CHAPTER 4
RESULTS

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

4.1.1 Evaluation of optimum cell density for MTT Assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay was performed using a flat-bottomed 96-well plate of 0.37 cm^2 growth area. The optimum cell density for the neural cell line, NG108-15 and lung fibroblast, MRC-5, was determined from the relation between the absorbance value and cell number per well. The plates were read using an ELISA microplate reader (EMax®, Molecular Device) at 540 nm, with a reference wavelength at 650 nm. The absorbance values used in calculations were the difference of measured absorbance at 540 nm and 650 nm. The cells were plated at cell number ranging from 1 000 – 20 000 cells per well and incubated for 50 hrs (the total duration of cytotoxicity assay).

A linear relationship was observed between the absorbance value and the cell number per well in NG108-15 cell line, where the absorbance value increased with higher cell density (**Figure 4.1**). The linear relationship between the two variables was expressed in the equation $y = 0.0001x + 0.1488$ with the coefficient of determination, $R^2 = 0.9716$. To assess drug sensitivity in MTT assay, the cells should remain in exponential growth throughout the assay (Plumb, 1999). The optimum cell density for NG108-15 was chosen at 5 000 cells per well where the absorbance value stated around 1.

A linear relationship was similarly observed between the absorbance value and the cell number per well for MRC-5 cell line (**Figure 4.2**). The absorbance value increased with increasing cell density but the absorbance values were generally lower compared to the NG108-15 cell line. The linear relationship between the two variables was expressed in the equation $y = 0.00003x + 0.0693$ with the coefficient of

determination, R^2 = 0.9542. As the MRC-5 cell line had a slower growth, the optimum cell density chosen was 10 000 cells per well.

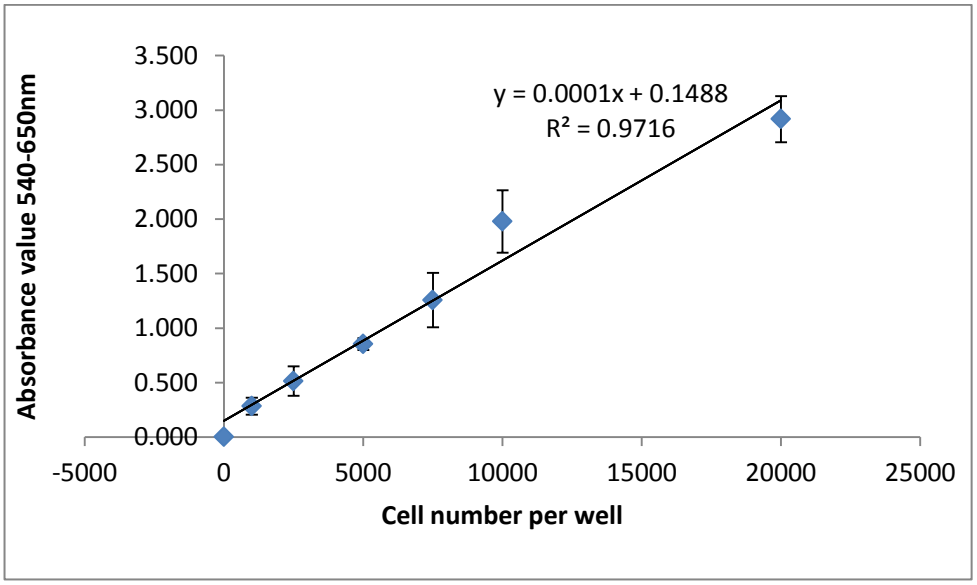

Figure 4.1: Relation between absorbance value and cell number in NG108-15 cells. The graph was plotted on the mean absorbance value ± SEM of two independent experiments carried out in triplicate. A linear relationship was observed between the absorbance value and cell number per well which can be expressed in the equation y = 0.0001x + 0.1488 (coefficient of determination, R^2 = 0.9716).

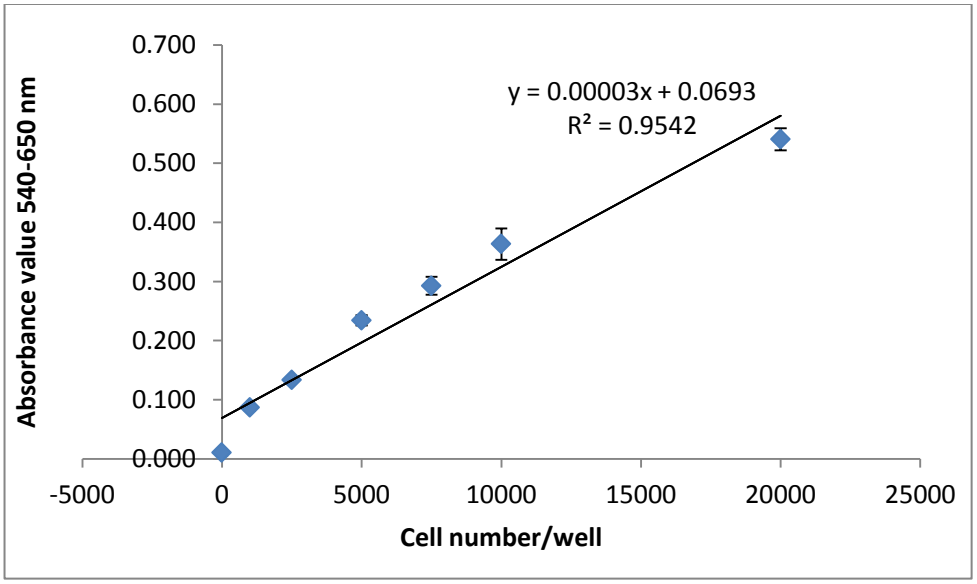

Figure 4.2: Relation between absorbance value and cell number in MRC-5 cells. The graph was plotted on the mean absorbance value ± SEM of two independent experiments carried out in triplicate. A linear relationship was observed between the absorbance value and cell number per well and expressed in the equation y = 0.00003x + 0.0693 (coefficient of determination, R^2 = 0.9542).

4.1.2 Cytotoxic activity of *H.erinaceus* extract in NG108-15 and MRC-5 cell lines

The cytotoxicity of *H.erinaceus* aqueous extract was evaluated using the MTT assay. The cell viability of human lung fibroblast, MRC-5, and neural hybrid cell, NG108-15, was assessed after an overnight incubation with various concentrations of *H.erinaceus* aqueous extract. Cell viability, expressed as a percentage, was defined as the ratio of absorbance of treated cells to untreated cells. The cell viability of untreated control cells were scored as 100%.

It was observed that varying concentrations of mushroom extracts had different effects on the cellular viability of MRC-5 fibroblast and NG108-15 neural cells for an incubation period of 24 hours (**Figure 4.3**). There was a concentration-dependent increase of cell viability in MRC-5 cells exposed to the extract concentrations from 0 – 1000 μg/ml. Cell viability of MRC-5 treated with 10 μg/mL – 1000 μg/mL mushroom extract was significantly different ($p < 0.05$) compared to untreated control cells (Appendix D, Table 1 & 2, pp.130)

At 1000 μg/mL, cell proliferation was observed at the end of 24 hr incubation, reflected by a 37.74% increment in cellular viability which was indicated as an increase in cell number. However, the cellular viability decreased at concentrations beyond 1000 μg/mL. At 50 000 μg/mL, the cellular viability of MRC-5 was greatly reduced to 11.08 ± 0.58 %. The 50% inhibitory concentration (IC_{50}) of *H.erinaceus* extract for MRC-5 interpolated from the response curve was 34 094.57 ± 1199.80 μg/mL (**Figure 4.3**).

The viability of NG108-15 cells was relatively constant when exposed to the mushroom extract at 0 – 1000 μg/mL. Further, there was no significant statistical difference between the groups at the range of concentrations tested (Appendix D, Table 3 & 4, pp.130-131). However, the cellular viability similarly decreased when the concentration of extract was increased (above 1000 μg/mL). At 50 000 μg/mL, the cellular viability of NG108-15 was significantly reduced to 33.40 ± 4.44 %. The

average IC_{50} value of *H.erinaceus* extract for NG108-15 cell line was 14 446.07 ± 1548.34 µg/mL.

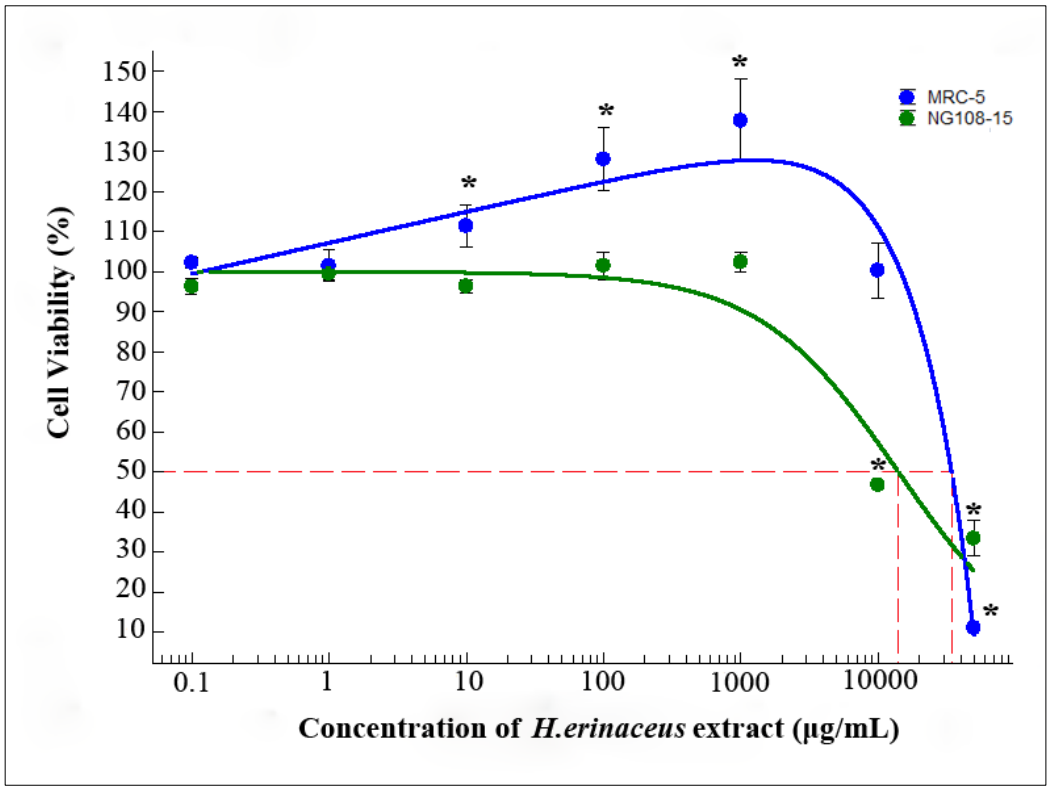

Figure 4.3: Cytotoxic effect of *H.erinaceus* extract *in vitro*. Each data point represents the mean cell viability ± SEM of three independent experiments carried out in triplicate. Untreated controls (0 mg/mL mushroom extract) were not plotted on log-scale graph. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. The 50% inhibitory concentration for the endpoint measured (IC_{50}) was interpolated from the response curve using IDBS XLfit® Software.

