Ooi & Liu, 2000). Higher anti-tumour activity is said to be correlated with higher molecular weight, lower level of branching and greater water solubility of β-glucans (Lindequist *et al.,* 2005). Since polysaccharides are soluble in water, they might not be fully extracted out in the dichloromethane extract. Thus, the dichloromethane extracts in the present study may not contain effective amounts of polysaccharides to exert anti-tumour activity.

The anti-tumour mechanisms from *Marasmius* spp. may not be directly cytocidal, but it may be host-mediated. Some mushrooms polysaccharides are known to act as an immune simulator to stimulate effector cells like macrophages, T lymphocytes, B-cells, and NK cells to secrete cytokines like TNF-α, IFN-1, IL-Iβ which are anti-proliferative and induce apoptosis and differentiation in tumour cells (Wasser, 2002). There is evidence that ß–D-glucans induce a biological response by binding to membrane complement receptor type 3 (CR3, alphaMß2 integrin or CD11b/cd18) on immune effector cells. The *Marasmius* dichloromethane extracts do not exert active cytotoxic effect against the cancer cell lines evaluated in this study. However further investigation is warranted to screen for their anti-tumour activity through other mechanisms such as via stimulation of the host immune system. It was reported that lentinan a polysaccharides from *Lentinus edodes* was not toxic to tumour cells but inhibits tumour growth by stimulating the immune system (Wasser & Weiss, 1999).

Cytotoxic activities of *Marasmius* spp. are critically based on their physiochemical structure and may vary with cultivation conditions, isolation methods and other factors. The growth condition can influence the chemical composition and as

a consequence, the nutritional value and the bioactivity of the cultivated mushrooms (Tshinyangu, 1996). Medium is important because it supplies necessary nutrient for the growth of mushroom mycelium. Based on a study conducted by Silva *et al*., (2002), *Pleurotus pulmonarius* was grown on three different substrates; substrate A (cotton waste), substrate B (leaves of *Cymbopogon citratus*) and substrate C (leaves of *Panicum maximum Jacq*.). The results showed a varied chemical composition in the mushroom. *Pleurotus pulmonarius* cultivated on substrate A has higher protein content (29.19%) and fibre (9.0%) compared to substrate B and C. In substrate B, the quantity of minerals was very low in comparison to the other substrates. Therefore, it was suggested that all these parameters might have an effect on the variability of the cytotoxic and/or anti-tumour activities of *Marasmius* extracts.

5.3 DPPH Radical Scavenging Activity of *Marasmius* spp.

Various methods have been suggested to evaluate the antioxidant potential of a sample (Amarowicz *et al.,* 2000). One important mechanism of antioxidation involves the scavenging of proton radicals. DPPH having a proton free radical was used in the DPPH scavenging assay to evaluate the proton-scavenging activity of the *Marasmius* dichloromethane extracts.

The DPPH radical scavenging assay is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. When compared with other lab generated free radicals such as the hydroxyl and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. The ability of antioxidants to scavenge DPPH radicals is due to their hydrogen donating ability (Baumann *et al*., 2002). The evolution of the different reaction kinetics depends on the nature of the antioxidant being tested. Obviously, the extracts contained antioxidant components that could

reduce the DPPH radicals in the test mixture, indicating presence of antioxidants which function as free radical inhibitor or scavenger. Various extracts might react with free radicals, particularly the peroxy radicals which are the major propagators of the autoxidation chain of fat; thereby terminating the chain reaction (Frankel, 1991).

