3.0 MATERIALS AND METHODS

3.1 *Auricularia auricula-judae* fruitbodies

Auricularia auricula-judae fresh fruitbodies (Plate 3.1) were obtained from a mushroom farm in Tanjung Sepat, Selangor, Malaysia.

Plate 3.1: Fresh fruitbodies of *A. auricula-judae*

3.2 Preparation of extracts

3.2.1 Hot water extract

The hot aqueous extract was prepared by boiling fresh fruitbodies at 100ºC of *A. auricula-judae* (1kg) previously cut into small pieces (Plate 3.1), in 1000 ml of distilled water for 30 minutes. The hot aqueous extract was collected by filtering out the supernatant from fruitbodies through Whatman No.1 filter paper. The dry weight of filtered supernatant was measured after freeze-drying. The dried hot water extract was stored at 4˚C for further

analyses of antioxidant, total phenolic content, cytotoxicity and anti Human Papillomavirus activity.

3.2.2 Polysaccharide extract and ethanol extract

One kilogram fresh fruitbodies of *A. auricula-judae* were extracted with two liters of 85% ethanol by boiling at 100ºC for three hours and filtered through Whatman No.1 filter paper. The filtered supernatant was evaporated to dryness at 26˚C ± 2˚C and 150rpm for 24 hours to obtain ethanol extract. The separated fruitbodies were boiled with three Liters of distilled water for three hours. After boiling, the mixture of fruitbodies and distilled water was filtered by Whatman No.1 filter paper. Polysaccharides was precipitated out with 500ml ethanol by allowing it to stand for 12 hours (Fan *et al.,* 2006). Polysaccharide precipitate was centrifuged at 3000 g for 5 min to obtain the polysaccharide extract (Fan *et al.,* 2006).

3.2.3 Methanol extract

One kilogram of *A. auricula-judae* sliced fresh fruitbodies was soaked in two liters of methanol for two days at 37˚C ± 2˚C (Plate 3.1). The methanol extract was collected by filtration through Whatman No.1 filter paper. Dried methanol extract was obtained by evaporation under vacuum using a rotary evaporator at 30˚C (Hobbs, 1995). Extracts were kept at 4˚C until further analysis.

3.2.4 Dichloromethane extract

The dichloromethane extract was prepared by soaking sliced fresh fruitbodies in two liters of dichloromethane for two days at 37˚C ± 2˚C (Plate 3.1). The fruitbodies was filtered with Whatman No.1 filter paper and the liquid fraction was collected and

evaporated under vacuum at 60˚C (Hobbs, 1995). The dichloromethanolic extract was stored at 4˚C prior to further analyses.

3.3 Cytotoxic Activity of A. *auricula-judae*

3.3.1 Preparation of stock solution

Twenty milligrams extract was weighed and dissolved in 1 ml of Dimethyl Sulfoxide (DMSO, Sigma) in a 1.5 ml provial to give stock solution of 20 mg/ml and the provial were stored at -20 ˚C for further analysis usage.

3.3.2 Cultivation of cell lines by Tissue Culture

Six cancer cells used in this study were cervical cancer cells (CaSki), ovary carcinoma cells (SKOV), colon cancer cells (HCT119), human breast cancer cells (MCF7), human intestinal colon cancer cells (HT29) and human mouth epidermal carcinoma cells (KB). All cells were purchased from the American Type Culture Collection (ATCC), USA

A provial of the cell line was transferred from the liquid Nitrogen tank into a beaker of ice. It was then placed into a 37˚C water bath (Grant Instruments) for quick thawing. The cells were transferred into a centrifuge tube (Falcon) containing 1ml of 20% supplemented medium 199 or Rosewell Park Memorial Institute (RPMI) 1640 in propylene tube and spun at 1000 rpm for 5 minutes . The supernatant was discarded and the cell pellet resuspended in 5 ml of 20% supplemented medium 199 or RPMI 1640 medium and incubated in a tissue culture flask at 37˚C in a 5% CO_2 water-jacketed incubator 37 ˚C.

