CHAPTER 3.0 MATERIALS AND METHODS

3.0 Experimental Plan

The flow chart of the experimental plan and the four objectives are shown in Figure 3.1.

3.1 *Ganoderma neojaponicum* strain

The strain of *G. neojaponicum* (KLUM61076) was collected from Bahau, Negeri Sembilan and deposited at the University of Malaya Culture Collection, Mushroom Research Centre (Kuala Lumpur, Malaysia). The *G. neojaponicum* was maintained as stock culture on malt extract agar (MEA, Oxoid) as in Appendix A1.

3.2 Screening of low cost materials as carbon and nitrogen sources by radial growth measurement

The low cost materials (brown sugar, spent yeast, molasses and corn steep liquor) were investigated as new sources for cultivation medium of *G. neojaponicum* growth using plate agar technique.

3.2.1 Carbon and nitrogen sources; and determination of carbon and nitrogen content

The source of molasses was a by-product of sugar refinery by-product collected from Central Sugar Refinery Sdn Bhd, Shah Alam, Selangor. Meanwhile, the source of spent yeast a by-product of brewery industry was from Carlsberg Brewery (M) Berhad, Petaling Jaya, Selangor. Brown sugar, a product cane industry by-product was obtained from Malayan Sugar Manufacturing Co. Bhd. While corn steep liquor, a by-product of corn industry was purchased from Sigma-Aldrich (M) Sdn. Bhd.
• Experiment 3.2: Screening of low cost materials as carbon and nitrogen sources by radial growth measurement
  Parameters: 1. Carbon sources: Molasses and brown sugar
  2. Nitrogen sources: Spent yeast and corn steep liquor

• Experiment 3.3: Determination of carbon and nitrogen concentrations level using glucose-yeast extract medium by FFD (in shake flask)
  Parameters: 1. Glucose concentration: 0-20% (w/v)
  2. Yeast extract concentration: 0-1.0% (w/v)

• Experiment 3.4: Selection of brown sugar-spent yeast concentrations by RSM (in shake flask)
  Parameters: 1. Brown sugar concentration: 2-10% (w/v)
  2. Spent yeast concentration: 0.02-1.0% (w/v)

• Experiment 3.5: Effect of surfactant on brown sugar-spent yeast medium for G. neojaponicum growth (in shake flask)
  Parameters: 1. Tween 80: 0-1% (v/v)
  2. Vegetable oil: 0-0.2% (v/v)

• Experiment 3.6: Optimisation of selected surfactant (Tween 80) and temperature in shake flask by RSM
  Parameters: 1. Tween 80: 0.1-0.5% (v/v)
  2. Temperature: 22-30 °C

• Experiment 3.7: Optimization of physical parameter on brown sugar-spent yeast medium in 2 L STR by RSM
  Parameters: 1. Temperature: 22-29 °C
  2. Aeration: 0.5-2 vvm

• Experiment 3.8: Profiling of G. neojaponicum using optimum medium composition and physical condition in 2-L STR reactor
  Parameters: 1. reducing sugar, 2. pH, 3. β-glucan, 4. mycelial dry weight

• Experiment 3.9: Evaluation of immunomodulatory properties on dried broth, dried mycelia and polysaccharides of G. neojaponicum via macrophage cell and human colon cancer cell lines
  Parameters: 1. cell proliferation, 2. cytotoxicity, 3. phagocytosis, 3. NFκB activity

• Experiment 3.10: Evaluation of acute toxicity test of dried mycelium of G. neojaponicum

Figure 3.1: The flow chart of experimental plan
The raw materials consisted of brown sugar, spent yeast, corn steep liquor and molasses were analysed for total carbon and nitrogen. The total carbon was determined using furnace method while total nitrogen was analysed using Kjeldhal Method (AOAC, 1980).