There are three types of kinetics that depend on the nature of the antioxidant being tested. The reaction kinetics is classified according to the time taken to reach steady state. A rapid kinetic behaviour is when the compounds react rapidly with the DPPH radicals in less than 1 minute to reach a steady state. Compounds that reached a steady state after approximately 5 to 30 minutes possess intermediate behaviour. Slower kinetics is when it takes about 1 to 6 hour for the compounds to reach a steady state. The graphs obtained for slower kinetics will all be in hyperbolic curves (Brand-Williams *et al*., 1995). In this study, all the eight dichloromethane extracts exhibited a slow kinetic reaction with the DPPH radicals since all the extracts took about 60 minutes to reach a steady state. As shown in Appendix B, the graphs with absorbance of 517 nm versus time were in hyperbolic curves. Scavenging effects on DPPH by the *Marasmius* dichloromethane extracts was concentration-dependent, with stronger inhibition occurring with increasing concentrations of the extracts.

Ascorbic acid shows rapid kinetic behaviour since each ascorbic acid reduces nearly two DPPH radical molecules (Brand-Williams *et al.,* 1995). In this study, the ascorbic acid acts as a positive control and showed good antioxidant activity with EC_{50} value of 0.078 mg/ml. At 0.2 mg/ml the scavenging activity of ascorbic acid on DPPH radicals was 91.4%. BHA also produces high scavenging ability of 85.8% at 0.2 mg/ml. However, this does not correlate with the results obtained from other previous studies. A study done by Mau *et al.,* (2004) reported that at 0.5 mg/ml the scavenging effect of ascorbic acid was only 41.8% while other studies by Lee *et al.,* (2007) claimed that at 0.1 - 20 mg/ml the scavenging abilities of ascorbic acid with DPPH were 77.9%, 81.0%

and 45.3%, respectively. Molyneux (2004) suggested that the stoichiometry for the reactions between DPPH solution and stock solution should be taken into consideration. Previous literatures showed that different authors used different initial DPPH concentration and reaction time. For this reason, a more accurate comparison of the antioxidant activity among the *Marasmius* with the mushrooms from previous studies can be done if the dilution factor between the DPPH solution and stock solution was taken into consideration.

In the present study, the EC_{50} values of the *Marasmius* were obtained on the basis of the linear regression plot from graphs of percentage of DPPH radicals inhibited at steady state. The EC_{50} is defined as the mount of antioxidants necessary to inhibit the initial DPPH radical concentration by 50% and this is determined from the plotted graph of scavenging activity against the concentration of extracts. However, the EC_{50} values for the *Marasmius* spp. tested were higher than 10 mg/ml. The values ranged from 51.58 mg/ml to 106.0 mg/ml. From previous studies, the EC_{50} values of other mushrooms were lower when compared with the EC_{50} values of *Marasmius* dichloromethane extracts in the present study. In a study conducted by Lee *et al.,* (2007), values of *Hypsizigus marmoreus* ethanolic, cold water and hot water extracts were 24.6, 36.2, 13.4 mg/ml respectively. In a study done by Mau *et al.,* (2004), the EC_{50} values for methanolic extracts from mycelia of *Morchella esculenta*, *Grifola frondosa* and *Termitomyces albuminosus*, were 3.71, 4.95 and 5.04 mg/ml, respectively.

The present study had identified a few extracts that were considered to be active by demonstrating the ability to decrease the initial amount of DPPH radicals. Results revealed that crude dichloromethane extracts of *Marasmius* were free radical inhibitors or scavengers with its antioxidative proton-donating ability. The ability of crude dichloromethane extracts of *Marasmius* to scavenge the stable chemical free radical 1,1-

diphenyl-2-picrylhydrazyl to 1-diphenylhydrazine indicated the H transfer reaction from crude extracts to the stable DPPH radicals.