3.3.3 Subculture of cells

The cells were examined daily using an inverted microscope (Olympus). This is done to observe the signs of bacterial or fungal contamination. The 20% supplemented RPMI 1640 medium or medium 199 in the flask was replaced with fresh 10% supplemented RPMI 1640 medium or medium 199 when colour change was observed. The cells were maintained in 10% supplemented RPMI 1640 medium or medium 199 in a 5% CO_2 incubator. Media change was performed twice-weekly or based on the colour changes from time to time. The cells were subcultured when the cell concentration exceeded 2 x 10^6 per ml.

Adherent cells attached and formed a single layer in a culture flask. The confluent cells were washed twice using PBS (Phosphate Buffer Saline) pH 7.2. The cells were detached from the flask by incubating in 1ml of 0.25% trypsin- Ethylenediaminatetraacetic acid (EDTA) solution and 3ml PBS solution for 5 minutes at 37˚C and then sharply tapped to detach the cells from the flask. The floating cells were transferred into a centrifuge tube and spun for 5 minutes at 1000 rpm. The supernatant was removed and 2 ml of 10% supplemented medium was added to the pellet. The cells were split and transferred into different flasks each containing 7 ml of culture media. The flasks were incubated in a 5% CO_2 incubator at 37 ˚C.

3.3.4 Freezing of cells

The cells with subconfluent or confluent monolayer were supplemented with fresh 10% RPMI 1640 medium or medium 199, 24 hours before freezing. The medium in the tissue culture flask was discarded. The cells then were washed with 10 ml of sterile PBS pH 7.2. The cells were detached from flask as described in section 3.3.3. After

centrifugation, the supernatant was discarded and the cell pellet was resuspended in 3 ml of freezing medium. The cell suspension was aliquoted in each 1ml amounts into 3 provials. The provials were left to stand in ice and kept in a polystyrene box. The box was kept in a -70˚C freezer overnight after which, the provials were transferred into canister and stored in a liquid Nitrogen tank.

3.4 Analysis of cytotoxic activity of *A. auricula-judae* extracts

3.4.1 Serial dilution of mushroom stock solution

About 0.02 g of the mushroom extract was weighed and dissolved in 1000 µl of 10% dimethyl sulfoxide DMSO to produce a stock solution of 20 mg/ml. It was then further diluted with 10% dimethyl sulfoxide to provide final concentration of 100 µg/ml and 50 µg/ml.

3.4.2 Incubation of cell lines with mushroom extract

The cells in confluent monolayer stage were detached from the flask as described in the Section 3.3.3. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 ml of 10% supplemented RPMI 1640, medium 199, Dulbeccos Modified Eagle Medium (DMEM) to obtain a stock cell suspension.

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 haemacytometer (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 haemacytometer was then examined under an inverted microscope and the unstained viable

cells were counted. The cell suspension at a concentration of 3 x 10^4 cells/ml (concentration of 5 x 10^4 cells/ml was used for HT119 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 haemacytometer

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

V_1 : Volume of stock cell suspension required

P_2 : The desired cell concentration in the cell suspension

V_2 : Volume of 10% supplemented McCoys medium used for seeding

200 µl of the cell suspension with known cell density was transferred into every well of a 96- wells plate (Nunc) and incubated overnight in a 5% CO_2 incubator at 37 ˚C. The plate was observed under microscope. The medium was discarded when the cells achieve 60-70% confluence. The cells were then incubated with fresh mushroom stock solution serially diluted in 10% supplemented McCoys medium at one concentration, 20 µg/ml for 72 hours in a 5% CO_2 incubator at 37 ˚C. In this study, 20 µg/ml concentration was used for prescreening method. This is important to test the mushroom extract effectivity towards cytotoxicity. If the results are less than 50 percent of inhibition, than the extracts were considered weak and further evaluation using other concentrations not needed. As for the active extracts were than further evaluated at varying concentrations (1, 10, 25, 50, 75 and 100 µg/ml). All the tests were performed in triplicates and untreated cells with just growth medium served as negative controls. Percentage inhibition were calculated and values were used to plot the dose-response curve. The ED_{50} value was extrapolated from the curve.

