CHAPTER 3
MATERIALS & METHODS

3.1 Preparation of Aqueous Extract of *H.erinaceus*

The mushroom extract was prepared according to Wong et al. (2007) with slight modification. The fresh fruiting bodies of *H.erinaceus* obtained from Vita Agrotech Sdn. Bhd. (a mushroom farm in Tanjung Sepat, Selangor) were freeze-dried at -50 ± 2 ℃ for 48 hr, ground and stored in air-tight containers. The mushroom powder was soaked in distilled water in the ratio of 1:10 (w/v) for 24 hr (27 ± 2 ℃). The mixture was boiled for 30 min with agitation and then cooled for 30 min. The cooled mixture was filtered using Büchner filtration system, where a vacuum suction was applied, to remove solid particles from the aqueous extract. The filtrate was freeze-dried and stored in air-tight bottles under -20 ℃. Prior to assay, the freeze-dried filtrate was dissolved in distilled water and further diluted in cell culture medium to the required concentration. The extract was then sterilized with a micropore filter of size 0.2 microns prior to use.

3.2 Cell Culture

The neuroblastoma-glioma hybrid NG108-15 was purchased from ATCC (American Type Culture Collection, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM, D5648, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS, PAA Lab GmbH, Austria), 100 U/mL penicillin/streptomycin (PAA Lab GmbH, Austria), 100 µM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine (HAT, Sigma-Aldrich, St. Louis, MO, USA). The human lung fibroblast MRC-5 was grown in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated foetal bovine serum (FBS, PAA Lab GmbH, Austria), 1mM sodium pyruvate (Sigma-Aldrich, St Louis, MO, USA), 1.5 g/L sodium bicarbonate ($NaHCO_3$, Merck, Darmstadt, Germany), 100 U/ml penicillin/streptomycin (PAA Lab GmbH, Austria) and 50 µg/mL

of amphotericin B (PAA Lab GmbH, Austria). The cell lines were cultured at 37℃ in a 5% CO_2 humidified incubator (Shel Lab, Oregon, USA). The medium was changed every 2-3 days as needed (Appendix A, pp.121). The cells were cultured to achieve at least 70% confluence prior to assay.

3.3 Assessment of Cytotoxic Activity of *H.erinaceus* Extract

The toxicity of *H.erinaceus* aqueous extract on the neural hybrid cell, NG108-15 and human lung fibroblast, MRC-5 was tested using MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] is a yellow tetrazolium salt reduced to insoluble purple formazan crystals by the mitochondrial dehydrogenases of viable cells. The normal lung fibroblast was chosen to test for the toxicity of the mushroom, if any, towards a normal cell line of human origin.

The cells were seeded in 96-well flat-bottomed microtitre plates (Orange-Scientific, Braine-I'Alleud, Belgium) at a seeding density of 5×10^4 cells/mL and incubated overnight to allow cell adherence. The mushroom extract of various concentrations was added to the cells and incubated for another 24 hr. MTT solution (5 mg/ml, Appendix B, pp.126) was added to each well and the plate was further incubated for 4 hr for formazan crystal formation. The medium was carefully removed without agitating the formazan crystals formed at the bottom of the wells. Dimethyl sulfoxide (DMSO, D5879, Sigma-Aldrich, St. Louis, MO, USA) was added to each well (100 µL) to dissolve the insoluble purple MTT-formazan crystals. The plate was read using an ELISA microplate reader (EMax®, Molecular Device Inc, USA) at an absorbance of 540 nm with a reference wavelength of 650 nm. The cell viability, expressed as a percentage, was defined as the ratio of absorbance of treated cells to untreated cells. The 50% inhibitory concentration (IC_{50}) was interpolated from the response curve using IDBS XLFit® Software.

3.4 Assessment of Neurite Outgrowth Stimulation in NG108-15 Cell Line

The neurite outgrowth stimulation activity in NG108-15 cell was studied by quantifying the percentage of neurite-bearing cells in culture, followed by measuring the extracellular NGF levels and immunofluorescence staining of neurofilament.