Radical scavenging activities have been detected in other mushroom such as the methanolic extract from *Agaricus blazei* that exhibited a high scavenging ability of 97.1% at 2.5 mg/ml (Huang *et al.,* 1999). Methanolic extract from *Pleurotus ostreatus* scavenged 81.8% of DPPH radicals at 6.4 mg/ml (Lin, 1999). With regards to hot water extracts, at 0.5 mg/ml, scavenging abilities of *Ganorderma tsugae* and *Agrocybe cylindracea* were 53.8 – 61.2% (Mau *et al*., 2005) and 47.3% (Tsai *et al.,* 2006) whereas at 5 mg/ml, that of *Pleurotus citrinopileatus* was 41.8% (Huang, 2003). At 10 - 20 mg/ml, scavenging abilities of ethanolic extracts of *Agaricus bisporus, Pleurotus eryngii*, *Pleurotus ferulae* and *Pleurotus ostreatus* were 69.2 - 74.4%, 78.7 - 92.2%, 69.5 - 81.9% and 75.5 - 77.9% respectively (Lo, 2005). This shows that the eight *Marasmius* dichloromethane extracts exhibited lower antioxidant activity compared to the other mushrooms from previous studies. This result may suggest that the bioactive compounds of the extract were not well extracted to inhibit the radicals.

In the present study, dichloromethane extracts of *Marasmius* was prepared by the hot extraction method using the soxhlet extractor system, which may contribute to the weak scavenging activity of DPPH radicals. The disadvantages of using the soxhlet extraction method is the long extraction time of up to 24 hours may be needed for full extraction and concentration of the analytes (Sporring *et al*., 2005), large volumes of solvent is required, and possibility of thermal decomposition of the active compounds as the extraction usually occurs at the boiling point of the solvent for a long period of time (Wang & Weller, 2006).

Previous study on other mushroom reported that the hot water extract from mature and baby Ling chih (*Ganorderma tsugae*) were shown to be less effective than the cold methanolic extracts in scavenging activities (Mau *et al*., 2005). Their study

revealed that the bound flavonoid contents of *Lentinula edodes* (shiitake) declined with the increasing both heating time and heating temperature. The DPPH radical scavenging activity of bound compound extract heated at 121°C for 30 minutes was significantly decreased ($P<0.05$) relative to those of raw shiitake or heat treated at 100°C for 15 and 30 minutes of 121°C for 15 minutes (Choi *et al*., 2006). This observation confirmed the evidence in a previous study that natural nutrients could be significantly lost during the thermal processing due to the fact that most of the bioactive compounds are relatively unstable to heat (Choi *et al*., 2006).

5.4 Reducing Power of *Marasmius* spp.

Reducing power assay is often used to evaluate the ability of natural antioxidant to donate electron (Dorman *et al*., 2003;Yildrim *et al*., 2000). For the measurements of the reductive ability, this study investigated the Fe^{3+}- Fe^{2+} transformation using the method of Oyaizu (1986). The presence of reductones (antioxidants) in the test sample would result in the reduction of the Fe^{3+}/ferriccyanide complex to the ferrous form. The Fe^{2+} can therefore be monitored by measuring the formation of Pearl's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates an increase in reducing power (Lih *et al*., 2001). Earlier authors (Duh, 1998; Duh *et al*., 1999; Yildirim, *et al*., 2001) have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, the reducing power capacity is ranked based on absorbance reading at 700 nm whereby higher absorbance reading indicates greater reductive power capacity.

Standard BHA and ascorbic acid possess strong reducing power capacity with the highest absorbance reading of 2.539 nm and 1.881 nm respectively. In comparison, the absorbance reading of crude extracts of *M. selangorensis* (KUM 20181) exhibited the highest reducing power of 1.57 nm at 10 mg/ml. *Marasmius kachingnensis* (KUM 20160), *Marasmius sp.* (KUM 20067) and *M. guyanensis* (KUM 20044) exhibited moderate reducing power abilities respectively nm at 10 mg/ml whereas the rest of the extracts exhibited poor reducing power abilities.

These results revealed that the crude dichloromethane extracts of *M. selangorensis* (KUM 20181) is an electron donor and might contain higher amount of reductone, which could react with free radicals, converting them to more stable products, and terminating radical chain reaction. However, the antioxidant activity of antioxidants have been attributed to various mechanisms among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Diplock, 1997). Like the radical scavenging activity, the reducing power of *Marasmius* spp. dichloromethane extracts increased with increasing concentration.

