Study on tau-aggregation inhibitors in Alzheimer's disease of methanol extracts of several medicinal plants collected in the Mekong Delta, Vietnam

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ABSTRACT

Tau protein and Aβ-amyloid have been studied as pathological aggregations, which form neurofibrillary tangles and amyloid plaques in Alzheimer's disease brain. Tau protein plays a critical role in neuron that binds to microtubules and assists with their formation and stabilization. However, unbinding of hyperphosphorylated tau and microtubules leads to unstable and disintegrating state of neuron. The free tau proteins form neurofibrillary tangles. The purpose of this study is to screen in vitro the the tau-aggregation inhibitory activity of nine methanol extracts of Psidium guajava leaf, Nelumbo nucifera leaf; wild Ipomoea aquatic, Cleome rutidosperma aerial parts, Artocarpus altilis leaf, cultivated Ipomoea aquatic, Centella asiatica leaf, Mimosa pudica L. aerial parts, Nelumbo nucifera seed pod collected in the Mekong Delta. Nine herbs were collected, dried and extracted with methanol. The half maximal inhibitory concentration (IC_{50}) of methanol extracts was measured by Thioflavin T assay at various concentrations. Silica gel column chromatography was employed to fractionate the Psidium guajava leaf crude extract. Nine methanol extracts were proved to reduce the tau aggregation in vitro. Extracts from leaves of Psidium guajava, Artocarpus altilis and Nelumbo nucifera impressively inhibited the tau aggregation with IC_{50} at 0.39 mg/mL, 1.05 mg/mL and 1.24 mg/mL, respectively. Methylene blue was used as a positive control, with IC_{50} at 1.35 µM. The five examined fractions of guava leaf were proved to inhibit the tau aggregation ranging from 33.70 % to 48.49 %, except the 100 % of hexane fraction showed almost no effect on the tau aggregation inhibitor.

***Keywords**: Artocarpus altilis, Alzheimer's disease, Nelumbo nucifera, Psidium guajava L, Thioflavin T, tau-aggregation*

INTRODUCTION

Tau is one of the microtubules associated proteins that has been reported to have a role in the stabilization of neuronal microtubules; these in turn provide the tracks for intracellular transport. It is abundant in both central and peripheral nervous systems [1-3]. The molecular weight of tau protein was between 55,000 and 62,000 Dalton. Tau protein has six isoforms which possibly have their particular physiological role and differential biological activities [4-7]. However, tau protein can be hyperphosphorylated by dynamic regulation of tau kinases and tau phosphatases, leading to the release tau and tau-supporting structures which will be disassembly [4]. Furthermore, the free tau is gradually accumulated into aggregates which are harmful for other cells in the human brain [3]. Neurofibrillary lesions made of

hyperphosphorylated microtubule-associated protein tau constitute one of the defining neuropathological features of Alzheimer's disease [8]. To date, the mechanism underlying tau release remains unclear [9]. However, inhibiting tau aggregation is a traditional tau-based therapeutic strategy. Treatment with blue methylene can preserve the cognition in a line of transgenic mice expressing human mutant tau [10]. Plants represent one of the important sources of leading compounds, with up to 40 % of modern drugs being derived from plant materials. Empirical knowledge based on the ethnomedical benefits of plants, coupled with bioassay-guided fractionation and isolation, has the potential to identify novel neuroprotection that could be used against tau protein aggregation [11]. Currently, herb and plant resources are relatively unlimited with respect to the search for functional phytochemicals but these resources are dwindling rapidly due to deforestation and advancements of industrialization [12]. Even though a number of studies have been performed using purified plant chemicals, very few studies have addressed the tau-aggregation inhibitors of plant crude extracts.

In this study, we screened tau anti-aggregation potential of methanolic extracts of nine plant samples collected in the Mekong Delta by using Thioflavin T method.

MATERIALS AND METHODS

Chemicals and reagents

Methanol, heparin sodium salt (Sigma-Aldrich Corporation, Japan), DMSO (Nacalai, Japan), thioflavin T (Sigma, St. Louis, Missouri, USA), Tau 3R MBD (kindly provided by Professor Hachiro Sugimoto, Doshisha University, Japan), blue Methylene (Sigma), Silica gel 70-230 mesh (Merck 107734.1000).

Plant materials

Nine medicinal plants were locally collected in Can Tho City and Ca Mau province with the descriptions from Cay Co Viet Nam [13] and based on their antioxidant and neuro-protection activity in treating various diseases [13]. The nine herbs were also selected because of its pharmaceutical values, popularity and their use as traditional medicine in the Mekong Delta (Table 1). Parts used of seven herbs were presented in Table 2.

