CHAPTER 4

Discussion and Conclusion

The incidence of Alzheimer's disease has been growing continuously. Finding an effective treatment became more important. Amyloid-β has been being the focus of research for several decades. An ever-growing incidence of AD has led researchers and clinicians to discover the cure. As amyloid plaques are increased in AD patients. Hence, the propose of drugs that prevented β-amyloid accumulation may be a potential treatment for AD. Recent researches have focused on natural products to avoid the side effect of the clinical used. Natural products such as flavonoids, alkaloids and curcuminoids have been extensively researched regarding to reduce the amyloid associated toxicity of Aβ. In this study, we have proposed phytochemicals as an *in-situ* inhibitor for amyloid protein fibrillation. Alkaloids molecules derived from *Stephania venosa* including crebanine, O-methylbulbocapnine, tetrahydropalmatine and N-methyltetrahydropalmatine, Polyphenols including quercetin and curcumin, and a paramagnetic agent called IronQ (complexation of Iron and Quercetin) were used as phytochemicals.

We first studied the interaction of phytochemicals with human insulin. The reasons why human insulin was chosen as the model protein in this study are as follows; 1) insulin and Aβ protein share a common characteristic. 2) Under appropriate conditions, they both aggregate into amyloid fibrils. 3) Although the proteins do not share sequence homology, they exhibit similar insoluble filaments and fibrillation responses [95, 96]. This suggests that the process of fibrillation may be common [97]. According to the process of insulin aggregation, it proceeds through the dissociation of oligomeric states into monomers, which then undergo conformational changes and make themselves into the stable state by forming of fibrous amyloid aggregates rich in

β-sheets [98]. In the present work, the aggregation kinetics of human insulin was studied at low pH and high temperatures. Decreased in Tyr fluorescence intensity, an intrinsic fluorophore, was monitored during insulin aggregation that accompany with insulin fibrillation using Thioflavin T. Our results clearly found that most alkaloids and polyphenols inhibited insulin aggregation and fibril formation as dose dependent manner. O-methylbulbocapnine is isomeric with crebanine with different position of the two methoxyl. Both alkaloids molecules exhibited the similar properties of anti-insulin fibrillation. Therefore, different position of the two methoxyl did not affect to the anti-amyloidogenic properties. Interestingly, we found the different properties of anti-insulin fibrillation between N-methyltetrahydropalmatine and tetrahydropalmatine. N-methyltetrahydropalmatine is an analogue of tetrahydropalmatine. We found that the methyl group on the nitrogen atom of N-methyltetrahydropalmatine decrease the capacity of insulin fibril formations. Therefore, the nitrogen atom on tetrahydropalmatine seems presumably to play a role as active site for an inhibitor of amyloid fibril formation. Among polyphenols, we found that IronQ and quercetin exhibit the most inhibitor of insulin fibrillation. Quercetin is the most inhibitor of insulin fibrillation. Our study was in good agreement with previously study. The study from Kuperstein *et al* found that quercetin at a dose of 10 μM has shown anti-amyloidogenic activity by inhibiting the accumulation of β-amyloids [99].

Partial unfolding of native protein structure is considered as the critical step, prior to amyloid formation. It is suggested that stabilization of the native state by compounds having aromatic rings might increase the activation energy barrier, thereby slowing the aggregation kinetics. In addition, it is believed that hydrogen bonds stabilize the core structure of all amyloid fibrils. Based on a report by Porat *et al*., all polyphenolic inhibitors have at least two phenolic rings, a linker and at least three OH groups on the aromatic rings. It has been emphasized that these structural elements are necessary for the non-covalent interaction with the β-sheet structures [100]. Considering to chemical structure of phytochemicals used in this study, quercetin is composed of at least two phenolic rings with two to six atom linkers, and a minimum number of three OH groups on the aromatic rings. We suggest that these structural are essential for the non-covalent interaction with β-sheet structures, which are common to all amyloidogenic structures. This interaction may explain the properties as an inhibitor of insulin fibrillation.

We successfully demonstrated the ability of phytochemicals to inhibit the kinetics of insulin aggregation. We postulated that a similar strategy could be used to study on amyloid β peptide. According to the evidence of the major form of the Aβ peptide that found in amyloid plaque that showed $A\beta_{40}$ and $A\beta_{42}$ form mixed aggregates. It attempted us to investigate the influence of each Aβ peptide on their aggregation kinetics behavior. Kinetic analysis found that $A\beta_{42}$ exhibited a fast fibrillation rate than $A\beta_{40}$. However, mixing of $A\beta_{40}$ to $A\beta_{42}$ seems to slow down the fibril growth rate of $A\beta_{42}$ when comparing with $A\beta_{42}$ alone. The study from Pauwels *et al*., used the NMR experiments for visualizing the spontaneous aggregation of mixing $A\beta_{40}$ to $A\beta_{42}$. It was showed that $A\beta_{40}$ slows down the aggregation kinetics of $A\beta_{42}$[101]. In the presence of phytomolecules, our studies demonstrated that phytochemicals did not significantly change the kinetic of amyloid beta fibrillations. However, it seems that A1, A3 and IronQ presumably inhibit the $A\beta_{40}$ fibrillation process, while A1, A2, A3, IronQ, Qct and Cur presumably inhibit in $A\beta_{42}$ fibrillation. In the mixing of $A\beta_{40}$: $A\beta_{42}$, only A1 and A2 presumably inhibit in the fibrillation process.

Since neurotoxicity is a consequence effect of Aβ aggregation. We further investigate the protective effect of phytomolecules from toxicity of different species of Aβ fibrillation process in human neuroblastoma cell line, SH-SY5Y. The cell viability assay indicated $A\beta_{40}$ or $A\beta_{42}$ alone did not cause any apparent cytotoxicity. Interestingly, mixing $A\beta_{40}/A\beta_{42}$ (ratio 1:4) was highly toxic compared to $A\beta_{40}$ or $A\beta_{42}$ treated alone, indicating that $A\beta_{40}$ influence to the behavior of $A\beta_{42}$ that increased the toxicity. Our study was in agreement with the study of Kuperstein *et al* that found samples of higher $A\beta_{42}/A\beta_{40}$ ratios with higher neurotoxicity [99]. Moreover, Pauwels et al. also found that $A\beta_{42}$ and $A\beta_{40}$ affect each other's aggregation rates and toxic activities [101]. However, treatment with phytochemicals did not significantly increase neuronal cell viability. However, coincubation of mixing of $A\beta_{40}$: $A\beta_{42}$ with A3, A4 and IronQ seems to protect neuronal cells from Aβ-mediated toxicity.

In conclusion, amyloid fibrillation could be monitored by using intrinsic Tyrosine fluorescence accompany with Thioflavin T assay. Phytochemicals have shown some promise against amyloid fibrils both in insulin and amyloid beta peptide. Most alkaloids group, except N-methyltetrahydropalmatine, exhibited potent properties of

anti-amyloidogenesis. In polyphenol, quercetin and IronQ exhibited potent properties of anti-amyloid fibrillation. These results suggest alkaloid and polyphenols as the natural compound for the development of drugs against amyloid protein aggregation for treatment of Alzheimer's disease.