Table 4.1: IC_{50} values of *H.erinaceus* aqueous extract in MRC-5 and NG108-15 cell lines. Values given represent the mean ± SEM of three independent experiments carried out in triplicate.

Cell Line	IC_{50} (µg/mL)
MRC-5	34 094.57 ± 1199.80
NG108-15	14 446.07 ± 1548.34

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

4.2.1 Quantification of neurite-bearing cells in culture

4.2.1.1 The effect of varying concentrations of NGF on neurite outgrowth stimulation

The neurite outgrowth stimulation effect of NGF on neuronal hybrid NG108-15 is presented in **Figure 4.4**. The neural cells NG108-15 were treated with NGF in the concentration range of 5 – 100 ng/mL. Percentage of neurite outgrowth activity was significantly higher ($p < 0.05$) than untreated control cells at all concentrations tested (Appendix D, Table 5 & 6, pp.131).

It was observed that there was a dose-dependent increase in the neurite outgrowth stimulation activity in NG108-15 cells when subjected to 5 – 20 ng/mL NGF. The addition of 5 - 20 ng/mL NGF increased the percentage of neurite-bearing cells to 28.2% - 33.7%, compared to untreated cells (23.5 ± 1.1 %). In this experimental model, the optimal concentration for maximal neurite stimulation (33.7 ± 2.9 %) was achieved at 20 ng/mL, attaining increment of 43.4% compared to untreated control.

Further, the neurite outgrowth stimulation activity appeared to be reduced at concentrations higher than 20 ng/mL. The percentage of neurite-bearing cells at 10 – 100 ng/mL was not significantly higher ($p < 0.05$) than the minimum NGF concentration tested (5 ng/mL), except for the optimal concentration (20 ng/mL).

4.2.1.2 The effect of varying concentrations of *H.erinaceus* aqueous extract on neurite outgrowth stimulation

The neurite outgrowth stimulation effect of *H.erinaceus* aqueous extract on neuronal hybrid NG108-15 is presented in **Figure 4.5**. The neural cells were treated with *H.erinaceus* extract in the concentration range of 1 – 500 µg/mL. Percentage of neurite outgrowth activity was significantly higher ($p < 0.05$) than untreated control cells at all concentrations tested (Appendix D, Table 7 & 8, pp.132).

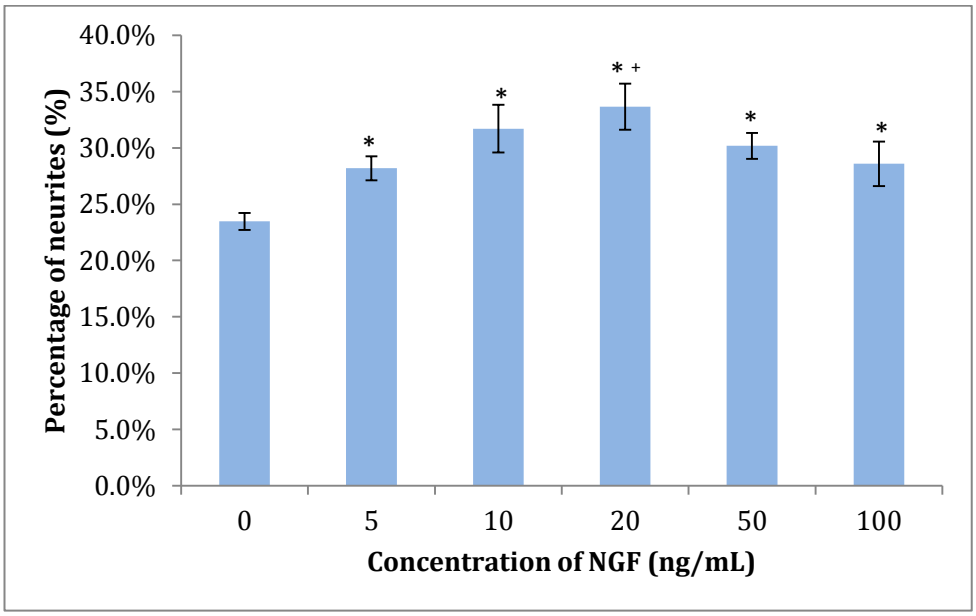

Figure 4.4: Percentage of neurite-bearing cells in NG108-15 cells treated with NGF. Data shown are mean ± SEM of three independent experiments carried out in duplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. A plus sign (+) denotes a significant difference ($p < 0.05$) in comparison to the 5 ng/mL treatment.

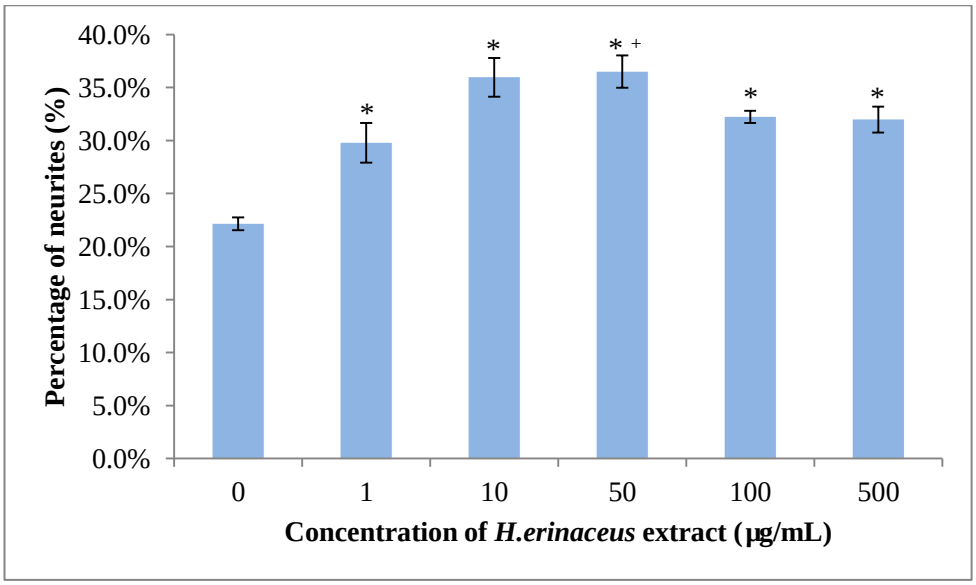

Figure 4.5: Percentage of neurite-bearing cells in NG108-15 cells treated with *H.erinaceus* aqueous extract. Data shown are mean ± SEM of three independent experiments carried out in duplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. A plus sign (+) denotes a significant difference ($p < 0.05$) in comparison to the 1 µg/mL concentration.

The mushroom extract, when applied at 1 – 50 µg/mL, induced neurite outgrowth activity in NG108-15 cells in a dose-dependent manner (**Figure 4.5**). Addition of *H.erinaceus* extract at 10 µg/mL gave a near-maximal response with the presence of 36.0 ± 2.6 % neurite-bearing cells. A peak response (64.8% increment) was achieved with 36.5 ± 2.2 % neurite-bearing cells at a concentration of 50 µg/mL *H.erinaceus* extract. The percentage of neurite outgrowth activity of 10 µg/mL and 50 µg/mL mushroom extract was significantly higher ($p < 0.05$) than the minimum mushroom extract concentration (1 µg/mL) tested.

Higher concentrations of the mushroom extract did not improve the neurite stimulation activity of NG108-15. Instead, a decline in the percentage of neurite-bearing cells was observed. When the mushroom extract concentration was doubled (100 µg/mL), the percentage of neurite-bearing cells was reduced to 32.2 ± 0.8 %. The neurite outgrowth stimulation activity reached a plateau at this concentration point despite increasing the extract concentration 5 fold to 500 µg/mL (32.0 ± 1.7 %).

4.2.1.3 The effect of varying concentrations of *H.erinaceus* aqueous extract combined with 5, 10 and 20 ng/mL NGF on neurite outgrowth stimulation

With the initial assessment of NGF on neurite outgrowth stimulation activity, the NG108-15 neural cells were subjected to 5, 10 and 20 ng/mL NGF combined with various concentrations of *H.erinaceus* aqueous extract. The concentration of *H.erinaceus* extract at 500 µg/mL was not further evaluated due to saturation of neurite stimulation activity at this concentration.

The treatment of NG108-15 cells with 5 ng/mL NGF stimulated 25.5 ± 1.9 % cells to extend neurites (**Figure 4.6**). The highest percentage of neurite-bearing cells (27.9 ± 1.8 %) was achieved in the combined treatment of 50 µg/mL mushroom extract and 5 ng/mL NGF. Although an increment of 42.4% (compared to control) was attained

with this combined treatment, there was only 9.5% increment compared to 5 ng/mL NGF treatment. This combined treatment was not significantly different ($p < 0.05$) compared to 5 ng/mL NGF individually (Appendix D, Table 9 & 10, pp.132-133). The addition of varying concentrations of mushroom extract (1 – 100 µg/mL) in combination with 5 ng/mL NGF did not significantly ($p < 0.05$) enhance the neurite outgrowth stimulation activity compared to treatment with 5 ng/mL NGF individually.

