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Decelerate amyloid fibrillation by the alkaloids extracted from *Stephania venosa*

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ABSTRACT

Background: Naturally occurring phytochemical compounds have received considerable attention as alternative candidates for anti-amyloidogenic agents.

Objectives: This study, utilizing human insulin and amyloid-β peptide as an *in vitro* model, determined the anti-amyloid effects of alkaloids extracted derived from *Stephania venosa*.

Materials and methods: Alkaloids extracts including crebanine, *O*-methylbulbocapnine, tetrahydropalmatine and *N*-methyltetrahydropalmatine were used. The Inhibition of amyloid protein aggregation was studied by fluorescence spectroscopy.

Results: Most alkaloids, except *N*-methyltetrahydropalmatine, exhibited inhibitory properties against amyloid fibrillation either insulin or amyloid-beta peptide. Among the alkaloids group, crebanine and tetrahydropalmatine showed potent properties of anti-amyloidogenesis.

Conclusion: These results suggest that alkaloids could be used as a natural compound for the development of drugs against amyloid protein aggregation for the treatment of amyloid-related diseases.

Introduction

Suppression of amyloid protein aggregation is considered a promising therapeutic approach to prevent or treat amyloidosis-related disorders. One of the current strategies aimed at finding the therapeutic compound against amyloidogenic activity is to inhibit the toxic amyloid formation and stabilize its native monomeric form or destabilize the fibrillated misfold form.[1] Knowing that protein aggregation is a shared property of all proteins, model proteins can be used to study this process. Various peptides and proteins can undergo self-aggregation that leads to the formation of amyloid fibrils. Human insulin is one protein that was chosen and widely used as a model protein for the study of amyloid formation *in vitro*. Recently, much attention has been paid to find out an inhibitor of insulin amyloid fibrils.

Natural products are a major class of amyloid inhibitors, and natural product-based amyloid inhibitors have been identified and characterized in recent years.[1] Several natural polyphenolic compounds have been well-studied as amyloid inhibitors such as epigallocatechin gallate (EGCG), curcumin, and resveratrol. Quinones

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show inhibitory effects to different extents on insulin oligomerization, especially for 1,4-benzoquinone and 1,4-naphthoquinone.[2] Quercetin dose-dependently inhibited the amyloid formation of insulin via destabilizing the preformed insulin fibrils and transforming the fibrils into amorphous aggregates.[3]

Alkaloids, a class of nitrogen-containing compounds, are found primarily in plants, especially in flowering plants.[4] Alkaloids have a broad spectrum of pharmacological effects including analgesic, antiasthmatic, antiarrhythmic, anticancer, etc.[4,5] Galantamine, the isoquinoline alkaloid family, inhibits Aβ-aggregation and cytotoxicity.[4,6] However, galantamine is associated with side effects, the most common being nausea, vomiting, diarrhea, and anorexia.[7] The *in vitro* and *in vivo* studies showed that Aβ and Aβ-induced neurotoxicity was reduced by the effect of caffeine.[8] In addition, caffeine also reduces levels of Aβ in neuroblastoma-2a cells stably expressing human Swedish mutant APP and protecting cerebellar granule neurons and basal forebrain neurons from neurotoxicity caused by Aβ.[8,9] Furthermore, it should find out an additional alkaloid, either novel compounds or an old one, that could be used for inhibiting Aβ-aggregation.

Stephania venosa (Blume) Spreng., belongs to the Menispermaceae family, and has been traditionally used as a tonic drug and treatment of various diseases in South East Asian countries.[10] It was found that alkaloids are the main phytochemical compound of this genus.[11] Their biological activities have been reported including anti-cancer activity chemosensitizer and acetylcholinesterase inhibition.[12-15] Crebanine, a major component of *S. venosa*, exerts anti-proliferative and anti-invasive effects on human cancer cells through the induction of cell cycle arrest at the G1 phases, induce apoptosis in K562, K562/adr, GLC4 and reduce the expression of MMP-2, MMP-9, uPA, and MT1-MMP.[13,16-18] In addition, there are several natural alkaloids, including *O*-methylbulbocapnine, tetrahydropalmatine, and *N*-methyl tetrahydropalmatine have been found in the tubes of *S. vernosa.*[12,13] Furthermore, tetrahydropalmatine inhibits LPS-induced IL-8 secretion by blocking MAPKs signaling pathway.[19] However, there is no report about the anti-amyloid effects of these alkaloids.