3.2.2 Experimental Design and statistical analysis

The selection of suitable combination of carbon and nitrogen sources as low cost substrates was designed by Design Expert software (Minitab Version 16) via full factorial design (FFD). Range of carbon investigated was as determined in Section 3.2.1. Two levels of full factorial designs constructed using MINITAB® software (Minitab Inc. USA) were used after a series of preliminary experiments. Table 3.1 shows the levels of four factors with a total of 60 runs were carried out in the experiment. Each factor was examined at high level (+1) and low level (-1). The experiments were conducted in one block with three center points and three replicates. The response variable was the radial extension rate (mm/day). Details for the factorial design and response surface were as described by Montgomery (1997).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>X1</td>
<td>% C</td>
<td>2</td>
</tr>
<tr>
<td>X2</td>
<td>% N</td>
<td>0.02</td>
</tr>
<tr>
<td>X3</td>
<td>Carbon source</td>
<td>MLS</td>
</tr>
<tr>
<td>X4</td>
<td>Nitrogen source</td>
<td>CSL</td>
</tr>
</tbody>
</table>

Table 3.1: Levels of factors used for selection of low cost materials in FFD

Abbreviations: MLS: Molasses BS: Brown sugar CSL: Corn Steep Liquor SY: Spent Yeast.
3.2.3 Low-cost medium formulation and experimental set up

The carbon and nitrogen content in low cost medium formulation were supplemented with a basal medium. The formulation of low cost media were calculated as Appendix A2-A5. The basal medium was consisted of 4% (w/v) bactoagar, 0.05% (w/v) KH$_2$PO$_4$, 0.05% (w/v) K$_2$HPO$_4$, 0.05% (w/v) MgSO$_4$.7H$_2$O (Appendix A7). The media were mixed and autoclaved at 121 °C for 20 minutes. The sterilized media were poured into petri dishes and allowed to solidify at room temperature.

*G. neojaponicum* inoculum was prepared as shown in Section 3.1 (page 36). One 5-mm disc containing mycelium was cut from the periphery of the colony and inoculated on the media in petri dish. The culture was incubated at 25±2 °C in static condition for seven days.

3.2.4 Measurement of radial growth rate

Radial extension rate was measured daily interval (every 24 hours) as a rate of change in the colony’s radius (mm/day). The size was defined as the average of four diameter measurements along lines crossing at right angles. The readings of colony radius were measured starting from the centre of the disk to the periphery of the colony until one of the strains reached the edge of the Petri dish. Radial growth rate was determined by the slope and intercept of a linear graph. Obtained linear equations were used to model linear growth as described by Baumer *et al.*, (2008).
3.3 Determination of carbon and nitrogen concentrations requirement as for carbon and nitrogen sources using glucose-yeast extract medium in shake flask

The commercial medium containing yeast extract (Merck, Germany) and glucose (Fluka, Germany) were used as reference to determine the carbon and nitrogen concentration requirement for mycelial growth yield of *G. neojaponicum*. The carbon source (glucose) at range of 10-20% (w/v) and nitrogen source (yeast extract) was tested at 0–1% (w/v). The medium was prepared as described in Appendix A6.

*G. neojaponicum* inoculum was prepared as described Section 3.1 (page 36). The inoculum was punched at 5 mm of agar plate (culture plugs) from the periphery of the colony. Ten plugs were inoculated into the 100 mL medium supplemented with basal medium in 250 mL Erlenmeyer flasks. The basal fermentation medium was consisted of 0.05% (w/v) KH$_2$PO$_4$, 0.05% (w/v) K$_2$HPO$_4$ and 0.05% (w/v) MgSO$_4$.7H$_2$O at pH 6.0. The medium was autoclaved at 121°C for 20 minutes. The culture was incubated at 25±2 °C, 160 rpm for six days.

3.3.1 Determination of mycelial dry weight

The culture broth was centrifuged at 10,000 rpm, 4 °C for 10 minutes using refrigerated ultracentrifuge (Beckmann Coulter, Germany). The supernatant was discarded and the filtrate was dried at 50±2 °C to obtain constant weight. The mycelial dry weight was expressed as gram of dry weight per litre (g/L).
3.3.2 Statistical analysis

Data analysis for selection of carbon and nitrogen levels in commercial medium was analysed by Duncan multiple range test via SAS Ver 9.3 (SAS Institute, Cary, USA) and differences between treatment means were considered significant at $P<0.05$.

3.4 Optimization of brown sugar and spent yeast concentrations in shake flask fermentation by RSM

Spent yeast and brown sugar were suitable as carbon and nitrogen sources for *G. neojaponicum* growth as determined from Experiment 3.3. The amount of carbon and nitrogen range for *G. neojaponicum* were set based on Experiment 3.3. The parameters investigated in this experiment were spent yeast and brown sugar concentrations ranges 2-10% (w/v) carbon content for brown sugar and 0.02-0.1% (w/v) nitrogen content for spent yeast.