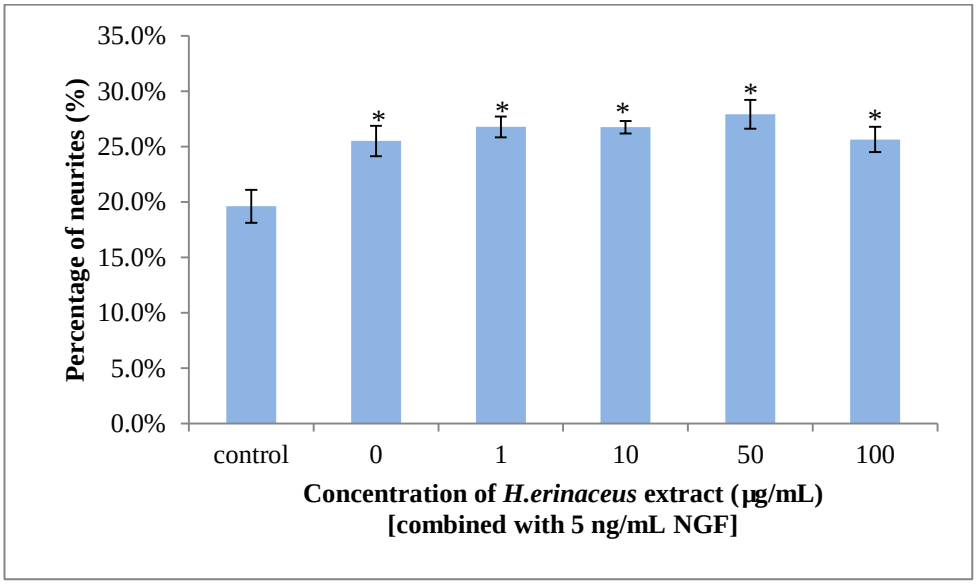

Figure 4.6: Percentage of neurite-bearing cells in NG108-15 cells treated with 5 ng/mL NGF and *H.erinaceus* aqueous extract. Data shown are mean ± SEM of three independent experiments carried out in duplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control.

The treatment of 10 ng/mL NGF stimulated 35.5 ± 1.4 % cells to extend neurites, attaining 33.3% increment compared to untreated control cells (**Figure 4.7**). The addition of 1 µg/mL mushroom extract to 10 ng/mL NGF markedly enhanced the neurite outgrowth stimulation activity with 42.7 ± 2.3 % cells showing extensions. A comparable increment was also seen in the treatment of 10 µg/mL mushroom extract added to 10 ng/mL NGF, with 42.3 ± 1.9 % of cells bearing neurite. The effect of neurite outgrowth stimulation was enhanced 1.2-fold in these combined mixtures when compared to 10 ng/mL NGF applied individually. The percentage of neurite outgrowth

stimulation activity in these combined mixtures was significantly higher ($p < 0.05$) compared to other combined concentrations tested (Appendix D, Table 11 &12, pp.133).

When the mushroom extract concentration was increased to 50 µg/mL, the enhancement of neurite outgrowth stimulation activity by the combined mixture was still observed but less pronounced. The percentage of neurite-bearing cells was 39.0 ± 1.0 % when the extract concentration was increased to 50 µg/mL. In this treatment, the neurite extension activity was enhanced 9.9% compared to 10 ng/mL NGF treatment alone. This enhancement was significantly different ($p < 0.05$) compared to 10 ng/mL NGF treatment. However, the enhancement of neurite outgrowth stimulation activity was not observed when the mushroom extract was further increased. The addition of 100 µg/mL mushroom extract to 10 ng/mL NGF reduced 2.3% of neurite outgrowth activity compared to 10 ng/mL NGF treatment alone. The percentage of neurite-bearing cells of this combined mixture (100 µg/mL mushroom extract + 10 ng/mL NGF) was not significantly different ($p < 0.05$) compared to 10 ng/mL NGF treatment alone.

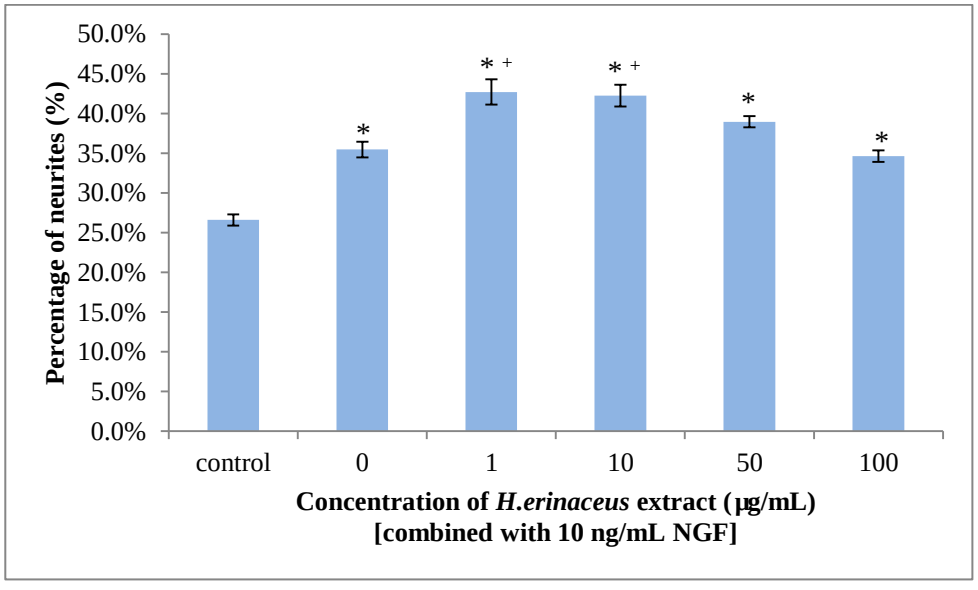

Figure 4.7: Percentage of neurite-bearing cells in NG108-15 cells treated with 10 ng/mL NGF and *H.erinaceus* aqueous extract. Data shown are mean ± SEM of three independent experiments carried out in duplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. A plus sign (+) denotes a significant difference ($p < 0.05$) in comparison to the 0 µg/mL mushroom extract concentration (with 10 ng/mL NGF only).

The treatment of NG108-15 cells with 20 ng/mL NGF stimulated 31.6 ± 2.9 % neurite-bearing cells, with 44.0% increment compared to untreated control cells (**Figure 4.8**). The addition of varying concentrations of mushroom extract (1 – 100 µg/mL) in combination with 20 ng/mL NGF did not significantly ($p < 0.05$) enhance the neurite outgrowth stimulation activity compared to treatment with 20 ng/mL NGF individually. Addition of 1 µg/mL and 10 µg/mL mushroom extract to 20 ng/mL NGF slightly increased the neurite outgrowth stimulation activity, with 32.2 ± 3.2 % and 32.6 ± 3.7 % neurite-bearing cells induced respectively. The addition of these combined mixture merely increased the neurite-bearing cells by 1.7% (1 µg/mL mushroom extract) and 3.2% (10 µg/mL mushroom extract) compared to 20 ng/mL NGF treatment alone.

When the mushroom extract concentration was increased (50 µg/mL – 100 µg/mL), the neurite outgrowth stimulation activity was reduced. The addition of 50 µg/mL mushroom extract to 20 ng/mL NGF reduced the percentage of neurite-bearing cells by 3.8% (30.4 ± 2.3 %) compared to 20 ng/mL NGF treatment alone. The neurite outgrowth stimulation activity further declined by 7.0% (29.4 ± 2.2 %) when the mushroom extract concentration was increased to 100 µg/mL. However, the decline of these treatment was not significantly different ($p < 0.05$) compared to 20 ng/mL NGF treatment (Appendix D, Table 13 & 14, pp.134).

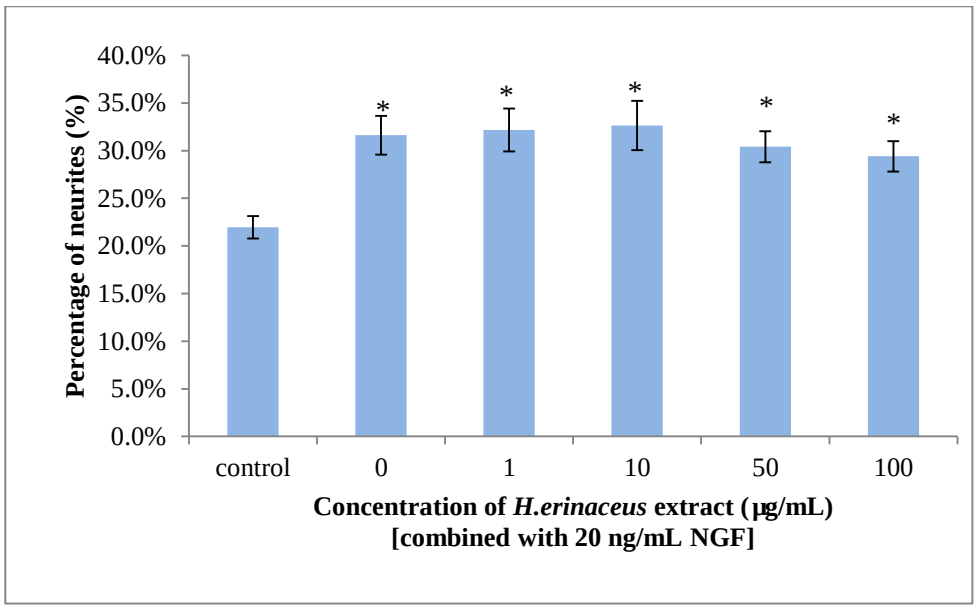

Figure 4.8: Percentage of neurite-bearing cells in NG108-15 cells treated with 20 ng/mL NGF and *H.erinaceus* aqueous extract. Data shown are mean ± SEM of three independent experiments carried out in duplicate. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control.