3.4.1 Quantification of neurite-bearing cells in culture

The NG108-15 cell was plated into 6-well poly-*D*-lysine coated (Appendix A, pp.125) cell culture plates (Orange-Scientific, Braine-I'Alleud, Belgium) at a cell density of 8×10^3 cells per well. The mushroom extract and nerve growth factor (NGF, Sigma-Aldrich, St. Louis, MO, USA) were then added to the wells either individually or as combined concentrations of extract. NGF was tested in the concentration range of 5 – 200 ng/mL, whereas the mushroom extract at concentration range 1 – 500 μg/mL. The mushroom extract (0 - 100 μg/mL) combined with NGF at concentrations 5 ng/mL, 10 ng/mL and 20 ng/mL were also tested. Control wells contained only cells with medium. The assay plates were incubated for 48 hr at 37℃ in a 5% CO_2 humidified incubator. After the incubation period, the cells were observed under a Nikon Eclipse TS100 microscope and images were captured with Nikon's Imaging Software, NIS-Elements. The scoring of neurite outgrowth was done with reference to criteria in Wong et al. (2007). Briefly, cells bearing neurites longer than one full diameter of cell body were scored as a positive score. Cell clumps containing more than five cells were excluded. The mean differentiation score was obtained for at least 300 cells in each well. Data are expressed as mean $\pm$ SEM for three experiments.

3.4.2 Measurement of extracellular NGF levels in cell culture

The NGF levels in cell supernatant were measured by enzyme-linked immunosorbent assays (ELISA) using NGF E_{max}® ImmunoAssay System (Promega Corporation, Madison, WI, USA). The assay was carried out according to the manufacturer's protocol.

The NG108-15 cell was seeded in 96-well plates (Orange-Scientific, Braine-I'Alleud, Belgium) at a seeding density of 1×10^4 cells per well and incubated overnight to allow cell adherence. The cells were treated with mushroom extract, or in combination with NGF, for 48 hr. Cell culture supernatant was collected, centrifuged at 1500 ×g for 15 min and maintained at 0 – 4 ℃ prior to assay.

To perform two-site immunoassays, 96-well flat-bottomed ELISA plate was coated with polyclonal NGF antiserum overnight at 4 ℃. The plate was washed the next day and incubated with Block & Sample Buffer (provided in the kit) for an hour to block nonspecific binding. Cell culture supernatant collected from combined treatments (*H.erinaceus* extract + 10 ng/mL NGF) was diluted with Block & Sample Buffer at a ratio of 1:125. Cell supernatant obtained from individual treatment (*H.erinaceus* extract only) was untreated. Prepared samples were added to the plate and incubated in a plate shaker for 6 hr at room temperature. The captured NGF was bound by monoclonal NGF antibody with an overnight incubation at 4 ℃. The amount of specifically bound monoclonal antibody was detected using horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody as a tertiary reactant, which required an incubation period of 2.5 hr. Unbound conjugates were removed by subsequent washing steps and followed by incubation with a chromogenic substrate, which allowed the measurement of colour change. The amount of NGF in the sample is proportional to the colour generated in the oxidation-reduction reaction. The plate was read using an ELISA microplate reader (EMax®, Molecular Device Inc, USA) at an absorbance of 450 nm. The amount of NGF secreted in the well was calculated using a standard curve generated with NGF standard provided in the system (Appendix C, pp.129).

3.4.3 Immunofluorescence staining of neurofilament 200 kDa subunit

The NG108-15 cell was grown in 6-well cell culture plate containing poly-*D*-lysine coated 12 mm glass cover slips (Appendix A, pp.125) at a cell density of 2.5 ×

10^4 cells per well. The mushroom extract and NGF was added as described in section 3.4.1 above. At the end of the incubation period, cells were fixed with 4% paraformaldehyde in warm PBS for 15 min, followed by permeabilization with freshly prepared 1.0% Triton X-100 in PBS for 5 min. The cells were washed once with washing buffer followed by incubation with blocking buffer for 5 min. The cells were then incubated with primary antibody, anti-neurofilament 200 (N4142, Sigma-Aldrich, St Louis, MO, USA) (1:200 dilution in blocking buffer) for 1 hr in a humidified chamber at room temperature. The cells were washed twice with washing buffer followed by reaction with FITC-conjugated sheep anti-rabbit IgG (F7512, Sigma-Aldrich, St Louis, MO, USA) (1:100 dilution in blocking buffer) for 1 hr in dark. The cell nuclei were then counterstained with ProLong® Gold antifade reagent with DAPI (P36931, Invitrogen) and mounted on glass slides for observation.