3.4.3 Neutral Red Cytotoxicity Assay

After the 72 hours incubation period, the media with or without mushroom extracts was removed and replaced with medium containing 200µl of Neutral Red (NR) into respective wells. The plates were incubated for another 3 hours in a 5% CO_2 at 37 ˚C incubator to allow the uptake of vital dye into the lysosomes of viable cells. After the incubation period, the media was removed and cells were washed with the neutral red washing solution. The neutral red dye was extracted from the viable cells by adding 200 µl of warm resorb solution into every well. It was than left to incubate at room temperature for 30 min in a benchtop incubator (LT Biomax 500). The plate was then gently agitated on a microplate shaker (LT Biomax 500) for 15 minutes and the optical density (OD) was determined spectrophotometrically at 540 nm using ELISA reader (Titertek Multiskan® MCC/340).

The average data from the triplicates for each mushroom extract was expressed in terms of inhibition percentage relative to negative controls. Percentage inhibition was calculated according to the down stated formula. The ED_{50} value refers to the effective dose (concentration of extracts in µg/ml) that inhibited 50 % of cell growth. Extracts having an ED_{50} value equal to or less than 20 µg/ml are considered active for cytotoxicity assay against cells (Geran *et al.,* 1972).

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

$$\textbf{Percentage of inhibition (\%)} = \frac{\textbf{OD control} - \textbf{OD sample}}{\textbf{OD control}} \times 100$$

*OD refers to Optical Density (nm)

3.5 Analyses for Anti- HPV E6 Oncoprotein Activity of *A. auricula-judae*

3.5.1 Cell line

The cell line used in this study was the cervical cancer derived HPV-16 containing CaSki cells**.**

3.5.2 Serial dilution of mushroom stock solution

Two different concentrations were used to study the anti- HPV 16 activity of mushroom extracts. 0.02g of mushroom extract was weighed out and diluted with 1000 µl for the stock preparation. 180 µl was taken from this mixture and diluted with 2820 µl of distilled water for the concentration of 1,200 µg/ml. Further two times and four times dilution with 1500 µl was carried out to provide 100 µg/ml and 25 µg/concentrations respectively.

3.5.3 Incubation of CaSki cells with mushroom extract

One millilitre of each serially diluted two stocks were added into two tissue culture flask containing cultured CaSki cells and two milliliter of fresh 10% supplemented RPMI 1640 medium to provide a final concentration of 100 µg/ml and 25 µg/ml respectively. The cells were left to incubate with the extracts in a 5% CO_2 incubator at 37 ˚C for 72 hours. CaSki cells not treated with mushroom extracts served as negative controls.

3.5.4 Fixation of CaSki cells into slides

Tissue culture flask containing CaSki cells were ready to be subcultured was taken out. The cells were detached from the flask as described in 3.3.3. After centrifugation, the

supernatant was discarded and the cell pellet was resuspended in 1.2 ml of PBS pH 7.2. 30 µl of cell suspension were transferred into every well of three teflon coated slides (Immuno – cell Int).The slides were left to dry overnight at room temperature. The slides were fixed with acetone (System) at -20 ˚C for 10 minutes. The slides were stored at -20 ˚C for few days until the staining process occurs.

3.5.5 Detection of Human Papillomavirus (HPV) E6 protein using immunoperoxidase method

The technique of immunocytochemical staining was carried out using the labelled Streptavidin Biotin Peroxidase kit (LSAB) and DAB substrate system (DAKO) according to specifications described by the manufacturer with some modifications. The washing steps required continuous and constant shaking and incubations with reagents were carried out in a humidified chamber.

The slides were immersed in decreasing concentrations of ethanol (Scharlau) – 100%, 95%, 90% and 80% for 2 minutes each and then washed in PBS pH 7.6 for 10 minutes on the rocker platform (Bellco Biotechnology). The surrounding areas of wells were blotted dry using filter paper (Whatman No.1). 50µl of 3% H_2O_2 was added onto every well of the slides. Incubation was carried out in CO_2 incubator (37 ˚C) for 10 minutes. The slides were washed again with 7.6 PBS (Phosphate Buffer Saline) for 5 minutes on the rocker. The surrounding areas of wells were blotted dry and 30 µl of diluted HPV-16E6 primary antibody (Chemicon), diluted 1:50 with sterile distilled water was added on one end of the slide (four wells). 30 µl of sterile PBS pH 7.6 was added to each of four wells on the other end of the slide. These cells which were untreated with the anti HPV-16E6 primary antibody served as negative controls. The slides were incubated in a benchtop incubator (LT Biomax 500) at room temperature for 1 hour and 30 minutes. After that slides were