4.2.1.4 Morphology of NG108-15 cells under different treatment conditions

NG108-15 is a somatic cell hybrid from a cross between mouse N18TG2 neuroblastoma cells with rat C6-BU-1 glioma cells. In normal untreated cultures, the cells appeared to be flat, round, growing in clusters and loosely adherent on cell culture surface. The poly-D-lysine coating served as a neutral substratum where the cells were observed to attach within the first hour of plating and this minimized the clustering of cells. Untreated control cells had short cellular processes but mostly did not reach the criterion length of one cell body diameter to be scored as a neurite-bearing cell.

When *H.erinaceus* aqueous extract was applied individually or in combination with NGF, visible neurite outgrowth was induced in the neuroblastoma-glioma hybrid NG108-15. Diverse and extensive neurite formation was observed in differentiated cells. Cells expressing ‘slow-onset’ neurites formed long, smooth, cable-like neurite extensions with a definite single axis of polarity (usually unipolar or bipolar). These cells usually appear as singlets, doublets or clusters of cells with neurite length of

several hundred μm (**Figure 4.9a**). On the other hand, cells expressing 'rapid-onset' neurites formed exuberant multipolar outgrowth with extensive branching. These neurite extensions were usually beaded with relatively short neurite length compared to 'slow-onset' neurites (**Figure 4.9b**). It was observed that neurite extensions became fused (fasciculation) and formed thick neurite bundles in some 'rapid-onset' neurites, especially in cells growing in clusters (**Figure 4.9c**).

Both distinct neurite outgrowth mode – 'rapid-onset' and 'slow-onset', was observed in mushroom-treated and NGF-treated cells. The cells exhibited extensive neurite networks with higher percentage of neurite-bearing cells present in culture when treated with a combination of *H.erinaceus* extract and 10 ng/mL NGF (**Figure 4.10**).

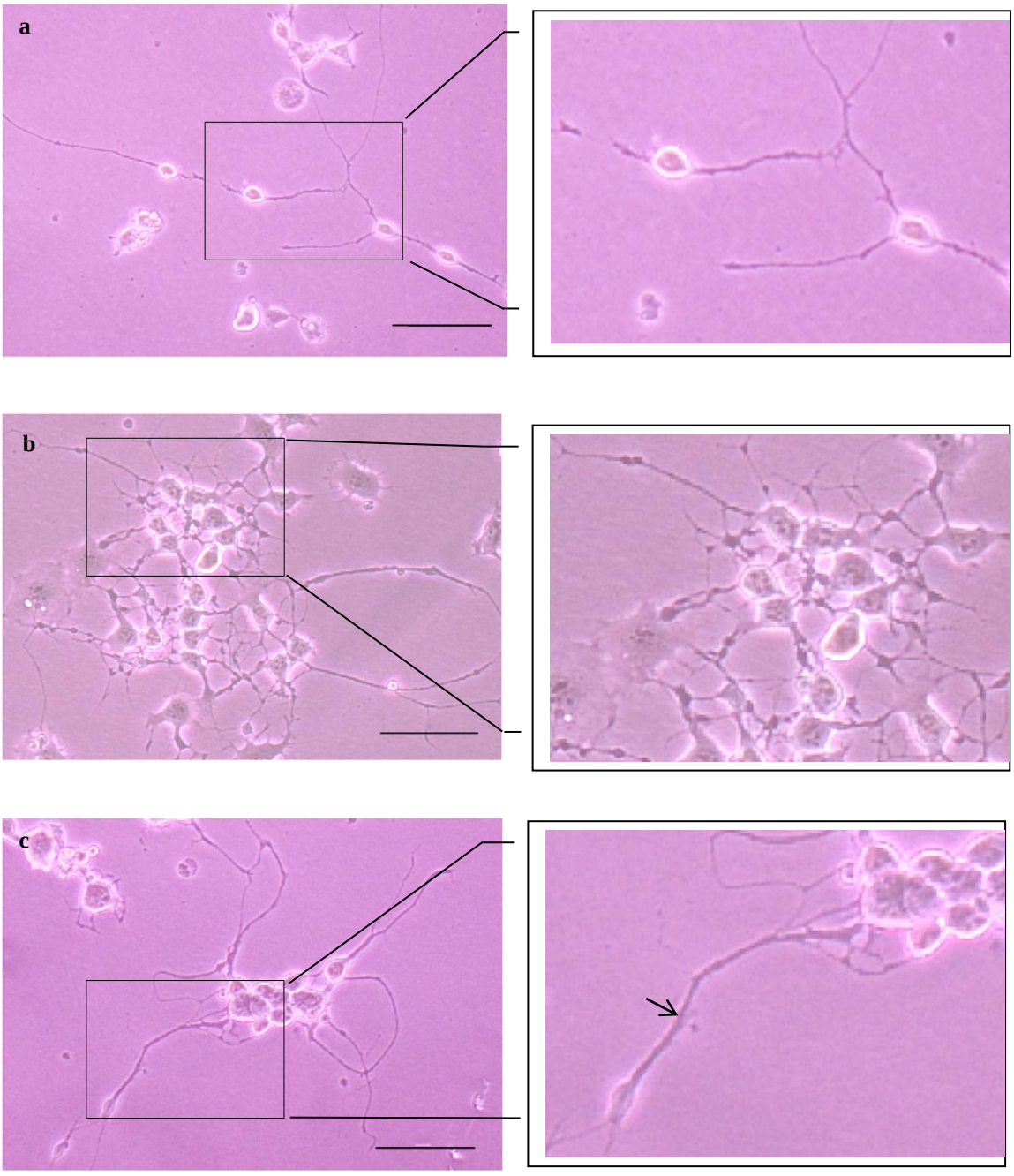

Figure 4.9: The prototypical slow-onset and rapid-onset neurite patterns observed in NG108-15 cells. (a) Cells expressing 'slow-onset' neurites formed long, unipolar, cable-like extensions. (b) Cells expressing 'rapid-onset' neurites formed short, irregular, branched neurites. (c) Neurite fasciculation (indicated by arrow) observed in culture. Scale bar = 100 µm.

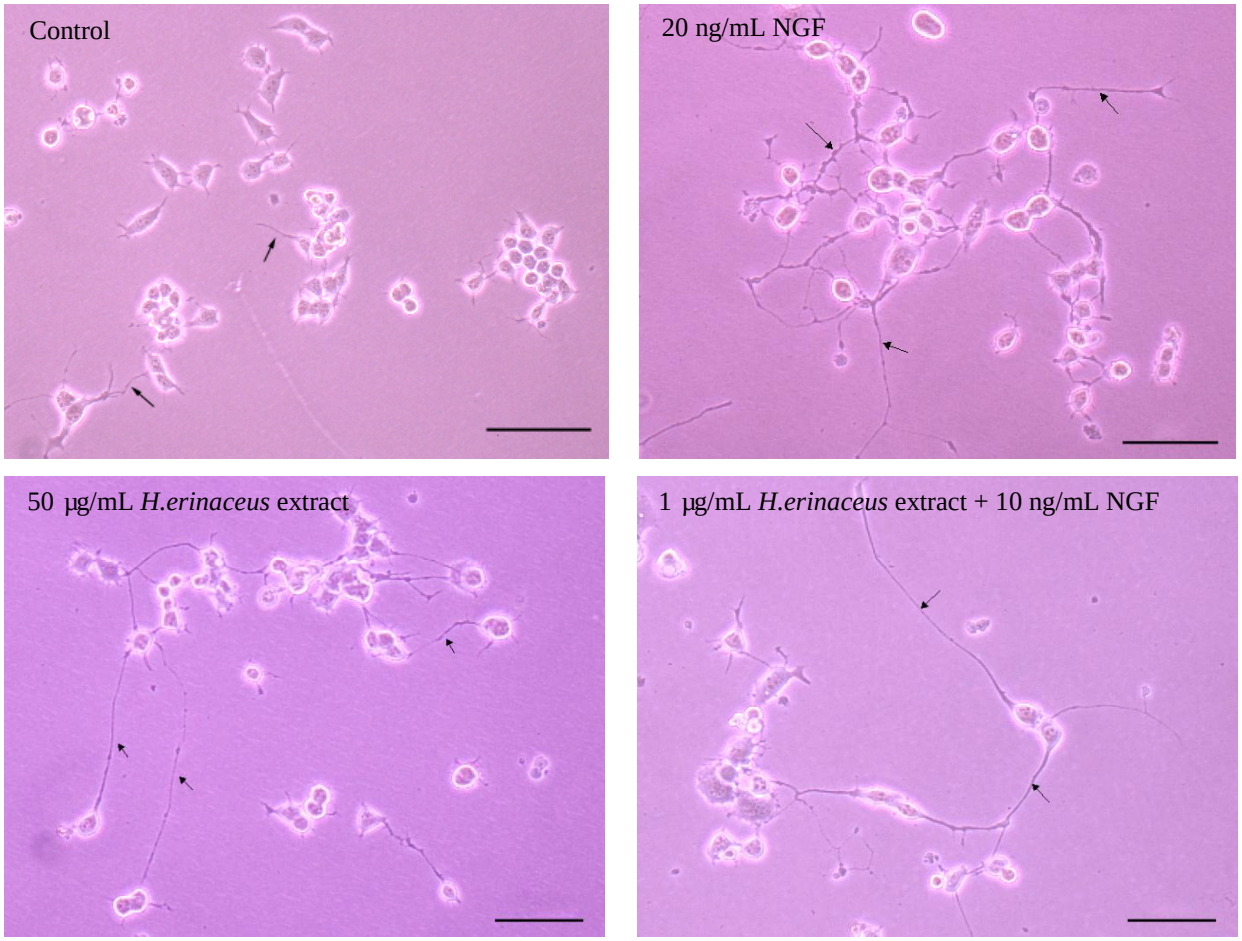

Figure 4.10: Representative images showing the morphology of NG108-15 cells treated with NGF, *H.erinaceus* aqueous extract or both.. The control group refers to untreated cells. The black arrows denote the presence of neurites in respective treatments. The prototypical slow-onset and rapid-onset neurite patterns are observed in cultures. Scale bar = 100 µm.

