CHAPTER 2

Materials and Methods

2.1 Phytochemicals and chemical reagents

Recombinant human insulin was purchased from Gibco, Life technology. Before the experiment, insulin solution was diluted in 0.025 M HCl, 0.1 M NaCl pH 1.6.

Amyloid β peptides, i.e. $A\beta_{40}$ and $A\beta_{42}$, were purchased from EZBiolab Laboratories and were initially solubilized, in 1,1,1,3,3,3 hexafluoro-2-propanol or hexafluoruroisopropanol (HFIP) (Fluka). Stock solution 1 mM in HFIP was aliquoted in 20 μL in each microtube and dried under N_2 gas atmosphere to undergo dried films, then stored at −20 °C. Before performed the experiment, aliquots were resuspended at a final concentration of 5 mM in DMSO (Sigma), sonicated using a bath sonicator for 10 min, and diluted to 100 μM with a PBS buffer + 0.05 % sodium dodecyl sulfate (SDS) (Sigma). Thioflavin T (ThT) (Sigma) was dissolved in PBS pH 7.4 and filtered through a 0.2 μm syringe filter. The concentration of Thioflavin T was determined using UV absorbance at 412 nm at extinction coefficient of 36,000 M^{-1} cm^{-1}.

Alkaloids molecules including crebanine (A1), O-methylbulbocapnine (A2), tetrahydropalmatine (A3) and N-methyltetrahydropalmatine (A4) were kindly provided by Associated Professor Dr. Wilart Pompimon, Department of Chemistry, Faculty of Science, Lampang Rajabhat University, Thailand. Quercetin and Curcumin were obtained from Sigma. All alkaloids molecules, Quercetin and Curcumin were dissolved in DMSO. IronQ was kindly provided by Assistant Professor Dr. Nathupakorn Dechsupa, Department of Radiologic Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand. IronQ was dissolved in double distilled water and prepared the stock solution at 2 $mg.mL^{-1}$.

Other chemical reagents include MEM, HAM/F12 medium with L-glutamine (Caisson, USA), DMEM with high-glucose and L-glutamine (Caisson, USA), Penicillin-Streptomycin (Caisson, USA), Fetal bovine serum (Gibco®, Invitrogen, USA), Dimethyl sulfoxide (DMSO).

2.2 Cell lines and cell culture

The neuroblastoma cell lines SK-N-SH and SH-SY5Y were used as models in this study. SK-N-SH were cultured in DMEM with high-glucose and L-glutamine, and SH-SY5Y cells were cultured in MEM:HAM/F12 (1:1) medium with L-glutamine. Both medium were supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum. Cells were cultivated at 37 ^{o}C in a humidified incubator of 95% atmosphere.

2.3 Spectroscopic analysis of Phytochemical-protein interaction

2.3.1 Interaction between insulin and phytochemicals

Fluorescence measurements were carried out in a spectrofluorometer (Perkin Elmer LS55), in a 1 cm path length quartz cuvette. The insulin formation was investigated by monitoring the fluorescence intensity of tyrosine residue (F_{tyr}). These fluorescence emission spectra were recorded at 25^{o}C using an excitation wavelength (λ_{ex}) at 276 nm. The fluorescence intensity (F_{tyr}) was investigated at 306 nm upon addition of phytochemicals (A1, A2, A3, A4, IronQ, Qct and Cur) at the same conditions.

2.3.2 The study of insulin fibrillation

1) Kinetics of insulin fibrillation monitoring by intrinsic Tyrosine fluorescence