In the present study, we measured protein aggregation of the model amyloid-forming protein using human insulin and amyloid beta peptide in the presence and absence of alkaloids. Human insulin fibril formation was generated by incubating at high temperatures and acid environment (pH 2.5 and 80 °C) and the effect of alkaloids on this fibrillation was investigated by intrinsic Tyrosine fluorescence assay and Thioflavin T assay. We reported here the capacity of alkaloids to interact with the insulins and thereby prevent their conversion to amyloid fibrils, indicating a therapeutic potential of the alkaloids in protein aggregation diseases.

Materials and methods

Chemical reagents

Recombinant human insulin was purchased from Gibco, Life Technology. Before the experiments, the 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 hexafluoroisopropanol (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 performing the experiments, 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 phosphate buffer solution (PBS) plus 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 ThT was determined using UV absorbance at 412 nm and calculated by using the extinction coefficient of 36,000 $M^{-1}cm^{-1}$. Four alkaloids 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[13] These alkaloids were extracted from the tuber of *S. venosa*. Tetrahydropalmation and crebanine are productions from ethyl acetate extraction. While *O*-methylbulbocapnine and *N*-methyltetrahydropalmatine were extracted by acetone. The extraction method was clearly described by Nantapap.[13] All alkaloid molecules were dissolved in DMSO (Figure 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).

Kinetics of insulin fibrillation by intrinsic Tyrosine fluorescence

The fluorescence intensity of tyrosine (Tyr) was used to investigate insulin fibrillation. The insulin fibrillation was performed by using the thermal-induced fibrillation method.[20] The experiment was assigned by incubating 2 mL of insulin (0.02 mg/mL) or insulin with alkaloids (0.002 mg/mL) at 80 °C for 24 hrs. The emission spectra of Tyr were recorded from 280 to 500 nm in a 1cm quartz cell by exciting at 276 nm. The fluorescence intensity of Tyr at 306 nm was plotted against the time of incubation. The efficiency of alkaloids to inhibit insulin fibrillation was assessed by two terms; 1) the half-time value of insulin fibrillation ($t_{0.5}^{ins}$) and 2) the altered fluorescence intensity of tyrosine (ΔF_{tyr}). The half-time value of insulin fibrillation ($t_{0.5}^{ins}$) is defined as the time when the signal has reached 50% of the amplitude of the transition ($A/2=(F_i-F_n)/2$) that as shown in Figure 2a. Where F_i and F_n are the fluorescence intensities at the initial reaction and steady state, respectively, and A is the amplitude of the reaction. 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 2a)

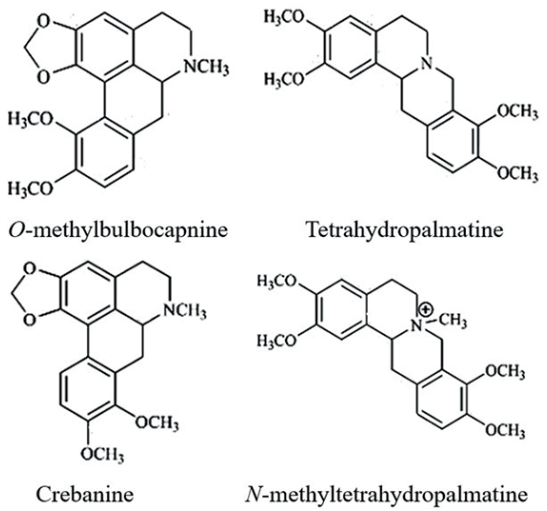

Figure 1 *Chemical structure of four alkaloids derived from Stephania venosa (Blume) Spreng.*