4.2.2 Measurement of extracellular NGF levels in cell culture

4.2.2.1 The extracellular NGF levels in NG108-15 cell line treated with varying concentrations of *H.erinaceus* aqueous extract

The aqueous extract of *H.erinaceus* was shown to induce neurite outgrowth stimulation activity in NG108-15 cells (**Figure 4.5**). As the neuroactive compounds in the *H.erinaceus* mushroom – hericenones and erinacines, were reported to induce NGF synthesis in mouse astroglial cells *in vitro* (Ma et al., 2010), the extracellular NGF level in NG108-15 cells treated with varying concentrations of *H.erinaceus* extract was measured using enzyme-linked immunosorbent assay (ELISA).

The NGF standard curve ranging 3.9 – 250 pg/mL was generated as a linear curve (Appendix C, pp.129). The NGF concentration (pg/mL) in test samples was interpolated from the standard curve using the average absorbance value of triplicate wells. The NGF level in NG108-15 cells treated with various concentrations of *H.erinaceus* aqueous extract measured using ELISA is presented in **Figure 4.11**.

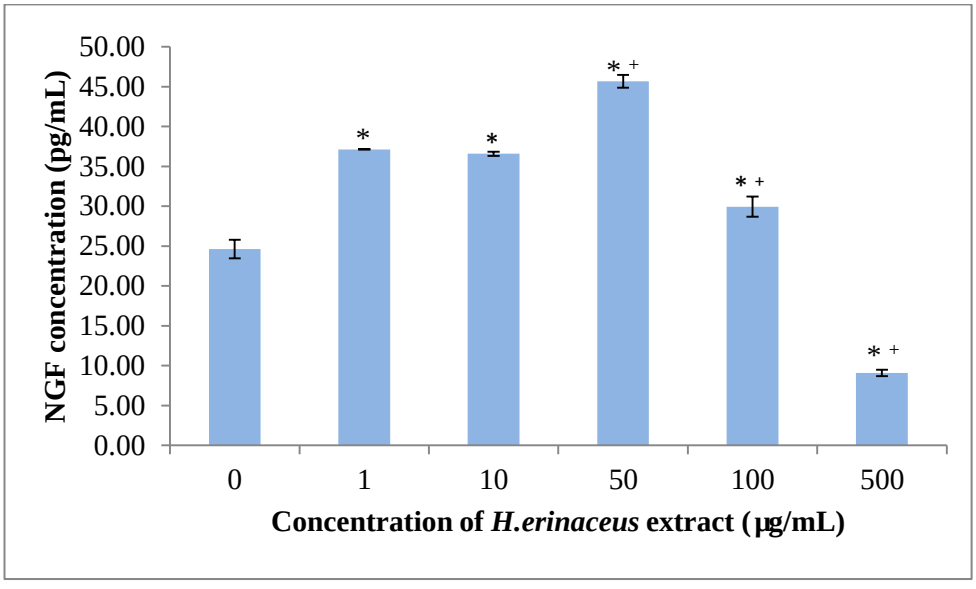

Figure 4.11: The extracellular NGF levels in NG108-15 cells treated with *H.erinaceus* aqueous extract. Data shown are mean ± SEM of two experiments carried out in triplicate wells. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. A plus sign (+) denotes a significant difference ($p < 0.05$) in comparison to the 1 µg/mL mushroom extract concentration.

It was observed that there was a significant increase ($p < 0.05$) in the NGF concentration when the cells were treated with 1 – 50 µg/mL *H.erinaceus* aqueous extract compared to untreated control (Appendix D, Table 15 & 16, pp.134). The addition of 1 µg/mL mushroom extract in the cells increased the NGF concentration by 50.8% (37.12 ± 0.03 pg/mL) compared to untreated control cells (24.62 ± 1.17 pg/mL). When 10 µg/mL of mushroom extract was applied, the amount of NGF was slightly reduced to 36.58 ± 0.27 pg/mL but this reduction was not statistically significant ($p < 0.05$). Maximum NGF secretion in the cell culture supernatant (45.67 ± 0.79 pg/mL) was observed in NG108-15 cells treated with 50 µg/mL *H.erinaceus* aqueous extract. As mentioned in section 4.2.1.2, maximal neurite outgrowth stimulation activity (36.5 ± 2.2 % neurite-bearing cells) was similarly observed at this treatment group.

A steep decline was observed in the NGF concentration in the cell culture supernatant when the concentration of mushroom extract was further increased beyond the optimal concentration (50 µg/mL). The amount of NGF was reduced to 29.94 ± 1.28 pg/mL when the cells were applied with 100 µg/mL *H.erinaceus* aqueous extract. When the mushroom extract was further increased to 500 µg/mL, the NGF concentration was drastically reduced to 9.07 ± 0.40 pg/mL, stating a 63.1% reduction compared to untreated control cells. The amount of NGF in the culture supernatant was significantly reduced ($p < 0.05$) when treated with 500 µg/mL *H.erinaceus* aqueous extract.

4.2.2.2 The extracellular NGF levels in NG108-15 cell line treated with varying concentrations of *H.erinaceus* aqueous extract combined with 10 ng/mL NGF

Earlier morphological differentiation studies showed that the combination of 10 ng/mL NGF and *H.erinaceus* aqueous extract could enhance neurite outgrowth stimulation activity in NG108-15 cells. Thus, this combined treatment group was chosen to measure the NGF concentration present in the cell culture supernatant.

The NGF level in NG108-15 cells treated with various concentrations of *H.erinaceus* aqueous extract combined with 10 ng/mL NGF is presented in **Figure 4.12**. The NGF concentration in the cell culture supernatant of NGF and/or mushroom extract-treated cells was significantly higher ($p < 0.05$) than untreated control cells (Appendix D, Table 17 & 18, pp.135). When the cells were treated with 10 ng/mL NGF for 48 hr, 661.35 ± 32.21 pg/mL NGF was detected to be present in the cell culture supernatant. However, when 1 µg/mL *H.erinaceus* aqueous extract combined with 10 ng/mL NGF was applied to the cells, there was a 281.5% increment (2523.23 ± 160.97 pg/mL) in the NGF concentration compared to 10 ng/mL NGF. A comparable increment (278.4%) of NGF concentration (2502.37 ± 94.28 pg/mL) was observed when the mushroom extract was increased to 10 µg/mL. The NGF concentration was significantly increased ($p < 0.05$) when the cells were treated with 1 – 10 µg/mL *H.erinaceus* aqueous extract combined with 10 ng/mL NGF. There was no significant difference ($p < 0.05$) between these two treatment groups.

Further, the NGF concentration was markedly enhanced when the *H.erinaceus* aqueous extract was increased to 50 – 100 µg/mL in the combined mixture (**Figure 4.12**). The addition of 50 µg/mL mushroom extract and 10 ng/mL NGF to the neural cells increased the NGF concentration by 18700.3 % (3175.02 ± 28.90 pg/mL) compared to untreated control cells. When the mushroom extract was increased to 100 µg/mL in the combined mixture, there was a slight decrease of NGF concentration (2978.32 ± 81.82 pg/mL) in the cell culture supernatant. However, the decrease was not statistically significant ($p < 0.05$) and there was no significant difference between these two treatment groups.

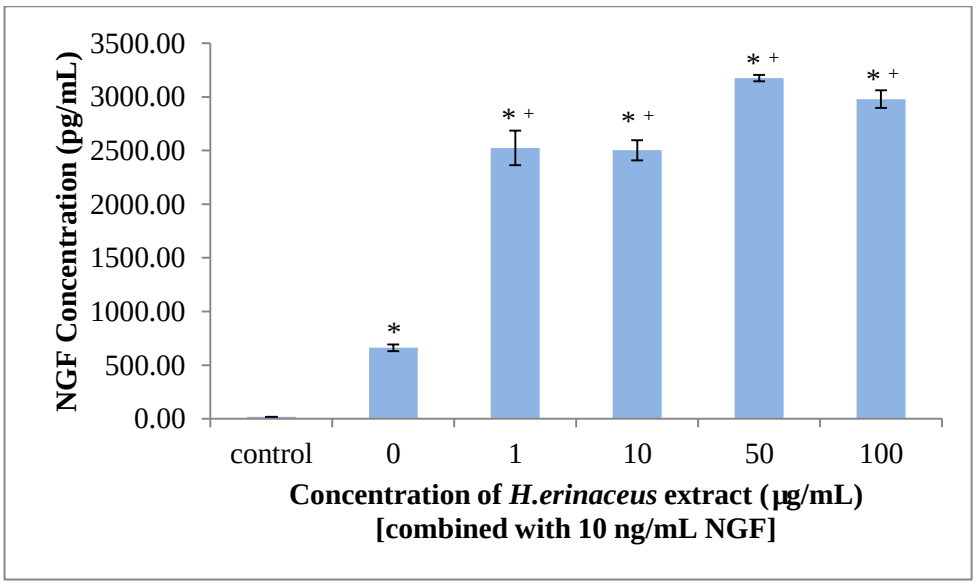

Figure 4.12: The extracellular NGF levels in NG108-15 cells treated with *H.erinaceus* aqueous extract combined with 10 ng/mL NGF. Data shown are mean ± SEM of three experiments carried out in triplicate wells. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for control. A plus sign (+) denotes a significant difference ($p < 0.05$) in comparison to the 0 µg/mL mushroom extract concentration (with 10 ng/mL NGF only).

4.2.3 Immunofluorescence staining of neurofilament 200 kDa subunit

As mentioned in earlier sections, the NG108-15 cells were able to extend neurites when treated with NGF and *H.erinaceus* aqueous extract either individually or in combination. Neurofilament 200 kDa subunit protein is an intermediate filament specifically expressed in neurons and often used as an axonal marker. Thus, the cells were stained for neurofilament to confirm the morphological extensions were of neuronal origin.

Untreated control cells remained undifferentiated throughout the 48 hr incubation period. The cells were flat, round in shape and lack of neurite extensions. However, these undifferentiated cells stained positively for neurofilament (**Figure 4.13**). Cells treated with *H.erinaceus* aqueous extract and/or NGF exhibited neuron-like morphology with extensive cellular processes. These differentiated cells similarly stained positive for neurofilament, indicating the visible neurite extensions were of neuronal origin.