3.4.1 Experimental design and statistical analysis

Response surface analysis was conducted using Central Composite Design (CCD) for two factors consisting of 39 runs with five centre points and three replicates. Table 3.2 shows the two levels of factors of carbon and nitrogen concentration medium used in this experiment. Each factor was examined at high level (+1) and low level (-1) as stated in Table 3.2. The experiments were conducted in one block with three replicates. The response variable was taken as mycelial dry weight. The determination of mycelial dry weight can be referred to Section 3.3.1 on page 40.
Table 3.2: Levels of factors in CCD for spent yeast and brown sugar

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Factor</th>
<th>Name</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown Sugar</td>
<td>$X_1$</td>
<td>Carbon Content (%) w/v</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g/L</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Spent Yeast</td>
<td>$X_2$</td>
<td>Nitrogen Content (%) w/v</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>g/L</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

3.4.2 Preparation of seed culture as inoculum

Stock culture was prepared as described Section 3.1 (page 36). The seed culture was prepared by inoculating ten plugs of stock culture to the 100 mL medium. The medium contained brown sugar-spent yeast supplemented with basal medium (0.05% (w/v) KH$_2$PO$_4$, 0.05% (w/v) K$_2$HPO$_4$, and 0.05% (w/v) MgSO$_4$.7H$_2$O) in 250 mL Erlenmeyer flask (Appendix A5). The medium was adjusted at pH 6.0 and autoclaved at 121°C for 20 minutes. The culture was incubated at 25±2°C, 160rpm for six days. Ten percent of seed culture (10% (v/v)) was then used as inoculums for subsequent experiment.

3.4.3 Model verification

To verify the model prediction of the response, comparison between experimental value and predicted value were performed under optimal condition as predicted by the model. The five replicates of experimental value were conducted using 6% (w/v) carbon content in brown sugar and 0.075% (w/v) of nitrogen content in spent yeast. Meanwhile, five replicates of predicted value were conducted using 6% (w/v) carbon content in brown sugar and 0.06% (w/v) of nitrogen content in spent yeast.
3.5 Effect of surfactants on brown sugar-spent yeast medium for *G. neojaponicum* growth and yield of polysaccharides in shake flask

The brown sugar (6% (w/v)) -spent yeast (0.075% (w/v)) medium formulation supplemented with basal medium (described Section 3.4.2 page 42) was further enhanced by addition of surfactants for increasing the *G. neojaponicum* growth and its polysaccharides. This experiment was carried out to select between two sources of surfactants namely vegetable palm oil (Seri Murni, cooking palm oil, Malaysia) at range (0-1% (v/v)) and Tween 80 (Sigma-Aldrich, USA) at range (0-1.2% (v/v)). The surfactant levels was selected as described by Dominguesa *et al.*, (2000) and Cassiano *et al.*, (2007).

The preparation of inoculum and seed culture *G. neojaponicum* can be refered to Section 3.4.2 (page 42). Ten percent of seed culture (10% (v/v)) was inoculated to the 100 mL brown sugar-spent yeast medium supplemented with surfactant in 250 mL Erlenmeyer flask (Appendix A5). The culture was incubated at 25±2 °C, 160 rpm for six days.

The determination of the mycelial dispersion of *G. neojaponicum* was observed based on size of the pellet formation in the cultivation flask. The mycelium and broth were separated by ultracentrifugation (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The sediment was washed twice with the same volume of distilled water and freeze dried for two days. This sediment was classified as dried mycelium (M). While, the supernatant was the culture broth of *G. neojaponicum* then also freeze dried for 2 days and classified as dried broth (B).
3.5.1 Extraction of polysaccharides from mycelium and broth of *G. neojaponicum*

The mycelium and broth of *G. neojaponicum* were separated by ultra centrifugation (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The broth was further partially purified using ethanol precipitation. The broth was added to double volumes of 95% (v/v) ethanol and left overnight at 4 °C to precipitate. Subsequently, were dried to remove the residual ethanol with a freeze dryer for 2 days. This extract was classified as ethanol precipitate of extracellular polysaccharides (EPS).