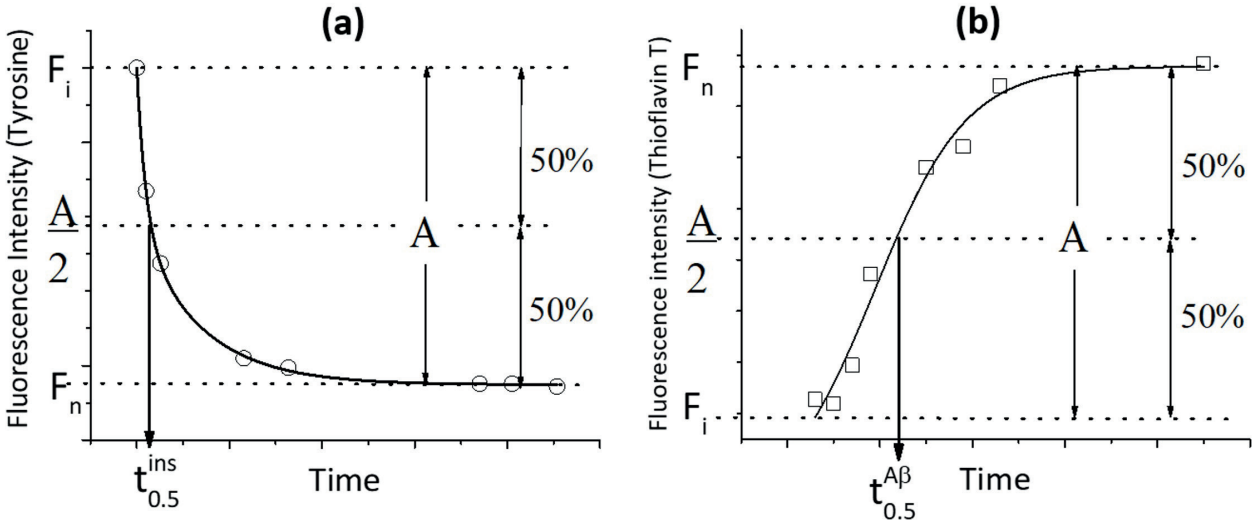

Figure 2 *Kinetic of insulin fibrillation monitoring by the fluorescence intensity of tyrosine (a). and the kinetic of amyloid beta fibrillation monitoring by the fluorescence intensity of Thioflavin T(b).*

Effect of alkaloids on insulin fibrillation

Various concentrations of alkaloids (0.002-0.01 mg/mL) were added into insulin solutions (0.02 mg/mL) prior to warming at 80 °C for 24 hrs. After incubation, 20 µM ThT was added, and the fluorescence emission spectra (excitation wavelength at 420 nm) were obtained. Relative ThT fluorescence values were calculated from the ratio of ThT fluorescence intensity of insulin in the presence of alkaloids and insulin control.

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 a spectrofluorometer (Perkin Elmer LS55) with an emission wavelength of 488 nm excitation wavelength of 420 nm.

Effect of alkaloids on Aб fibrillation

The experiments were performed by co-incubating of $Aβ_{40}$ (1 µM), $Aβ_{42}$ (1 µM), or $Aβ_{40}$:$Aβ_{42}$ (0.2:0.8 µM) with alkaloids (0.002 mg/mL) at 40 °C and used 10 µM of ThT for fibrillation analysis. The fluorescence intensity of ThT was measured at 488 nm when excitation was at 420 nm using a spectrofluorometer. 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 2b. The fibrillation rate presented in the half-time value ($t_{0.5}^{Aβ}$) was used for data analysis. The efficiency of alkaloids to inhibit the Aβ fibrillation was assessed by two terms; 1) the half-time value ($t_{0.5}^{Aβ}$) defined as the time when the signal has reached 50% of the amplitude (A) of the transition ($A/2=(F_n-F_i)/2$). Where F_i and F_n are the fluorescence intensities at the initial reaction and at steady state, respectively, and A is the amplitude of the reaction. (Figure 2b)

Statistical analysis

All data are expressed as mean±SD. Statistical significance was determined using Student's t-test between the groups treated and the control. A probability $p<0.05$ was considered statistically significant.

Results

Alkaloids extracts inhibited the kinetic of insulin aggregation.