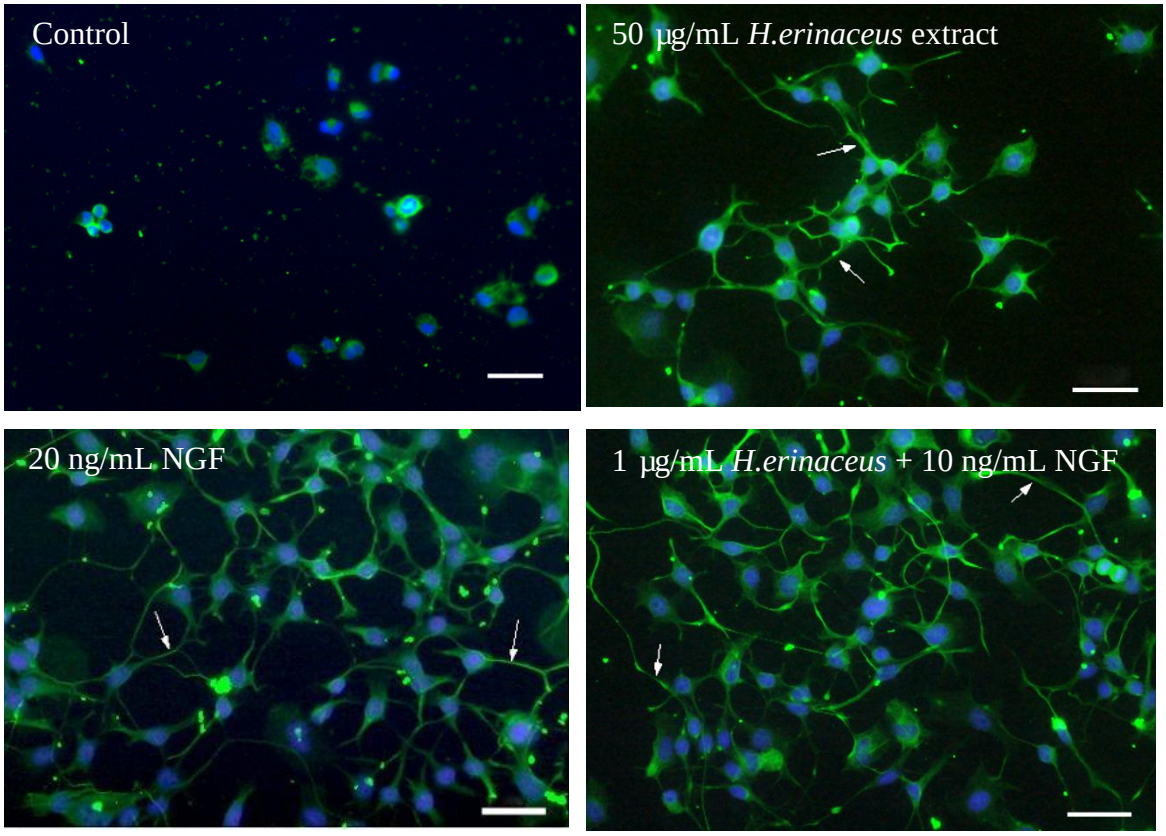

Figure 4.13: Immunocytochemical staining of NG108-15 cells for neurofilament 200 kDa subunit. The neuronal cells were labelled with antibodies against neurofilament followed by FITC (shown in green). Cell nuclei were counterstained with DAPI (blue) to facilitate cell counting. The neurites stained positive for neurofilament-200 (white arrows) after treated with *H.erinaceus* extract, NGF, or both. Scale bar = 50 µm.

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

4.3.1 Assessment of cellular viability using MTT assay

4.3.1.1 Evaluation of cytotoxic activity of H_2O_2 in NG108-15 cells

The neural cells NG108-15 were subjected to various concentrations of H_2O_2 (0 – 500 µM) for 2 - 24 hr to evaluate the cytotoxic activity of H_2O_2. The viability of untreated control cells at each incubation time was scored as 100%.

The cellular viability was greatly influenced by two factors: H_2O_2 concentration and incubation period. From **Table 4.2**, it was shown that H_2O_2 at low concentrations (0.5 – 10 µM) did not greatly affect the cellular viability even with increased incubation period up to 24 hr. The cellular viability was maintained at 90% and above when incubated with 0.5 – 10 µM H_2O_2 for 2 – 24 hr. When 0.5 – 10 µM H_2O_2 was applied to the cells, the viability was not statistically different ($p < 0.05$) compared to the corresponding untreated control at each incubation period except for 4 hr treatment group (Appendix D, Table 19-30, pp.135-140).

When the cells were subjected to 50 µM H_2O_2, the cellular viability was reduced to 84.27 ± 4.36 % (2 hr incubation) and further dropped to 36.98 ± 1.44 % after 24 hr incubation. At 50 µM H_2O_2, the reduction of cellular viability was statistically significant ($p < 0.05$) at all incubation time except for 8 hr treatment group. The neural cells were 54.89 – 60.50 % viable when incubated with 100 µM H_2O_2 for 2 – 4 hr, but viability was drastically reduced (13.04 – 35.74 %) when the incubation period was further increased (6 – 24 hr). The cellular viability significantly declined ($p < 0.05$) when the H_2O_2 concentration was further increased to 250 – 500 µM.

From the evaluation of cytotoxic activity of H_2O_2, the treatment condition of 100 µM H_2O_2 and 2 hr incubation was chosen to further evaluate the neuroprotective activity of *H.erinaceus* aqueous extract.

Table 4.2: Cellular viability of NG108-15 cells treated with varying concentrations of H_2O_2 at different incubation period. Untreated cells were taken as control and cellular viability was scored as 100%. Values given represent the mean ± SEM of two independent experiments carried out in duplicate. Means with different letters in a same column are significantly different ($p < 0.05$).

H_2O_2 Concentration (μM)	Cell Viability (%)					
	2 hr	4 hr	6 hr	8 hr	12 hr	24 hr
0	100.00 ± 4.98^{d}	100.00 ± 2.32^{d}	100.00 ± 4.54^{e}	100.00 ± 4.44^{de}	100.00 ± 2.63^{d}	100.00 ± 3.64^{d}
0.5	94.04 ± 4.87^{d}	103.82 ± 3.78^{d}	91.46 ± 4.59^{de}	99.32 ± 1.84^{de}	95.91 ± 7.14^{d}	94.74 ± 2.36^{d}
1	102.30 ± 7.22^{d}	99.45 ± 3.67^{d}	89.13 ± 2.82^{d}	88.96 ± 4.00^{c}	98.38 ± 8.75^{d}	97.18 ± 1.75^{d}
5	94.82 ± 3.89^{d}	97.79 ± 4.31^{d}	94.33 ± 6.84^{de}	107.33 ± 2.93^{e}	94.93 ± 7.44^{d}	99.18 ± 6.07^{d}
10	100.73 ± 4.32^{d}	112.56 ± 3.38^{e}	93.96 ± 4.32^{de}	104.40 ± 1.95^{e}	102.85 ± 2.35^{d}	95.03 ± 1.85^{d}
50	84.27 ± 4.36^{c}	78.04 ± 4.86^{c}	61.40 ± 9.82^{c}	92.20 ± 9.67^{cd}	66.25 ± 2.81^{c}	36.98 ± 1.44^{c}
100	54.89 ± 4.40^{b}	60.50 ± 3.82^{b}	32.56 ± 4.99^{b}	35.74 ± 5.46^{b}	19.98 ± 3.24^{b}	13.04 ± 1.29^{b}
250	19.03 ± 2.36^{a}	5.68 ± 0.87^{a}	8.75 ± 0.71^{a}	10.41 ± 3.81^{a}	2.09 ± 0.40^{a}	2.42 ± 0.32^{a}
500	16.57 ± 1.54^{a}	3.77 ± 0.32^{a}	9.12 ± 0.61^{a}	10.20 ± 3.56^{a}	1.79 ± 0.23^{a}	2.26 ± 0.30^{a}

4.3.1.2 Cellular viability of NG108-15 cells pre-treated with *H.erinaceus* extract prior to H_2O_2-induced oxidative stress

To assess the neuroprotective effect of *H.erinaceus* extract against oxidative stress, pre-treatment and co-treatment of extract application were studied. In pre-treatment experiments, NG108-15 cells were incubated with extract prior to the introduction of oxidative stress. The neural cells were treated with *H.erinaceus* aqueous extract at varying concentrations for 2 hr and 24 hr respectively prior subjected to 100 µM H_2O_2 for 2 hr (**Figure 4.14**). The cellular viability of untreated control cells was scored as 100%. All treatment groups were significantly different ($p < 0.05$) from untreated control (Appendix D, Table 31 - 34, pp.141 - 142).

The viability of NG108-15 cells decreased to ~ 60% (57.11% – 61.04%) when subjected to 100 µM H_2O_2 for 2 hr. The pre-treatment of *H.erinaceus* extract (1 – 1000 µg/mL) for 2 hr did not improve cellular viability and there was no significant difference ($p < 0.05$) between the treated cells and the non-treated cells. The cellular viability remained within the range of 58.00% - 63.48%.

The cells were then further incubated with the mushroom extract for 24 hr in hoping for cellular protection upon longer incubation period. However, the viability of extract-treated cells was not improved with extended hours of extract pre-treatment. The viability of treated cells was not significantly different ($p < 0.05$) from non-treated cells. (Appendix D, Table 33 & 34, pp.142). Further, cells incubated with high concentrations of extract (500 – 1000 µg/mL) for 24 hr showed a significant reduction in cellular viability (38.67 – 42.01 %) compared to lower concentrations (< 500 µg/mL).