The mycelium was washed twice with the same volume of distilled water and freeze dried. 1 g of the freeze dried mycelium was ground into powder and dissolved with 10 mL of distilled water (1:10 w/v) pre-sterilized at 121 °C for 30 minutes. Double volumes of 95% (v/v) ethanol was added to aliquots of the extract and left overnight at 4 °C to precipitate the ethanolic extract of IPS. This extract was classified as ethanol precipitate of intracellular polysaccharides (IPS). This procedure was carried out as described by Hseih *et al.*, (2005).

3.5.2 Determination of total carbohydrate in dried mycelial (M), dried broth (B) and polysaccharides extracts (IPS and EPS).

The content of total carbohydrate was determined by phenol-sulphuric acid assay according to Dubois *et al.* (1956). 0.1 g of M, B, IPS and EPS were diluted into 1 mL distilled water in test tube. Then, 1 mL of 5% (v/v) phenol solution and 5 mL of concentrated sulphuric acid were added to each tube. The mixtures were allowed to stand at room temperature for 10 minutes. The prepared aliquots were read using spectrophotometer (Cary WinUV, Agilent,
USA) at 490 nm absorbance. The percentage of total carbohydrate was determined using standard curve of glucose as in Appendix B1. The standard curve obtained was a linear graph of glucose concentration ranging from 0-1000 mg/mL.

3.5.3 Determination of β-glucan content in dried mycelial (M), dried broth (B) and polysaccharides extracts (IPS and EPS).

The Mushroom and Yeast β-glucan kit (K-YBGL, Megazyme, Ireland) was used in determining the β-glucan content. The contents of total glucan, α-glucan and β-glucan were calculated as in Appendix D1. For total glucan, 0.1 mL of prepared extract were mixed with exo-1,3,β-glucanase and β-glucosidase in 40 °C of water bath for 60 minutes. Then, 3 mL of glucose oxidase was added to each tube and incubated for another 20 minutes. The aliquots were measured at an absorbance of 510 nm. The content of β-glucans was calculated as a difference between the total glucan and α-glucan. Meanwhile the solubilisation and partial hydrolysis of α-glucan were as described in Appendix C1. For α-glucan, 0.1 mL of prepared aliquots was added to 0.1 mL sodium acetate buffer followed by the addition of 3 mL of glucose oxidase to each tube and incubation at 40 °C for another 20 minutes. The absorbance of the α-glucan solution was read at 510 nm against a reagent blank. The β-glucan content was calculated by subtraction of total glucan and α-glucan as detailed in Appendix D1.

3.5.4 Statistical analysis

The analysis of data for selection of types and concentration of surfactants were performed using Duncan multiple range test by SAS Version 9.3 (SAS
Institute, Cary, USA) and differences between treatment means were considered significant at $P<0.05$.

3.6 Optimisation of Tween 80 concentration and temperature in shake flask by RSM

The range of Tween 80 concentrations was prepared as determined in Section 3.5. The optimisation of Tween 80 concentration and temperature were conducted in this experiment by RSM in shake flask fermentation. The temperature range was set in this study was based on literature study as summarized in Table 2.2 on page 11. The parameters investigated in this experiment were temperature and Tween 80 concentration on spent yeast-brown sugar medium.

3.6.1 Statistical experimental design analysis

This experiment was designed by Central Composite Design (CCD) via Response Surface Methodology (RSM) (Minitab Ver. 16). The experiment was conducted with two factors in one block consisting of 39 runs with five center points and three replicates. Each factor was examined at high level (+1) and low level (-1) as referred to in Table 3.3. Table 3.3 shows the levels of factors used in the experiment. Response variables used in the response surface model were mycelial dry weight and β-glucan content.

Table 3.3: Levels of factors used for Tween 80 and temperature by CCD

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1^a$</td>
<td>Tween 80 Concentration</td>
<td>% w/v</td>
</tr>
<tr>
<td>$X_2^b$</td>
<td>Temperature</td>
<td>°C</td>
</tr>
</tbody>
</table>
3.6.2 Experimental set up

The preparation of inoculum and seed culture can be referred to Section 3.4.2 (page 42). The determination of mycelial dry weight is as described to Section 3.3.1 page 40. The determination of β-glucan content is as illustrated in Section 3.5.3 on page 45.