When comparing the reducing power values of *Marasmius* extracts with other mushroom extracts, the methanolic extract from *Antrodia camphorata* showed an excellent reducing power of 0.96 - 0.97 at 10 mg/ml (Huang, 2000). Methanolic extracts from other medicinal mushrooms including *Ganorderma lucidum* and *Ganoderma tsugae* exhibited a strong reducing power of 0.99 and 1.26 at 2 mg/ml, respectively (Lin, 1999). In another previous study, reducing powers of methanolic extracts from *Grifola frondosa*, *Hericium erinaceus* and *Tricholoma giganteum* increased slowly with the increased concentrations and were 1.18, 1.01 and 0.63 at 9 mg/ml and 2.50, 1.78 and 1.11 at 24 mg/ml respectively (Mau *et al.,* 2002).

5.5 Metal Chelating Property of *Marasmius* spp.

Antioxidants inhibit interaction between metal and lipid through formation of insoluble metal complexes with ferrous ion (Hsu *et al*., 2003). The iron-chelating capacity assay measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion (Elmastas *et al*., 2006). The chelation of ferrous ions by the extract was evaluated by the method of (Dinis *et al*., 1994). In this assay, the *Marasmius* extracts interfered with the formation of ferrous and ferrozine complex, suggesting that they possess some chelating activity and captured ferrous ion before ferrozine. The absorbance of Fe^{2+}-ferrozine linearly decreased dose-dependently (1, 2, 3, 4, 5 mg/ml). Ferrous ions could stimulate lipid peroxidation by Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1991).

It was reported that chelating agents may serve as secondary antioxidants because they reduce the redox potential by forming σ-bonds with a metal, thereby stabilizing the oxidized form of the metal ions (Gordon, 1990). Accordingly, it is suggested that the low-to moderate ferrous ions chelating effects would be somewhat beneficial to protect against oxidative damage. The data obtained from Figure 4.3 revealed that all the crude dichloromethane extracts of *Marasmius* have a weak capacity for iron binding when compared to EDTA at all concentration tested. Apparently the eight extracts are weak chelator of ferrous ions and not as effective chelators as EDTA. EDTA as the positive control exhibited a significantly high chelating ability of 95.68% at a concentration as low as 1 mg/ml. Based on the results, *Marasmius selangorensis* (KUM 20181) exhibit higher percentage of metal chelating capacity compared to the rest of the extracts.

In previous studies, the percentages of metal chelating capacity of other mushrooms are much higher than what was found in *Marasmius* dichloromethane extracts. Studies done by Huang *et al.,* (1999) found that the methanolic extract from *Agaricus blazei* produced a high chelating ability of 98.6% at 2.5mg/ml. Tsai *et al.,* (2006) recorded that the methanolic extract from *Agrocybe cylindracea* strain B chelated ferrous ions by 90.6% at 5 mg/ml. Ethanolic extracts of *Agaricus bisporus, Pleurotus eryngii, Pleurotus ferulae* and *Pleurotus ostreatus* chelated ferrous ions by 59.5%, 41.4%, 51.0% and 64.0% at 5 mg/ml (Lo, 2005). In another study, the chelating abilities of water extracts from *Pleurotus citrinopileatus* at 5 mg/ml were 46.4%, 66.6% and 82.1% for ethanolic, cold water and hot water extracts from mycelia, respectively (Huang, 2003).

Among other factors contributing in the significant variations of antioxidant effect of *Marasmius* extracts might be due to the different phenolic compositions or the presence of non-phenolic antioxidants (Velioglu *et al*., 1998) and the quantitative and qualitative nature of the phenolic content (Aljadi & Kamaruddin, 2004), which needs further study. This is in the agreement with other researchers who found variations between different classes of phenolics in terms of their antioxidant activities (Hirano *et al*., 2001). Mujić *et al*., (2010) found that high content of total phenols and total flavonoids for *Lentinus edodes* showed relatively strong DPPH radical scavenging effect while the *Hericieum erinaceus* extract which contained lower total phenols than *Lentinus edodes* showed higher reducing power capabilities. These findings are in accordance with a report by Mau *et al*., (2002) who revealed that some other components also existed and contributed in part to the antioxidant properties of medicinal mushrooms. Based on this finding, it is possible to suggest that some other antioxidant compounds not only total phenols are the sole contributor that may account for the antioxidant activities.