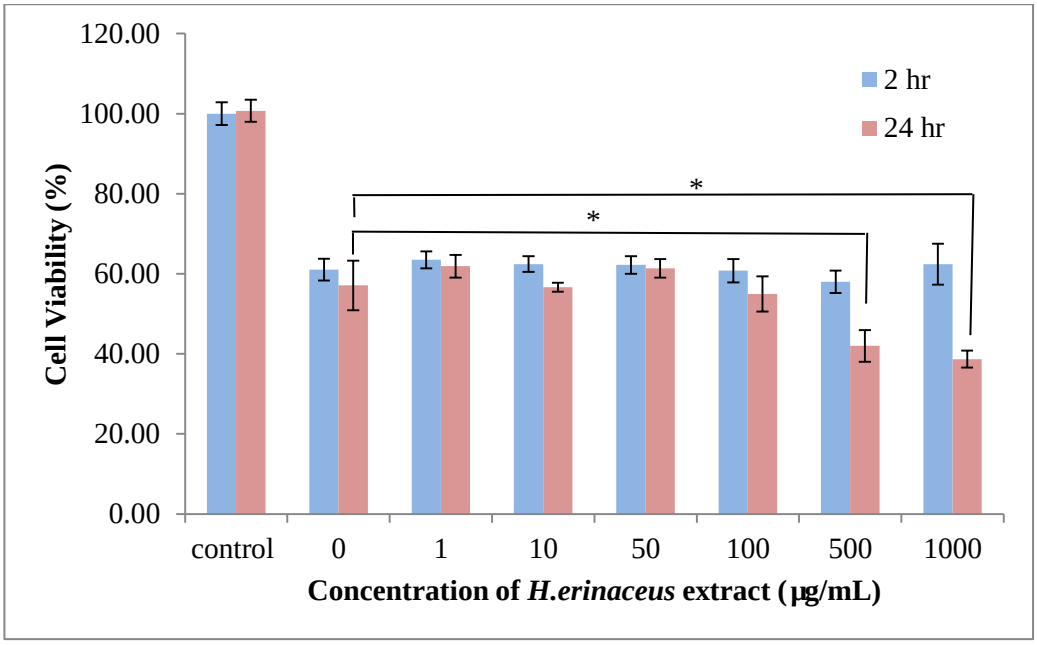

Figure 4.14: Cellular viability of NG108-15 cells pre-treated with *H.erinaceus* aqueous extract prior to H_2O_2-induced oxidative stress. Untreated control cells were set at 100% viability. All treatment groups were significantly different ($p < 0.05$) from untreated control. An asterisk (*) denotes a significant difference ($p < 0.05$) from the corresponding value for 0 µg/mL extract concentration (treated with 100 µM H_2O_2 only).

4.3.1.3 Cellular viability of NG108-15 cells co-treated with *H.erinaceus* extract and H_2O_2

To assess the neuroprotective effect of *H.erinaceus* extract in the presence of H_2O_2, co-treatment of extract application was studied. The mushroom extract was added simultaneously with 100 µM H_2O_2 in NG108-15 cells and incubated for 2 hr (**Figure 4.15**). The cell viability of untreated control cells was scored as 100%. All treatment groups were significantly different ($p < 0.05$) from untreated control (Appendix D, Table 35 & 36, pp.143).

The cell viability of NG108-15 was reduced to 56.67 ± 4.23 % when subjected to 100 µM H_2O_2 for 2 hr. The simultaneous addition of *H.erinaceus* extract within the concentration range of 1 – 1000 µg/mL did not improve cell viability in the 2 hr oxidative assault. The cell viability declined within the range of 51.49 – 59.68 % when co-incubated

with *H.erinaceus* extract and H_2O_2 for 2 hr. The cell viability of extract-treated groups was not statistically different ($p < 0.05$) from cells not treated with the extract (0 µg/mL).

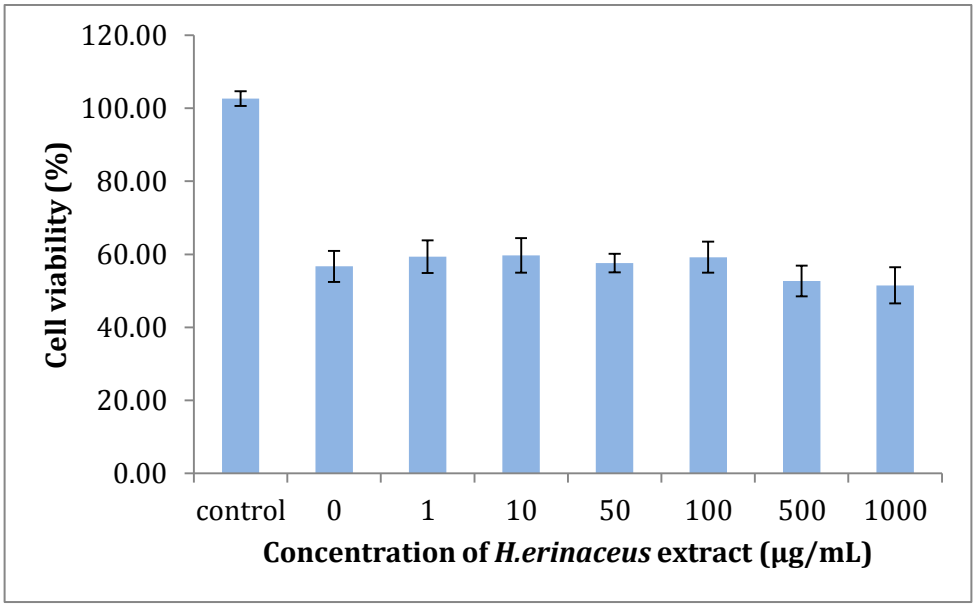

Figure 4.15: Cellular viability of NG108-15 cells co-treated with *H.erinaceus* aqueous extract and H_2O_2. Untreated control cells were set at 100% viability. All treatment groups were significantly different ($p < 0.05$) from untreated control.

4.3.2 Assessment of cellular viability using trypan blue exclusion assay

4.3.2.1 Cellular viability of NG108-15 cells pre-treated with *H.erinaceus* extract prior to H_2O_2-induced oxidative stress

The neuroprotective activity of *H.erinaceus* extract was evaluated using trypan blue exclusion assay. NG108-15 cells were pre-treated with varying concentrations of extract for 2 hr and 24 hr prior subjected to 100 µM H_2O_2 for 2 hr (**Figure 4.16**). All treatment groups were significantly different ($p < 0.05$) from untreated control (Appendix D, Table 37 - 40, pp.144 - 145).

The cell viability of NG108-15 decreased to 56.8 - 61.5 % when subjected to 100 µM H_2O_2 for 2 hr. Pre-incubation of *H.erinaceus* extract (1 – 1000 µg/mL) for 2 hr did not

improve cell viability of NG108-15; there was no significant difference ($p < 0.05$) between extract treated and non-extract treated cells. The cell viability remained within the range of 50.4 – 59.9%. However, the extended incubation period of *H.erinaceus* (24 hr) significantly improved the cellular viability of NG108-15. When the cells were pre-incubated with 1 – 50 μg/mL extract for 24 hr, the cellular viability was increased to 74.6 – 79.2 %. The pre-treatment of *H.erinaceus* extract at 100 μg/mL gave the highest level of cellular protection with cell viability maintained at 80.5 ± 3.4 %. This treatment increased the cell viability by 30.9% compared to cells without extract treatment (0 μg/mL). Cell viability of NG108-15 treated with 100 μg/mL *H.erinaceus* extract prior H_2O_2 assault was significantly different ($p < 0.05$) from all other extract-treated groups except 10 μg/mL treatment.

The cell viability of NG108-15 cells improved, though at a lesser degree, when the concentration of *H.erinaceus* extract was further increased. The cell viability was maintained at 72.6 – 75.1 % when pre-treated with 500 – 1000 μg/mL extract. There was no significant difference ($p < 0.05$) between these two treatment groups.

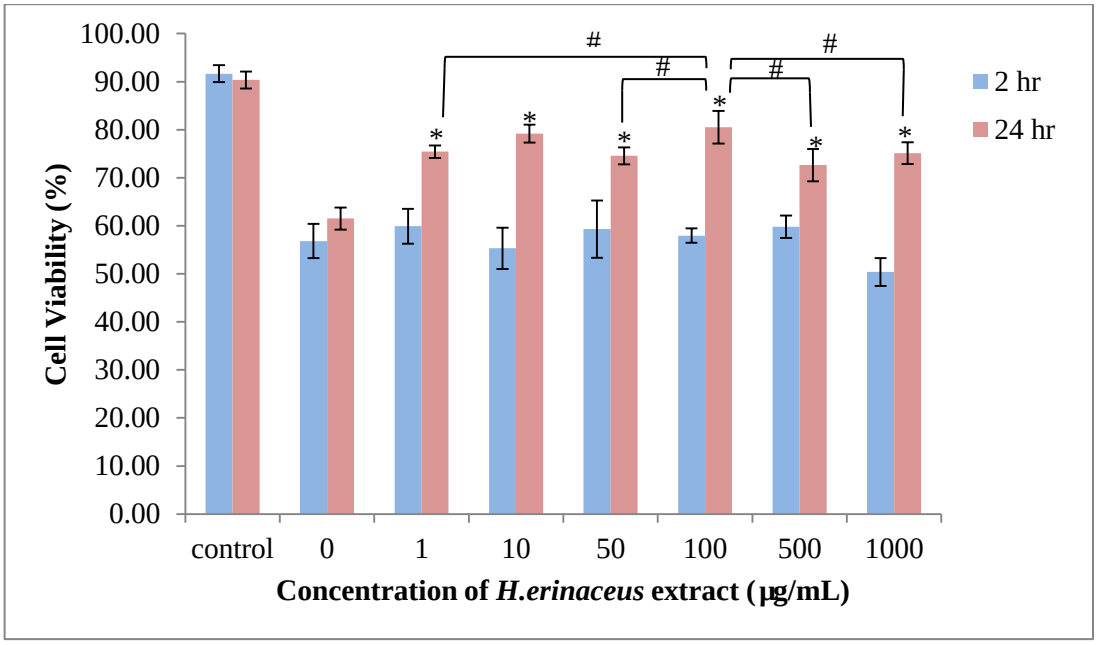

Figure 4.16: Cellular viability of NG108-15 cells pre-treated with *H.erinaceus* aqueous extract prior to H_2O_2-induced oxidative stress. All treatment groups were significantly different ($p < 0.05$) from untreated control. An asterisk (*) denotes a difference ($p < 0.05$) from the corresponding value in 0 µg/mL extract concentration (treated with 100 µM H_2O_2 only). A hash sign (#) denotes a significant difference ($p < 0.05$) between the marked treatment groups.