3.6.3 Model verification

To verify the model prediction of the response, comparison between experimental value and predicted value were performed under optimal conditions as predicted by the model. The five replicates of experimental value were conducted using 0.046% (w/v) at a temperature of 26 °C. Meanwhile, five replicates of predicted value were conducted using 0.05% (w/v) Tween 80 and at a temperature of 26 °C.

3.7 Optimization of physical parameter of growth and polysaccharides yield of G. neojaponicum in brown sugar-spent yeast medium in a 2-L stirred tank reactor (STR) by RSM

Physical condition is an important parameter to be optimized in submerged fermentation. This study selected temperature and aeration rate as the most crucial physical factors for scale-up process for G. neojaponicum as referred to Felse & Panda, (2000). Meanwhile the selection of aeration rate range in this experiment was based on the summary reviewed by Wagner (2003b) while, the selection of temperature for G. neojaponicum as determined in Section 3.6 on page 46.
3.7.1 Experimental design

This experiment was based on the designed by Central composite Design (CCD) via Response surface methodology (RSM) (Minitab Ver .16). Response surface analysis was run on two factors consisting of 39 runs with five centre points and three replicates. Table 3.4 shows the levels of factors used in the experiment. The response variable was mycelial dry weight.

Table 3.4: Levels of factors used in the Central Composite Design for aeration and temperature

<table>
<thead>
<tr>
<th>Factor</th>
<th>Name</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>X₁</td>
<td>Aeration</td>
<td>0.5</td>
</tr>
<tr>
<td>X₂</td>
<td>Temperature</td>
<td>22</td>
</tr>
</tbody>
</table>

3.7.2 Fermentation condition of STR reactor

The *G. neojaponicum* was investigated for upsccaling production using 2-L STR reactor (Biostat ®A plus, B. Braun International, Germany) (Figure 3.2). The STR reactor (Biostat ®A plus, B. Braun International, Germany) is a stainless steel top plate fermenter that equipped with inlets for pH and oxygen electrodes, inoculation, sampling and temperature controlling sensor, air sparger, acid, alkali and antifoam inlets. Two six-bladed turbine impellers with a diameter of 52 mm mounted on the agitator shaft were used for agitation (Figure 3.3). During the fermentation process, the pH profile was measured using a pH probe. The probe was calibrated using buffers at pH 4.0 and 7.0 prior to the fermentation sterilisation. A dissolved oxygen probe was used to measure the Dissolved Oxygen Transfer (DOT).
Figure 3.2: A Biostat A plus 2-L stirred tank reactor
Figure 3.3: A schematic diagram of 2-L stirred tank reactor
3.8 Profiling of reducing sugar, growth and polysaccharides yield of *G. neojaponicum* using optimum medium composition and physical condition in 2-L bioreactor

The growth profile of *G. neojaponicum* was conducted in optimum spent yeast-brown sugar medium to obtain suitable cultivation time in 2-L STR reactor. The optimum cultivation medium and physical condition was prepared as obtained from previous Experiment 3.7. The optimum medium formulation was 5.74% (w/v) brown sugar, 0.06% (w/v) spent yeast, 0.46% (v/v) Tween 80 with constant basal medium. The optimum physical condition was at temperature 26.71 °C, aeration 1.33 vvm, pH 6 and 160 rpm. The cultivation medium and physical condition of bioreactor experiment was prepared as shown in Appendix A7. The preparation of inoculum and seed culture can be refered to Section 3.4.2 (page 42). Ten percent of seed culture (10% (v/v)) was inoculated to a 1-L working volume in 2-L stirred tank reactor (STR). *G. neojaponicum* was profiled based on mycelial growth, pH and β-glucan and reducing sugar content. The profiles were conducted at every two-day time interval during six days of cultivation period.

### 3.8.1 Extraction of polysaccharides and determination of polysaccharide yield

The mycelium and broth of *G. neojaponicum* were separated by ultracentrifuge (Beckman Coulter, Germany) at 10,000 rpm for 10 minutes. The mycelium was washed twice with the same volume of distilled water and freeze dried. The culture broth of *G. neojaponicum* was freeze dried for 2 days. The freeze dried mycelium (M) and dried broth (B) were further extracted for EPS and IPS using ethanol precipitation as described in Section 3.5.1 on page 44.
The content of total carbohydrate was determined by phenol-sulphuric acid assay according to Dubois et al., (1956) as in Section 3.5.2 on page 44. The determination of β-glucan content was conducted as in Section 3.5.3 on page 45.