To determine whether alkaloids extract inhibited insulin fibrillation, tyrosine emission spectra of insulin (0.02 mg/mL) in the presence of alkaloids (0.002 mg/mL) were observed for 24 hrs. The results showed a decrease in Tyr fluorescence intensity during insulin fibrillation. This becomes apparent on plotting the emission intensity at 306 nm against time with the half-time ($t^{ins}_{0.5}$) of insulin equal to 0.55±0.11 hrs. However, the addition of A1, A2, A3, and A4 to the insulin did not decrease in intensity of the emission at 306 nm over the time of incubation. Therefore, the half-time ($t^{ins}_{0.5}$) of A1, A2, and A4 significantly increased except A3.

Alkaloids extracts inhibited insulin fibril formation.

To confirm the presence of insulin fibril formation. ThT fluorescence assay was performed. It was found that ThT binds specifically to the cross-β sheet structure of amyloid fibers and gives more intense once bound. In this experiment, after incubation for 24 hrs, 20 mM ThT was added and measured the fluorescence intensity at 488 nm after exciting with 420 nm. The fluorescence intensity of ThT of insulin incubated with A1, A2, and A3 after incubation was lower than that of insulin control with the relative ThT fluorescence equal to 0.44±0.06, 0.59±0.05 and 0.57±0.07, respectively (Table 1). Interestingly, the ThT fluorescence intensity of A4 incubated with insulin did not change when compared with insulin control. Therefore, all alkaloid extracts, except A4, inhibited insulin fibril formation.

Table 1 Effect of alkaloids on insulin fibrillation detected by Tyr fluorescence and Thioflavin T versus insulin control.

Alkaloids	Tyrosine fluorescence		Relative ThT fluorescence
	Half time ($t^{ins}_{0.5}$), h	ΔF_{tyr}	
Insulin	0.55±0.11	88.0±4.7	1.00
Insulin +A1	0.81±0.01*	25.2±4.6*	0.44±0.06*
Insulin +A2	0.89±0.09*	16.9±4.6*	0.59±0.05*
Insulin +A3	0.69±0.07	61.5±10.6*	0.57±0.07*
Insulin +A4	0.79±0.09*	33.5±4.5*	1.29±0.27

*Note: *p<0.05*

Alkaloids extracts inhibited the insulin fibril formation in a dose-dependent manner.

The previous data demonstrated that most alkaloids, except A4, had the potential to be an inhibitor of insulin fibrillation. We performed a further experiment to determine whether alkaloid extracts affect the insulin fibrils formation in a dose-dependent manner, different concentrations of A1-A4 (0.002-0.01 mg/mL) were added into insulin (0.02 mg/mL) before warming them to 80°C for 24 hrs. After incubation, 20 µM ThT was added and the fluorescence intensity at 488 nm (excitation wavelength at 420 nm) was obtained. Relative ThT fluorescence values were calculated and derived from the ratio of ThT fluorescence intensity of insulin in the presence of alkaloids and the ThT fluorescence intensity of fibrils insulin control. Increased concentration of alkaloid extracts was found that A1, A2, and A3 potently inhibited insulin fibril formation in a dose-dependent manner, while A4 did not. Among alkaloid molecules (at 0.002 mg/mL), A1 might be the most inhibitor of insulin fibrillation. (Figure 3)

Alkaloids extracts inhibited the Aβ fibrillation.