4.3.2.2 Cellular viability of NG108-15 cells co-treated with *H.erinaceus* extract and H_2O_2

Trypan blue exclusion assay was similarly used to assess neuroprotective activity of *H.erinaceus* extract when co-incubated with 100 µM H_2O_2 for 2 hr (**Figure 4.17**). All treatment groups were significantly different ($p < 0.05$) from untreated control cells (Appendix D, Table 41 & 42, pp.145).

The cell viability of NG108-15 was reduced to 66.60 ± 4.56 % when subjected to 100 µM H_2O_2 for 2 hr. When varying concentrations of mushroom extract were added simultaneously with H_2O_2 through the incubation period, significant improvement in cellular viability was not observed. The cell viability declined within the range of 65.86 – 68.87 % when treated with 1 – 50 µg/mL extract together with H_2O_2 for 2 hr. There was no significant difference ($p < 0.05$) among these treatment groups.

Improvement in cellular viability was not observed when the mushroom extract was further increased to 100 – 1000 µg/mL. Instead, a slight decrease was observed in cell viability (57.13 – 61.94 %). There was no significant difference ($p < 0.05$) among these concentration treatments. The decline in cell viability (57.13 ± 6.29 %) when treated with 1000 µg/mL extract was significantly different ($p < 0.05$) compared to cells not treated with extract (0 µg/mL).

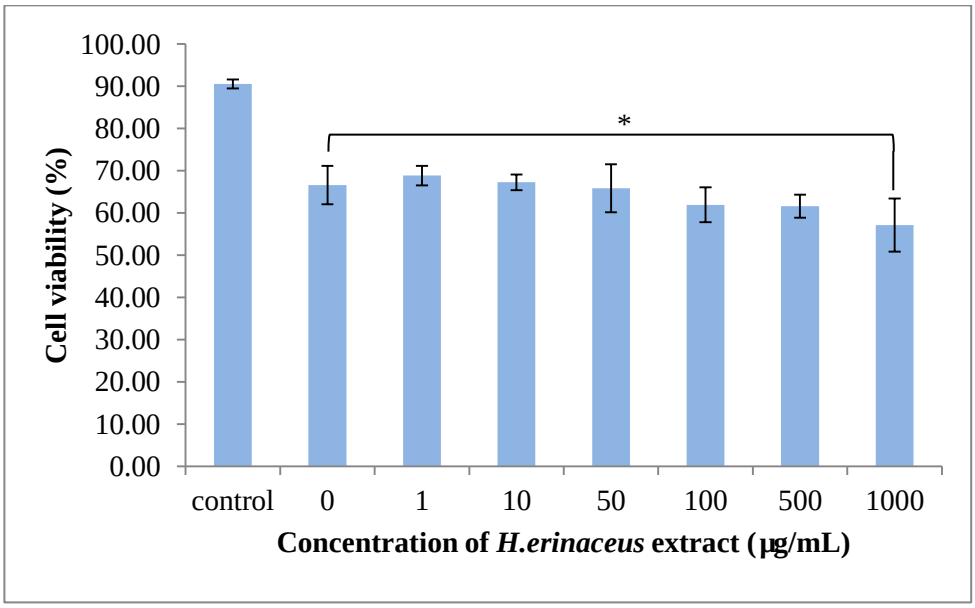

Figure 4.17: Cellular viability of NG108-15 cells co-treated with *H.erinaceus* aqueous extract and H_2O_2. Data shown are mean ± SEM of two experiments carried out in duplicate wells. All treatment groups were significantly different ($p < 0.05$) from untreated control. An asterisk (*) denotes a significant difference ($p < 0.05$) from 0 µg/mL extract concentration (treated with 100 µM H_2O_2 only).

4.3.3 Assessment of apoptosis using TUNEL assay

4.3.3.1 Evaluation of apoptotic cells in NG108-15 cells pre-treated with *H.erinaceus* aqueous extract

The pre-treatment of *H.erinaceus* extract (100 µg/mL) for 24 hr prior H_2O_2 exposure showed significant protection when assessed using trypan blue exclusion assay.

This treatment was further evaluated to assess the neuroprotective activity in *H.erinaceus* extract using TUNEL assay. The TUNEL method identifies apoptotic cells present in the cell culture.

NG108-15 cells were treated with 100 µg/mL extract (24 hr), 100 µM H_2O_2 (2 hr), or in pre-treatment mode [100 µg/mL extract (24 hr) followed by 100 µM H_2O_2 (2 hr)]. When the cells were incubated with 100 µg/mL *H.erinaceus* extract for 24 hr, 11.89 ± 1.81 % apoptotic cells were present in the culture (**Table 4.3**). A higher fraction of apoptotic cells (17.68 ± 3.41 %) was detected when the cells were incubated with 100 µM H_2O_2 for 2 hr. Pre-incubation of *H.erinaceus* aqueous extract for 24 hr slightly reduced the apoptotic cells to 15.85 ± 3.05 %. However, this reduction was not significantly different ($p < 0.05$) when compared to H_2O_2 treatment alone. All treatment groups were significantly different ($p < 0.05$) compared to the untreated control (Appendix D, Table 43 & 44, pp.146).

A positive control for TUNEL was prepared using bovine DNase I (nuclease) to induce DNA strand breaks (**Figure 4.18**). The apoptotic cells in the positive control slide were intensely stained green and showed typical apoptotic features such as cellular fragmentation. Similar morphological patterns were observed in cells treated with H_2O_2 alone and pre-treatment of extract followed by H_2O_2. In cells treated with extract alone, the apoptotic cells were moderately stained green. It was observed that there was a lower occurrence of cellular fragmentation in this treatment group.

Table 4.3: Evaluation of apoptotic cells in NG108-15 cells subjected to different treatments using TUNEL assay. Cells were grown in Lab-Tek™ 8-well chamber slide and treated with extract, H_2O_2 or both in succession. Values given represent the mean ± SEM of two independent experiments carried out in duplicate. Means with different letters in a same column are significantly different ($p < 0.05$).

Treatment	Apoptotic Cells (%)
Control	2.1 ± 0.6^{a}
100 µg/mL extract	11.9 ± 1.8^{b}
100 µM H_2O_2	17.7 ± 3.4^{c}
100 µg/mL extract – 100 µM H_2O_2	15.9 ± 3.1^{bc}

* Values are given in 1 decimal point as counting of TUNEL-positive cells was done manually.

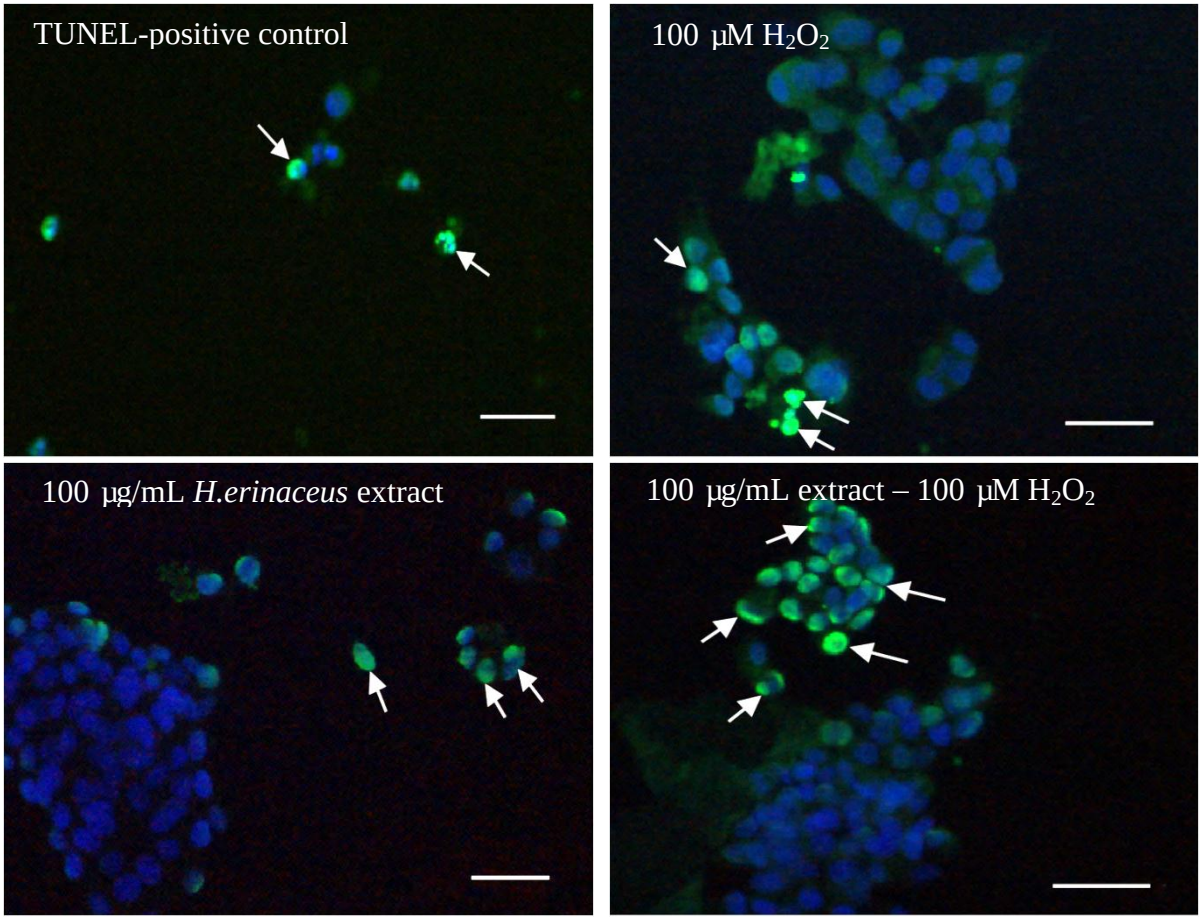

Figure 4.18: Morphology of apoptotic cells stained using TUNEL assay. The TUNEL-positive control was stained using DNase I provided in the kit. Apoptotic cells were stained green (avidin-FITC) and display morphological properties such as cellular condensation and fragmentation. The white arrows denote the presence of apoptotic cells in each photo. Scale bar = 100 µm.