The fluorescence intensity of Tyr was used to investigate the insulin fibrillation. The insulin fibrillation was performed by using the thermal-induced fibrillation method. The experiment was assigned by incubating 2 mL of insulin (0.02 mg.mL^{-1}) or insulin with phytochemicals (0.002 mg.mL^{-1}) at 80°C over a period of 24 h. The emission scans of Tyr were recorded from 280 to 500 nm in a 1-cm quartz

cell by exciting at 276 nm. The fluorescence intensity of Tyr at 306 nm was plotted against time of incubation. Efficiency of phytochemicals to inhibit the insulin fibrillation was assessed by two terms; 1) the Half time value ($t_{0.5}^{ins}$) and 2) the altered fluorescence intensity (ΔF_{tyr}). The Half time value ($t_{0.5}^{ins}$) defined as the time where the signal has reached 50% of the amplitude of the transition ($A/2=(F_i-F_n)/2$) that as shown in Figure 2.1. The altered fluorescence intensity (ΔF_{tyr}) signified the amount of insulin fibril formation and defined the percentage of the diminution of fluorescence intensity (A/F_i*100).

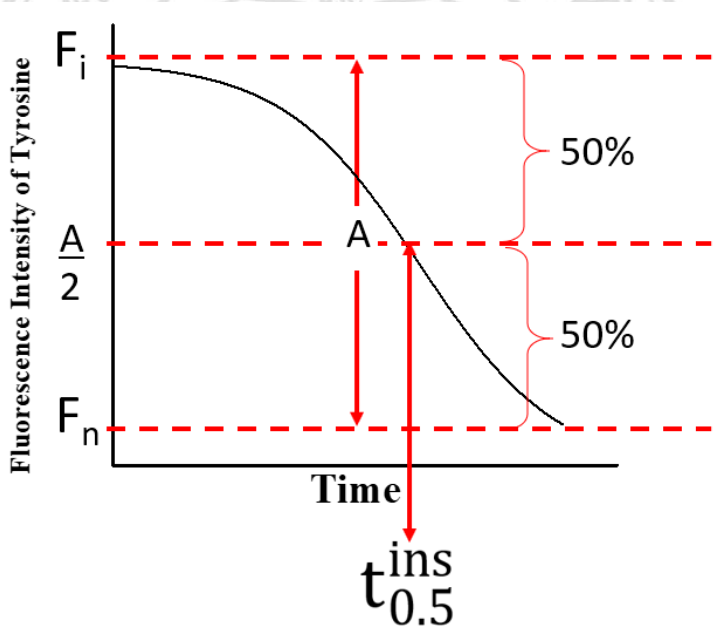

Figure 2.1 The model of kinetic of insulin fibrillation monitoring by the fluorescence intensity of Tyrosine

2) Effect of phytochemicals on insulin fibrillation by Thioflavin T

To determine whether phytochemicals affect the formation of insulin. Different concentration of phytochemicals (0.002-0.01 $mg.mL^{-1}$) were added into insulin solutions (0.02 $mg.mL^{-1}$) prior to warm them at 80 °C for 24 h. After incubation, 20 µM ThT was added and the fluorescence emission spectra (excitation wavelength at 420 nm) were obtained. Relative ThT fluorescence value were calculated from the ratio

of ThT fluorescence intensity of insulin in the presence of phytochemical and insulin control.

2.3.3 Kinetic analysis of amyloid fibrillation

The Aβ fibrillation was performed in 1 μM $Aβ_{40}$, 1 μM $Aβ_{42}$, and a combination of $Aβ_{40}$:$Aβ_{42}$ (0.2 μM:0.8 μM). All samples were added in 250 μL of PBS buffer pH 7.4 with 0.05% SDS containing 10 μM of ThT, and then incubated at 40°C. The fluorescence intensity of ThT was taken using spectrofluorometer (Perkin Elmer LS55) with the emission wavelength at 488 nm excitation wavelength at 420 nm.

2.3.4 Effect of phytochemicals on amyloid beta fibrillation

In order to elucidate the effect of phytochemicals on the formation of Aβ fibrillization. The experiments were performed by co-incubating of $Aβ_{40}$ (1μM), $Aβ_{42}$ (1μM) or $Aβ_{40}$:$Aβ_{42}$ (0.2:0.8 μM) with phytochemicals (0.002 $mg.ml^{-1}$) at 40 ºC and used 10 μM of ThT for fibrillation analysis. The fluorescence intensity of ThT was measured at 488 nm when excitation at 420 nm using spectrofluorometer (Perkin Elmer LS55). The kinetics of Aβ fibrillization could be described as sigmoid curves and the aggregation parameters were determined by fitting the plot of fluorescence intensity versus time as indicated in Figure 2.2. The fibrillation rate presented in the haft time value ($t_{0.5}^{Aβ}$) was used for data analysis.