3.5 Assessment of Neuroprotective Activity of *H.erinaceus* Extract on NG108-15 Cell Line

The neuroprotective effect of *H.erinaceus* extract on NG108-15 cells was evaluated using three assays namely MTT, trypan blue exclusion assay and TUNEL assay.

3.5.1 Assessment of cellular viability using MTT assay

The MTT assay was carried out as mentioned in section 3.3, with slight modifications. Briefly, the NG108-15 cell was seeded in poly-*D*-lysine coated 96-well plates (Appendix A, pp.125) at a cell density of 1×10^4 cells per well and incubated overnight to allow cell adherence. The cells were treated with *H.erinaceus* extract under two treatment modes: pre-treatment and co-incubation. In the pre-treatment mode, the cells were pre-incubated with mushroom extract followed by H_2O_2 exposure for 2 hr. The mushroom extract was introduced to the cells during the 2 hr H_2O_2 exposure in co-incubation mode. The cells were incubated in phenol-red free medium (Hyclone®

DMEM, Catalogue No.: SH30284.01) when H_2O_2 was introduced. MTT solution (5 mg/mL) was added to each well and the plate was further incubated for 4 hr. The medium was carefully removed using a syringe and DMSO was added to dissolve the formazan crystals. The plate was read at absorbance 540 nm, with a reference wavelength of 650 nm. The cell viability, expressed as a percentage, was defined as the ratio of absorbance of treated cells to untreated cells.

3.5.2 Assessment of cellular viability using trypan blue exclusion assay

The NG108-15 cell was plated into 12-well cell culture plates (Orange-Scientific, Braine-I'Alleud, Belgium) at a cell density of 5×10^4 cells per well and incubated overnight to allow cell adherence. The cells were similarly treated with two treatment modes, as mentioned in section 3.5.1, where the cells were either pre-treated with extract prior H_2O_2 damage or both agents were added simultaneously. After the incubation period, the cells were collected and centrifuged at 100 ×g for 5 min. The cell pellet was resuspended with serum-free complete medium and mixed with 0.4% trypan blue in the ratio of 1:1. The cell suspension was examined under phase contrast microscopy using a haemocytometer. Viable healthy cells appear as clear white disks that have excluded trypan blue (unstained cells). Early apoptotic cells exclude trypan blue but with irregular shape or shrunken nucleus. Cells in end-stage apoptosis or necrosis appear as irregular, blue-stained cells or remnants of dead cells. A minimum of 200 total cells were counted and the percentage of viable cells were calculated as follows:

$$\% \text{ viable cells} = \frac{\text{Total no. of live cells}}{\text{Total no. of cells counted}} \times 100$$

3.5.3 Assessment of apoptosis using TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay detects cells that exhibit DNA fragmentation associated with apoptosis.

The TUNEL assay was performed using TUNEL Apoptosis Detection Kit (Millipore Corporation, Temecula, CA, USA) according to the manufacturer's protocol.

The NG108-15 cell was plated in Lab-Tek^{TM} II – CC$^{2\ TM}$ 8-well chamber slide (Nalge Nunc International, Naperville, IL, USA) at a cell density of 2×10^4 cells per well and incubated overnight to allow cell adherence. The cells were treated with mushroom extract (24 hr), H_2O_2 (2 hr), or both in succession. At the end of the incubation period, cells were fixed with 4% paraformaldehyde in 0.1 M NaH_2PO_4 for 15 min, followed by 0.5% Tween®-20, 0.2% BSA in PBS for 15 min. The fixed cells were incubated with TdT end-labelling cocktail in a moist chamber for 60 min. A positive control slide was prepared in parallel by incubating the cells with DNase I in PBS containing Ca^{2+} and Mg^{2+}. After washing with PBS, cells were incubated with blocking buffer for 20 min. This was followed by incubation with avidin-FITC solution in a dark moist chamber for 30 min. The cells were counterstained with ProLong® Gold antifade reagent with DAPI (P36931, Invitrogen) and mounted on glass slides for observation.

3.6 Statistical Analysis

The data were statistically analyzed by one way analysis of variance (ANOVA) and the significant differences between means were determined by Duncan's multiple range test. Values with $p < 0.05$ were regarded as statistically significant (Appendix D, pp.130-146).