### 3.8.2 Determination of reducing sugar

The reducing sugar was assayed according to DNS method (Miller, 1959). The di-nitrosalicylic (DNS) acid solution was prepared by adding 1g of DNS (Fluka, Germany) to 20 mL of 1 N NaOH (Merck, UK) in the 100 mL of volumetric flask. This was followed by the addition of 50 mL of distilled water to the flask. Thereafter, 30 g of potassium tartrate (Fluka, Germany) was added for colour stabilization and the volume made up to 100 mL distilled water. To 1 mL of sample, 1 mL of DNS solution was added in a test tube followed by the addition of 2 drops of 1 N NaOH (Merck, Germany). The test tube was then heated in boiling water for 5 minutes and cooled under running water. 10 mL distilled water was added to the content of the test tube before reading in a spectrophotometer (Cary Win UV, Agilent, USA) at 540 nm absorbance. The percentage of reducing sugar was determined using a standard curve of glucose as in Appendix B3. The standard curve was a linear graph of glucose concentration that ranged from 0-1000 mg/mL.

### 3.8.3 Statistical analysis

The Statistical Analysis System (SAS) Version 9.3 (SAS Institute, Cary, USA) was employed in performing statistical analysis. Data were provided as Mean±SD; t-tests were employed in carrying out statistical analyses and statistical significance was considered only at P-values less than 0.05.
3.9 Production of *G. neojaponicum* polysaccharides at optimum condition and evaluation of its immunomodulatory properties

The production of *G. neojaponicum* polysaccharides were produced at optimum condition using 2-L STR reactor as described previously in Experiment 3.8 page 51 at day 4 of cultivation time. Four *G. neojaponicum* extracts consisted of dried broth (B), dried mycelium (M), IPS and EPS extracts were extracted as described in Section 3.5.1 page 44 and evaluated for immunomodulatory properties. The positive control used was yeast β-glucan extract (Megazyme, Germany) and the negative control used was cell culture medium without sample. The evaluations of immunomodulatory and antitumour properties of *G. neojaponicum* were conducted using *in-vitro* test via macrophage (RAW264.7). Human colon cancer cell lines (HT29) against cell proliferation, cytotoxicity, phagocytosis and NFκB activity.

3.9.1 Cell culture

Two types of cell cultures were used macrophage cell (RAW264.7) and human colon cancer cell (HT29) for immunomodulatory assay. The RAW264.7 cells was obtained from ATCC (USA) and was maintained in DMEM medium (Invitrogen, Carlsbad, USA) containing 100 U/mL penicillin (PAA, Austria), 100 ug/mL streptomycin (PAA, Austria) and 10% v/v (PAA, Austria) fetal bovine serum. The cells were cultured to confluence in sterile tissue culture flasks; they were then carefully detached by scraping. Meanwhile, the human colon cancer cell of HT29 (ATCC, USA) was maintained in RPMI1640 (PAA, Austria) containing 100 U/mL penicillin (PAA, Austria), 100 µg/mL streptomycin (PAA, Austria) and 10% v/v (PAA, Austria) of fetal bovine serum. Both cells were grown in an incubator at 37 °C in a humidified atmosphere containing 5% CO₂.
(Shel Lab, USA). The protocol for the maintenance and aseptic handling of cells is detailed in Appendix C1-C6.

### 3.9.2 Proliferation and cytotoxicity effects

The proliferation activity of *G. neojaponicum* extracts was investigated of RAW264.7 macrophages while, cytotoxicity effect of *G. neojaponicum* extracts were investigated of human colon cancer cells (HT29). 1 x 10^6 cells/well of HT29 or RAW264.7 cells were nurtured in 96-well microplates with complete medium. The cells were treated for 24 h with 100-1000 µg/mL of *G. neojaponicum* extracts. After incubation, 20 µL of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA) at 5 mg/mL concentration was added into 170 µL of fresh medium in each well and incubated for 4 h at 37 °C and 5% CO₂ (Shel Lab, USA). Thereafter, the medium was removed from each well and DMSO (100 µl) (Merck, Germany) extraction buffer was added to each well to extract and solubilise the formazan crystal. The solution was vigorously mixed to dissolve the reacted dye and incubated for 20 minutes at 37 °C and 5% CO₂. Finally, the plate was read on an ELISA Reader (Versamax with SoftMax Pro 5.3, USA) with 570 nm wavelength. The calculation was as below:

\[
\text{Cytotoxicity / proliferation rate (\%) } = \left( \frac{\text{Absorbance of sample} - \text{Absorbance control}}{\text{Absorbance of control}} \right) \times 100\%
\]