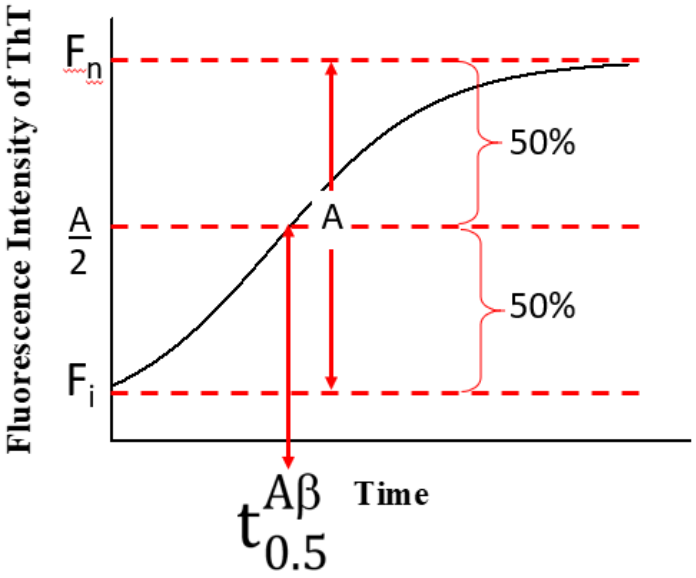

Figure 2.2 The model of kinetic of amyloid beta fibrillation monitoring by the fluorescence intensity of Thioflavin T

2.4 Effect of phytochemicals on cell viability of human neuroblastoma cell lines

Human neuroblastoma cell lines, SK-N-SH and SH-SY5Y at $5x10^4$ cells per well were seeded in 96-well plates and incubated with increasing concentrations of phytochemicals for 3 days. After incubation, 20 µL of resazurin stock solution (0.02 $mg.mL^{-1}$) was added to each well. The plates were further incubated for 4 h in at 37°C, 5% CO_2 incubator. Cell viability was assessed by the ability of the remaining viable cells to reduce resazurin to resorufin. The fluorescence intensity of resorufin (570 nm excitation/590 nm emission) was measured with a spectrofluoroscopic microplate reader (PerkinElmer LS55 spectrofluorometer). The 50% inhibitory concentration (IC_{50}), defined as the drug concentration causing 50% reduction in cell viability, was determined by plotting the concentration of the drug in the x-axis and the percentage of the cell viability in the y-axis. The experiments were run in triplicate.

2.5 Protective effect of phytochemicals against the neurotoxicity-induced by amyloid protein aggregation

To determination of protective effect of phytochemicals from amyloid fibril aggregation induced neurotoxicity *in vitro*. Cotreatment of phytochemicals (0.002 $mg.mL^{-1}$) with $A\beta_{40}$(1µM), $A\beta_{42}$(4µM) and $A\beta_{40}/A\beta_{42}$ (1:4 µM) were performed. The neuroblastoma cell lines SH-SY5Y ($5x10^4$ cells per well) were plated in 96 well plates. Next, cells were treated with phytochemicals alone or amyloid proteins alone and combination of phytochemicals with amyloid fibril protein. After 72 hours of treatment, 20 µL of 0.05 $mg.mL^{-1}$ of resazurin were added to each well and incubated at 37 ^oC, 5% CO_2 atmosphere for 4 hours. The fluorescence intensity of the wells indicated the relative fluorescent units (RFU) using a plate reader at excitation wavelength of 570 nm and emission wavelength at 590 nm. The average values were determined from triplicate readings. The percentage of cell viability were obtained.