CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

Gallic acid (3,4,5-trihydrobenzoic acid, FM=$C_7H_6O_5$,FW=170.12), ascorbic acid, folin–ciocalteu reagent 2.0N, stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl) and ferrous sulphate ($FeSO_4$) were purchased from Sigma Chemical Co. (Aldrich), catechin hydrate, sodium carbonate (Na_2CO_3), quercetine dihydrate (3,3,4,5,7-pentahydroxyflavone), dimethyl sulfoxide (DMSO, FM=$(CH_3)_2SO$, FW=78.13) from Fisher Scientific, and thiochloroacetic acid (TCA, FM=$C_{13}CCOOH$, FW=163.4) from AppliChem, Darmstandt. All other reagents and solvents used were of analytical grade.

3.2 Preparation of *L. squarrosulus* inoculum and solid substrate fermentation for antioxidant production

Lentinus squarrosulus (KUM50016) was obtained from Mycology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya. Mycelial culture was maintained on Glucose-Yeast-Malt-Peptone (GYMP) agar and stored at 4°C. GYMP was prepared as described in Appendix A. Mycelial inoculum was prepared by inoculating one plug taken from the outer rim of a mycelial colony onto the centre of GYMP agar plates. The inoculated plates were then incubated at 25°C for 5-7 days.

Substrates investigated consist of maize, soya bean and rice supplemented with nitrogen sources consisting of peptone, malt extract and yeast extract. Rice, maize and soya bean were purchased from a local supermarket. White rice was of the Jasmine Sunwhite (Grade: Floral Siam wangi) brand, whereas maize (Grade: USA) and soya bean (Grade: Canada) were of the Kian Hin Dried Condiments brand. All substrates (rice, maize and soya bean) were washed and soaked with tap water for 24 hours and then coarsely ground using mortar and pestle. Approximately 50g of each substrate were put into 250ml Erlenmeyer flasks supplemented with malt extract, yeast extract and peptone separately at the concentrations of 0.04%, 1.0% and 2.0% respectively. All substrate formulations were autoclaved for 20 minutes at 121°C. Five 7mm-in-diameter plugs of 5 to 7 days old inoculum were inoculated into each of the cooled substrates and then incubated at 27°C for 14 days. Substrates without the mycelia plug served as controls. Both fermented and unfermented formulated substrates were lyophilized before antioxidants extraction.

3.3 Liquid fermentation of *L. squarrosulus* for the production of antioxidants

A hundred millilitres of Glucose-Yeast-Malt-Peptone (GYMP) medium (Appendix A) was put into each 500ml Erlenmeyer flasks. The flasks were then covered with non-absorbent cotton plugs and aluminium foil and autoclaved for 20 minutes at 121°C. Ten 7mm-in-diameter plugs of 5 to 7 days old inoculum were inoculated into the cooled medium and then incubated at 27°C for 14 days.

3.4 Extraction of antioxidants using methanol and dichloromethane

All fourteen day old *L. squarrosulus* fermented rice, maize and soya bean substrates was supplemented with malt extract, yeast extract and peptone separately at different concentrations (0.04%, 1.0% and 2.0%) was broken up and soaked in 200ml methanol in a 500ml Erlenmeyer flask. The mycelia grown in the GYMP medium and supplemented with 0.04% peptone was also soaked in the methanol. The mixture was shaken in a rotary shaker rotating at 100 rpm at room temperature 37°C for 48 hours. The mixture was then filtered through a Whatman No. 1 filter paper. The methanolic supernatant collected was subsequently rotary-evaporated using Buchi Rotavapor R-114 at 50°C to yield total soluble antioxidant extracts. Dichloromethane (DCM) extract was prepared similarly, replacing methanol as the solvent. All the methanolic and DCM extracts were stored at -20°C until DPPH analysis.