To characterize the process of Aβ fibrillation, the kinetic fibrillization process of Aβ peptide with different mixing ratios of $Aβ_{40}$ to $Aβ_{42}$ was performed. Our model is based on the finding that there are two main Aβ peptides of different lengths involved in Alzheimer's disease, $Aβ_{40}$, and $Aβ_{42}$ residues. It was found that the mixing of $Aβ_{40}$ and $Aβ_{42}$ enhanced toxicity in the early onset of some familial Alzheimer's diseases.21 Otherwise, our previous study found that the ratio of $Aβ_{40}$: $Aβ_{42}$ (1:4) increased the toxicity in a neuroblastoma cell line, SK-N-SH, higher than treated with $Aβ_{40}$ or $Aβ_{42}$ alone (data not shown). To mimic the pathology of AD, three Aβ peptides were prepared in 1 µM $Aβ_{40}$, 1 µM $Aβ_{42}$, and a combination of $Aβ_{40}$:$Aβ_{42}$ (0.2 µM:0.8 µM). The fibrillization of Aβ was observed by an increase in ThT fluorescence due to the binding of the dye to the fibrils. The representative fibrillization curves were shown in Figure 4. Our results showed that $Aβ_{40}$, $Aβ_{42}$, and $Aβ_{40}$:$Aβ_{42}$ demonstrated similar fibrillization kinetics which exhibit a sigmoidal appearance. It seems to be that the fibril growth rate which is represented by $t^{Aβ}_{0.5}$ for $Aβ_{42}$ was shorter than that of $Aβ_{40}$, indicating $Aβ_{42}$ exhibited a fast fibrillation rate than $Aβ_{40}$. Mixing of $Aβ_{40}$ to $Aβ_{42}$ seems to decrease the fibril growth rate of $Aβ_{42}$ compared with $Aβ_{42}$ alone (Figure 4d).

In the presence of alkaloid extracts (0.002mg/mL), the results showed that different alkaloid extracts showed the different effects on the kinetic of amyloid beta fibrillation as indicated by half-time ($t^{ins}_{0.5}$) and the relative ThT fluorescence value is shown in Table 2. Increased half-time and decreased relative ThT fluorescence value represent the decreased amyloid fibrillation formation. The half-time of amyloid formation for $Aβ_{40}$ was increased from

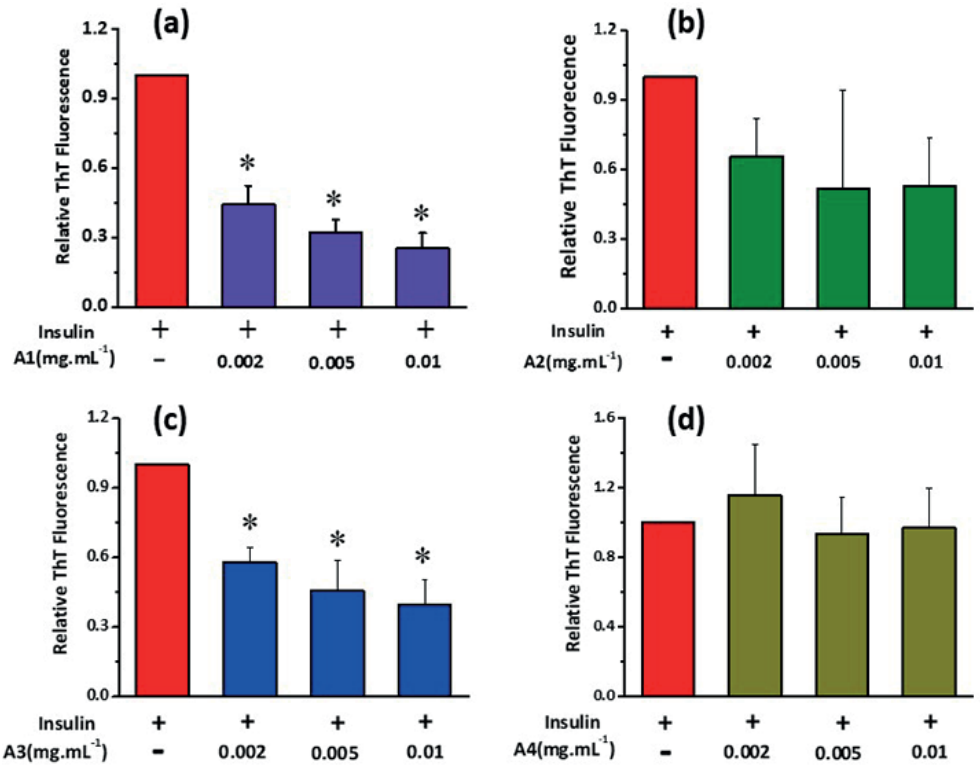

Figure 3 *Effect of various concentrations of alkaloid extracts, crebanine (a), O-methylbulbocapnine (b), tetrahydropalmatine (c) and N-methyltetrahydropalmatine (d), on insulin fibrillation, detected by thioflavin T. The data were presented as mean±S.D. (N=3), *p<0.05 versus insulin control.*