Apart from that, antioxidants concentration, extraction medium, temperature, pH of medium (Gazzani *et al*., 1998), chemical structures and position in the molecule (Prior *et al*., 2005) are pertinent factors that could also be taken into account to evaluate the antioxidant activity of an extract. The use of solvent is also important in ensuring that the active compound is being extracted. Solvent extraction is frequently used for isolation of antioxidants because both extraction yield and antioxidant activity of extracts are strongly dependent on the solvent (Marinova & Yanishlieva, 1997).

The types of solvents used play an important role in ensuring the maximum extraction of the mushroom's phytochemicals. There is a general principal where 'like dissolve like' thus non-polar solvents will extract non-polar substances while polar substances will be extracted by polar solvents. Hence, the active compound must be soluble in the solvent used according to polarity. Higher yield of total phenols are obtained by using polar solvents for instance water, methanol and ethanol (Goli *et al*., 2005) compared to non-polar solvents. In addition, Kitzbeiger *et al*., (2007) also found that methanol and ethanol solvent is important to extract out polar components such as sugar, amino acids and glycosides and that most substances that show high antioxidant activity are polar components.

In the present study, dichloromethane solvent was used for the extraction of *Marasmius*. Dichloromethane is a non polar solvent resulting in the extraction of mainly non polar components with low antioxidants (alkaloids, aglycones and volatile oils) (Houghton & Raman, 1998). These compounds are thought to account weak radical scavenging activity. During the hot extraction method, some of the natural antioxidants could be significantly lost because most of the bioactive compounds are relatively unstable to heat. The high temperature affects compounds stability via chemical and enzymatic decomposition, losses by volatilization or thermal decomposition (Choi *et al*., 2006). Thus, optimization of extraction techniques, application of other extraction

methods such as cold extraction and further purification can be carried out in the future. Further studies should be carried out in order to investigate the potential of the *Marasmius* extracts such as screening for their total phenolic content and also for other possible antioxidant and anti-cancer properties in order to have a better understanding on the different antioxidant action mechanisms that might be present in *Marasmius* sample. Besides, isolation and identification of the active compound in dichloromethane extracts of *Marasmius* can be carried out by using gas chromatography, thin layer chromatography (TLC) and high performance liquid chromatography (HLPC) analysis.

5.6 Conclusion

In the present study, all the crude dichloromethane extracts of the eight *Marasmius* were found to exhibit weak cytotoxic activity and varying degrees of inhibition against the human cancer derived cell lines; CaSki, KB, HT 29, HCT 119, SKOV 3, MCF 7 and also on normal human fibroblasts cell MRC 5. The extracts showed low inhibitory activity by producing less than 50% of killing percentages at 20 µg/ml therefore their EC_{50} values could not be determined.

The scavenging activity of the antioxidant compounds showed slow kinetic behaviour with the DPPH radical molecule and reached a steady state at 60 minutes. Overall the EC_{50} values of the dichloromethane extracts of *Marasmius* spp. were found to be higher than 10 mg/ml therefore they exhibited lower antioxidant activity. These EC_{50} values can be further reduced by optimizing the growth medium for the production of mycelia and purification of the extracts is needed to enhance the activity of the compound.

In the present study, all the extracts possessed weak metal chelating activities. In the reducing power assay, *M. selangorensis* (KUM 20181) showed the highest absorbance peak compared to the rest of the extracts. Overall in this study, the

dichloromethane extracts of the eight *Marasmius* spp. showed low activity in all the bioassays tested.