washed again, twice with PBS for 15 minutes each time. 45 µl of HRP (Horse Raddish Peroxides) was added on the slides and incubated for 30 minutes. The slides were washed again with PBS for 5 minutes. After blotted dry, 40µl mixture of chromogen and 2ml of DAB was added on the slides and incubated for 20 minutes (35 ˚C). The slides were rinsed with distilled water and then counterstained with Mayer's Haematoxylin solution (Fluka) for 2 minutes (Mizuno *et al.,* 1995). The slides were rinsed with distilled water and then immersed into 30% ammonia solution (Systerm) for 10 seconds. After rinsing procedure, the slides were mounted with prewarmed glycergel (53 ˚C, Dako) and were left to dry overnight in the dark. The stained slides were then visualized under the light microscope (Olympus).

3.6 Antioxidant activity of A. *auricula-judae*

3.6.1 Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical

The scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, were determined by the method described by Brand Williams *et al.,* (1995). Each mushroom extracts (20 – 200 mg/ml) in methanol was mixed with 3.9 ml methanolic solution containing DPPH radicals (Sigma) resulting in final concentration of 0.06 mM DPPH. The mixture was shaken vigorously and left to stand for 60 minutes in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm against methanol as a blank. The decrease in absorbance was recorded at 0,1,2 minutes and every 15 minutes until the reaction reached plateau. The time needed to reach steady state was determined by one-way analysis of variance (ANOVA) to compare the decrease in absorbance values.

The antioxidant activity was compared with L-ascorbic acid (0.1 mM-1.0mM) and BHA (Butylated hydroxyanysole) as positive standards. All determinations were performed in triplicate. The scavenging activity on DPPH was expressed as percentage of radical scavenging calculated as follows:-

$$\text{Scavenging activity (\%)} = \frac{A_0 - A_S}{A_0} \times 100\%$$

A_0 refers to the absorbance of 0.06mM DPPH methanolic solution whereas As is the absorbance of mixture.

3.6.2 Free Reducing Antioxidant Power (FRAP) Assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of each mushrooms crude extracts (0.05 – 0.20 mg/ml) in one ml methanol was mixed with 2.5ml of one percent (w/v) solution of Potassium Ferricyanide (Sigma). The mixture was incubated at 50 ºC for 20 minutes. After incubation, 2.5 ml of ten percent trichloroacetic acid were added, and the mixture was centrifuged at 650 rpm at ten minutes. The upper layer (2.5 ml) was mixed with 2.5 ml of deionised water and 0.5 ml of 0.1 percentage of ferric chloride, and the absorbance was measured at 700 nm against a blank. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. BHA and L-ascorbic acid were used as standards.

3.6.3 Total Phenolic Content (TPC) assay

Phenolic compounds in the mushroom extracts were estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications (Singleton & Rossi, 1965). Briefly, one ml of mushroom extract was mixed with 250 µl of 10% of Folin and Ciocalteau phenol reagent. After three minutes, one ml of saturated sodium carbonate (Na_2 CO_3) solution was added to the mixture and it was adjusted to ten ml with distilled water. The reaction was kept in the dark for 90 minutes at 40 ˚C after which the intensity of blue color was measured at 750nm. Gallic acid was used to calculate the standard curve (1-10µg/ml). The absorbance values were converted to total phenolics content. The calculation was done on the basis of the gallic acid calibration curve and expressed as gallic acid equivalent (GAEs) in mg/g dried extracts. Estimation of the phenolic compounds was carried out in triplicate. Methanol was used as blank; BHA and ascorbic acid were used as positive controls.

3.7 Statistical Analysis

The means of data were subjected to a one way analysis of variance (ANOVA) and the significance of the difference between means was determined by the Duncan's multiple range tests at 95% least significance difference ($p< 0.05$) (Appendix D). P – Values less than 0.05 were considered statistically significant. The data were analyzed by using the STATGRAPHICS plus for Windows Version 3. All analyses was performed in triplicates except for the anti HPV- 16E6 which was done in duplicates.