### 3.9.3 Phagocytotic ability

The ability of phagocytosis was assessed by neutral red uptake as previously reported (Weeks *et al.*, 1987). RAW264.7 cells were seeded at a
density of 1 x 10\(^6\) cells per well in 96-well microplates. The calculation of cells was followed as Appendix A8. The cells were treated with 100-1000 µg/mL of \textit{G. neojaponicum} extracts for 24 hours. Then, 0.075\% (w/v) of neutral red was added. The plates were kept in an incubator for 30 minutes. Next, the cells were rinsed to remove leftover dye and were then blotted dry. After that, the cells were resuspended in ethanol (50\% v/v) containing glacial acetic acid (1\% v/v), and absorbance was measured at 540 nm in a microplate reader (Versamax with SoftMax Pro 5.3, USA).

The calculation of absorbance was translated into phagocytotic ability as calculation below:

\[
\text{Phagocytotic ability} = \frac{\text{Absorbance Sample} - \text{Absorbance control}}{\text{Absorbance Control}} \times 100\%
\]

### 3.9.4 Inhibition of NF-κB

The 1.5 x 10\(^6\) cells/mL of HT 29 were stimulated with 500 µg/mL and 1000 µg/mL of the polysaccharides in 6-well multiwell cell culture plates and were subsequently incubated at 37 °C overnight. The quantification of NF-κB was carried out by NF-κB/p65 ActivELISA (Imgenex, San Diego, USA). The NF-κB ActivELISA system is a sandwich ELISA. It quantified the nuclear levels of p65 that may have positive relationship with the activation of NF-κB pathway. The free p65 was captured by anti-p65 antibody followed by alkaline phosphatase (AKP)-conjugated secondary antibody using calorimetric detection in ELISA microplate reader (Versamax with SoftMax Pro 5.3, USA) at a wavelength of 405 nm. Details of the protocols of NF-κB ActivELISA is described in Appendix.
D2. The inhibition and activation of the cells were determined by comparing them with the negative control (cells without polysaccharides). The protein in NF-κB / p65 was measured using BCA protein kit (Pierce, Thermo Fisher, USA) (Appendix D3).

3.9.5 Statistical analysis

The analysis of bioactivity data was performed using t-tests SAS Version 9.3 (SAS Institute, Cary, USA) (SAS, 1990). Statistical significance was considered at $P$-values less than 0.05.

3.10 Oral acute toxicity of dried mycelium of G. neojaponicum

The dried mycelium of G. neojaponicum was selected as a representative sample of G. neojaponicum extracts produced in this study to evaluate its safety analysis using in-vivo acute oral toxicity test. The dried mycelium of G. neojaponicum was prepared using optimum medium and physical conditions obtained from previous experiment using 2-L STR reactor (as described in Section 3.8 on page 51). 50 g of dried mycelium of G. neojaponicum was then sent to an independent specialist services laboratory at SIRIM, Selangor, Malaysia for in-vivo toxicology analysis. The blood samples of the rats were sent to the pathology laboratory in UPM, Selangor for haematology analysis. The data were analysed based on Compilation Report No. R220/11/B19/03 (Appendix E7).

The dried mycelium of G. neojaponicum at 2000 mg/kg concentration was given orally force-fed to Sprague Dawley rats. The rats were observed over 14 days for mortality and physical/ behavioural changes as described by OECD (1989). Two groups consisting of control and test group with ten Sprague Dawley rats were used for acute
oral toxicity testing. The test group (five rats) were administered a single dose of the dried mycelium of *G. neojaponicum* (2000 mg/kg) orally. The dried mycelium were solubilised in water and were administered orally to the test group while, the control group (five rats) were given a normal diet without sample. The volume administered was 10 mL/kg body weight. After the extract was administered, the animals were closely monitored for 14 days for any symptoms of toxicity or mortality.