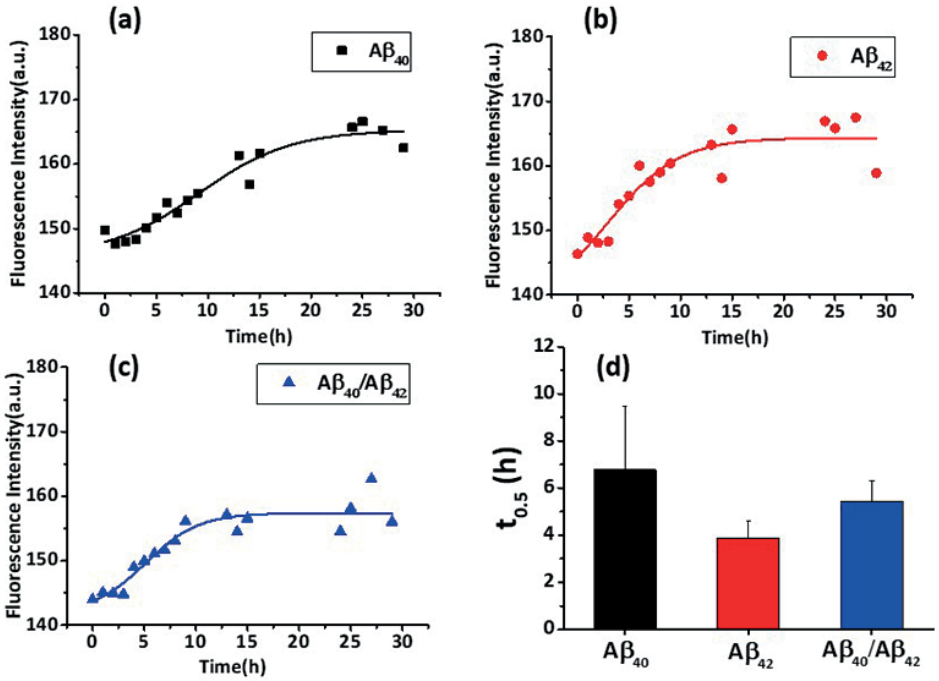

Figure 4 *Kinetic of amyloid-β fibrillation detected by Thioflavin T. ThT fluorescence emission at 488 nm was monitored upon excitation at 420 nm. The Aβ concentration was performed in 1 μM $Aβ_{40}$ (a), 1 μM $Aβ_{42}$(b), and a combination of $Aβ_{40}$:$Aβ_{42}$ (0.2 μM:0.8 μM)(c). All samples were added to 250 μL of PBS buffer pH 7.4 with 0.05% SDS containing 10 μM of ThT, and then incubated at 40 °C.*

Table 2 Effect of alkaloids (0.002 mg/mL) on Aβ fibrillation detected by Thioflavin T assay versus insulin control.

Phytochemicals	Thioflavin T assay					
	Half time ($t_{0.5}^{A\beta}$) hr.			Relative ThT fluorescence		
	$A\beta_{40}$	$A\beta_{42}$	$A\beta_{40:}$ $A\beta_{42}$	$A\beta_{40}$	$A\beta_{42}$	$A\beta_{40:}$ $A\beta_{42}$
Aβ Control	6.8±2.7	3.9±0.7	5.5±0.9	1.00	1.00	1.00
Aβ+A1	8.8±1.9	3.9±1.2	5.0±1.8	0.20±0.03*	0.70±0.05	1.02±0.11
Aβ+A2	6.4±2.7	8.4±8.0	6.3±1.2	0.55±0.12*	0.93±0.38	1.03±0.18
Aβ+A3	7.7±3.3	5.2±0.9	9.3±2.7	0.67±0.07*	1.01±0.06	1.54±0.74
Aβ+A4	9.0±0.6	4.4±3.4	4.3±1.2	1.25±0.05	1.35±0.25	1.58±0.04