3.5 Determination of antioxidant capacity using DPPH (2,2-Diphenyl-1-picrylhydrazyl) free radical scavenging activity

The ability of the extracts to donate a hydrogen atom was assessed on the basis of the scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical according to a procedure based on Williams *et al*. (1995). The hydrogen atom or electron donation ability of the antioxidant compounds was measured from the bleaching of the purple-coloured methanol solution of DPPH. Methanolic and dichloromethane antioxidants extracted from solid substrate fermentation of *L. squarrosulus* on rice, maize and soya bean supplemented with nitrogen sources (malt extract, yeast extract and peptone) were analyzed for the DPPH radicals scavenging ability. Stock solutions of methanolic and

dichloromethane extracts were prepared by weighing 0.05g in 1ml methanol to obtain a concentration of 50mg/ml. For the ascorbic acid (2mM), quercetin dihydrate (0.1mM) and BHA (1mM) stock solution was prepared by adding 0.0035g of ascorbic acid powder into 10ml methanol.

Briefly, 3.9ml of 0.06mM DPPH solution was added to 0.1ml of methanol diluted crude extracts to obtain final concentrations of 5-300mg/ml. The mixture was mixed vigorously and the absorbance was then measured at 515nm against methanol as the blank. Triplicate tubes were prepared for each extract. 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 the steady state was determined by one-way analysis of variance (ANOVA) to compare the decrease in absorbance values. The DPPH scavenging activity was expressed as percentage radical scavenging activity and calculated as follows:

% radical scavenging effect, RSE = Ac – As / Ac*100

Where, RSE: Percentage radical scavenging activity, Ac: Absorbance of 0.006mM methanolic DPPH and As: Absorbance of methanolic DPPH with sample extract

The extract concentration exhibiting 50% radical scavenging effect (RSE) (IC_{50}) was calculated from the graph of percentage RSE versus extract concentration. IC_{50} value (mg extract/ml methanol) is the effective concentration at which 50% DPPH radicals were scavenged and was obtained by interpolation from linear regression analysis. Antioxidant activity was compared with ascorbic acid, BHA and quercetin dihydrate (0.1mM-1.0mM) as a positive standard. All the determinations were performed in triplicates.

3.6 Inhibition of lipid peroxidation of selected antioxidant extracts using buffered egg yolk and cooking oil

Lipid peroxidation can be evaluated by the thiobarbituric acid (TBA) reactive substance method. This method evaluates the inhibition of lipid peroxidation assayed for malondialdehyde (MDA), the final product of lipid breakdown. Determination of MDA level in proteins was used as an index of the extent of lipid peroxidation. The crude extract, which showed the highest DPPH scavenging ability was analysed for the inhibition of lipid peroxidation. In the lipid peroxidation assay of egg yolk, reaction mixture contained 1ml egg yolk (Brand: Telur Megaria from Liang Kee Farming) emulsified with 1M phosphate buffer, pH=7.4 and final concentration of 25g/l buffered egg yolk. Then, 100µl of 1000µM $FeSO_4$ and 100µl of diluted extracts (1-30mg/ml) were added into the reaction tubes. Each set was prepared in triplicates. The mixture was incubated for 1 hour at 37°C and later treated with freshly prepared 0.5ml 15% thiochloroacetic acid (TCA) and 1.0ml 1% thiobarbituric acid (TBA). The tubes were incubated in the boiling water bath for 10 minutes at 100°C to develop the rose-pink colour by the reaction between MDA and TBA. After the incubation process, each tube was centrifuged at 3500rpm for 10 minutes to remove precipitated protein. The formation of MDA-TBA complex was measured by removing 200µl of supernatant and its absorbance was measured at 532nm using a Power Wave X340 microplate reader (Bio-Tek Instruments, Inc.).