*Note: *p<0.05*

6.8 hrs to 8.8 hrs and 7.7 hrs in the presence of A1 and A3 respectively. Accordingly, with half-time value, the relative ThT fluorescence values were decreased in the presence of A1 and A3 compared with Aβ control. Therefore, A1 and A3 inhibited the amyloid fibrillation for $A\beta_{40}$. It was also found that amyloid formation for $A\beta_{42}$ was inhibited by A2. However, it seems to be that A2 inhibited amyloid formation for $A\beta_{40}$ and A1 inhibited amyloid formation for $A\beta_{42}$ as indicated by the decreased relative ThT fluorescence value compared with its control.

Discussion

The previous study in our laboratory found that at concentrations ranging from 0.002 to 0.01 mg/mL of crebanine, O-methylbulbocapnine, tetrahydropalmatine, and *N*-methyltetrahydropalmatine exhibited a cytotoxic effect of less than 10% on the neuronal cell lines (SK-NSH and SH-SY5Y). However, it's important to note that this data has not been published. The incidence of amyloid-related diseases has been growing continuously. Finding an effective treatment became more important. Amyloid fibrillated protein has been the focus of research for many years.[22] An ever-growing incidence of amyloid-related diseases has led researchers and clinicians to discover a cure. Hence, the purpose of drugs that prevented amyloid accumulation may be a potential treatment. Recent research has focused on natural products to avoid the side effect of clinical use.[23] Natural products such as flavonoids, alkaloids, and curcuminoids have been extensively researched regarding reducing the amyloid-associated toxicity of Aβ.[24,25] In this study, we have proposed alkaloids as an *in-situ* inhibitor for amyloid protein fibrillation. Four alkaloids derived from *Stephania venosa* including crebanine, *O*-methylbulbocapnine, tetrahydropalmatine, and *N*-methyltetrahydropalmatine were used as interested molecules.

We first studied the interaction of alkaloids 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.[26,27] 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 a stable state by forming fibrous amyloid aggregates rich in β-sheets.[28] In the present work, the aggregation kinetics of human insulin was studied at low pH and high temperatures. Decrease in Tyr fluorescence intensity, an intrinsic fluorophore was monitored during insulin aggregation that accompanies insulin fibrillation using Thioflavin T. Our results clearly found that most alkaloids inhibited insulin aggregation and fibril formation in as dose-dependent manner. *O*-methylbulbocapnine is isomeric with crebanine with different positions of the two methoxyl.[13] Both alkaloid molecules exhibited similar properties of anti-insulin fibrillation. Therefore, different positions of the two methoxyl did not affect to the anti-amyloidogenic properties. Interestingly, it was 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 decreases the capacity of insulin fibril formations. Therefore, the nitrogen atom on tetrahydropalmatine seems presumably to play a role as an active site for an inhibitor of amyloid fibril formation.

We successfully demonstrated the ability of alkaloids to inhibit the kinetics of insulin aggregation. We postulated that a similar strategy could be used to study amyloid β peptide. According to the evidence of the major form of the Aβ peptide found in amyloid plaque that showed $A\beta_{40}$ and $A\beta_{42}$ form mixed aggregates.[29] It attempted to investigate the influence of each Aβ peptide on their aggregation kinetics behavior. The kinetic analysis found that $A\beta_{42}$ exhibited a fast fibrillation rate than $A\beta_{40}$. However, the mixing of $A\beta_{40}$ to $A\beta_{42}$ seems to slow down the fibril growth rate of $A\beta_{42}$ when compared 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 shown that $A\beta_{40}$ slows down the aggregation kinetics of $A\beta_{42}$.[30]

Conclusion

In conclusion, amyloid fibrillation could be monitored by using intrinsic Tyrosine fluorescence to accompany with Thioflavin T assay. Alkaloids 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. These results suggest alkaloids can be used as the natural compound for the development of drugs against amyloid protein aggregation for the treatment of Alzheimer's disease.

Conflict of interest

The authors declare no conflict of interest.

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