Inhibition of lipid peroxidation by BHA, quercetin dihydrate and ascorbic acid were measured as a positive control. A stock solution of 6000µg/ml was prepared separately by dissolving 0.06g each of the positive control in 10ml distilled water. This stock solutions

were then diluted within the range of 5-500µg/ml. The percentage inhibition ratio was calculated using the following equation:

% Inhibition of lipid peroxidation (ILP) = Ao – As / Ao*100

Where, ILP: Percentage ratio of lipid peroxidation inhibition, Ao: Absorbance of methanolic extract and As: Absorbance of methanolic antioxidant extract

The percentage inhibition of lipid peroxidation was plotted against extract concentration in order to determine the effect on MDA-TBA complex formation and the concentration required to achieve 50% inhibition of phospholipid oxidation. The efficacy of inhibiting lipid peroxidation of the extracts was determined using a Power Wave X340 microplate reader (Bio-Tek Instruments, Inc.) at 532nm.

Lentinus squarrosulus extract showing the best antioxidant potential was selected to evaluate the ability to inhibit lipid peroxidation of cooking oil. The dried extract (25mg) was dissolved in 6ml analytical grade dimethyl sulfoxide (DMSO, FM=$(CH_3)_2SO$, (FW=78.13) from Fisher Scientific. Then, the mixture was diluted to the concentrations of 1.0 and 5.0mg/ml accordingly with DMSO and 1ml of diluted mixture was transferred to a measuring flask containing 12ml palm cooking oil (Labour brand from Lam Soon Edible Oils) to yield the final extracts with 13ml in each flask. Palm oil was chosen because it was reported to have ability to reduce 15% of blood pressure compared to olive oil with only 5% (Bill, 2005). The oil supplemented with extracts was then heated with a Bunsen burner for 20 minutes in Erlenmeyer flasks to simulate cooking or frying condition. Besides that, Erlenmeyer flasks containing fresh oil only, fresh oil supplemented with DMSO, fresh oil supplemented with quercetin dihydrate, fresh oil supplemented with ascorbic acid and fresh

oil supplemented with BHA were prepared. After the heating process, 500µl of heated oil was taken out and transferred into screwcap tube and the capacity of extracts to inhibit lipid peroxidation in oil was determined as mentioned in egg yolk assay. The formation of MDA-TBA complex in the aqueous phase (lower part) was measured by sampling out 200µl of its lower part and its absorbance was measured at 532nm. BHA, quercetin dihydrate and ascorbic acid were used as positive controls. The amount of MDA-TBA complex was represented in terms of absorbance and was plotted against sample concentration in order to determine the inhibition of lipid peroxidation in cooking oil.

3.7 Determination of total phenolic content (TPC)

The total phenolic content (TPC) in the selected extract was determined spectrophotometrically according to the Folin–Ciocalteu procedure (Mau *et al*., 2004), using gallic acid as a standard (concentration ranges between 5–25mg per 100ml). The results were expressed as gallic acid equivalents (GAE). Solutions were prepared in distilled water at room temperature. The 250µl of 10% Folin–Ciocalteu reagent was added to 250µl of dried extracts (0.1 mg/ml) in 1.5ml disposable plastic cuvettes and were then mixed. After 3 minutes of incubation, 500µl of 10% sodium carbonate was added. Mixtures were left to stand at room temperature for an hour in the dark before measuring the intensity of the blue colour at 750nm using a spectrophotometer. The determination of TPC for each concentration was done in triplicates and a standard calibration curve was derived using gallic acid with a concentration of 0-10µg/ml. The blank for the test consisted of distilled water and reagent without any extract samples. The absorbance value was converted to total phenolic content calculated on the basis of the gallic acid calibration curve and expressed as gallic acid equivalents (GAE) in mg/g dried extract.

3.8 Statistical analysis

The results were reported in mean ± standard deviation (SD). The significance of differences among treatment means was determined by analysis of variance (one-way ANOVA) using STATGRAPHIC plus for Windows 3.0 system (Statistical Graphics Corp.). P-values less than 0.05 were considered statistically significant. All analyses were performed in triplicates and correlations from regression analysis between the parameters were also determined.