Table 1. Research studies of selected medicinal plants in the Mekong Delta in relation to inhibition activity against tau aggregation

Plants names	Family	Research studies related to brain protection	Reference
Psidium guajava	Myrtaceae	Anti-epileptic activity	[14]
		Antioxidant activity and free radical-scavenging capacity	[15]
Nelumbo nucifera	Nelumbonaceae	Cognitive enhancing and neuroprotective effect	[16]
		Anti-Cholinesterase and antioxidant activity	[17]
		BACE1 and cholinesterase inhibitory activities	[18]
Ipomoea aquatic	Concolvulaceae	Nervous debility	[19]
Artocarpus altilis (Park.) Fosb	Moraceae	Xanthine oxidase inhibitory activity of ethanolic extract from leaf	[20]
Centella asiatica	Apiaceae	Neuroprotective effect	[21, 22]
Mimosa pudica L.	Fabaceae	Neuroprotective effect of ethanolic extract of *Mimosa pudica* in D-galactose induced Alzheimer's model treat neurological problems	[23] [24]
Cleome rutidosperma	Caparaceae	Antioxidant and free radical scavenging activities	[25]

Preparation of methanolic extracts

Five hundred mg of finely ground samples were extracted in 4 mL of methanol, which were suspended in water bath at 55 ºC for 5 hours. The suspensions were then centrifuged at 4 ºC for 10 min at 15000 × g, after which the supernatants were filtered through a 0.2 µm cellulose acetate membrane syringe filter and stored in the dark at 4 ºC. These methanol: extracts were diluted in methanol throughout this study [26].

Thioflavin T (ThT) fluorescence

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils and is commonly used to diagnose amyloid fibrils, both *ex vivo* and *in vitro* [27, 28]. Methylene blue, is a type of phenothiazine, may act as a destabilizing agent of tau aggregates [29] and is used as a positive control.

The reaction mixture consists of 150 µL of Tris-HCl buffer (50 mM, pH 7.6) were mixed with 10 µL of each methanol extract. Then, 20 µL of 100 µM tau protein (3R-MBD) was added, followed by 20 µL of heparin (100 µM). The mixtures were incubated at 37 ºC for 16 hours without being exposed to the light. After incubation, 135 µL of reaction solution was measured base fluorescence values, symbolized as ThT (-) by microplate reader Perkin Elmer AROV Wallac 1420. Subsequently, 15 µL of thioflavin T (100 µM) was mixed with the solution and then to measure ThT (+). The fluorescent intensity was measured with the excitation wavelength at 440 nm and the emission wavelength at 486 nm. Each extract was examined at four different concentrations and replicated 3 times. The percent inhibition (%) and the half maximal inhibitory concentration (IC_{50}) were obtained by the following equations:

$$\text{Inhibition (\%)} = 100 - \frac{100 \times [\text{Average } (S_1 - S_o)]}{\text{Average } (T_1 - T_o)}$$

S_1: the average ThT (+) of sample; S_o: the average of ThT (-) of the sample

T_1: the average ThT (+) of negative control (methanol); T_o: the average ThT (-) of methanol

IC_{50} (mg/mL) = 10^log(C_1/C_o)*(50-I_o/((I_1 – I_o) + logC_o)

C_1: Concentration inhibiting less than 50 %; C_o: Concentration inhibiting higher than 50 %

I_1: Inhibitory rate which is higher than 50 %; I_o: Inhibitory rate which is lower than 50 %

Statistical analysis

The mean and standard deviation values were calculated based on the data from at least 3 independent experiments. Descriptive statistics and ANOVA analysis using Minitab Statistical Software (version 16.0) (Minitab Inc., State College, PA, USA) was used in Figure 1 and 2 to identify statistical significance ($p<0.05$). Bar charts and graphs were designed in Microsoft Excel.

Column chromatography

The column chromatography (length: 450 mm; bore 30 mm) was performed using 70-230 mesh silica gel to elute out individual components from the crude plant extract. The column was rinsed with η-hexane and completely dried before use. The column was filled 3/4th with η-hexane and the silica gel was packed approximately 2/3rd of the column length with simultaneous draining of the solvent to aid proper packing. The packing was performed after activating the silica gel at 100 ºC for 1 hour and gently poured on the top of the column with constant tapping to avoid air bubbles and cracks after mixing with hexane. The column was run with 200 mL of varying solvent polarities (η-hexane in ethyl acetate in different ratios as specified in Table 3) after loading with 0.1 g crude extract mixed with 5 g of activated silica gel [30]. The fractions collected were evaporated and diluted with 500 µL DMSO

(dimethyl sulfoxide) in order to test for their tau aggregation inhibitor activity.

RESULTS AND DISCUSSION

The nine methanolic extracts were tested for tau-aggregation inhibitors using Thioflavin T method in 96-well microplate. Blue methylene was used as the standard tau aggregation inhibitor in this study. The inhibition curve of methylene blue was presented in Fig. 1.

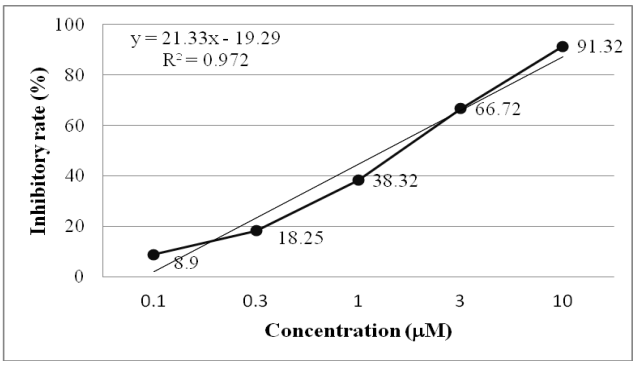

Fig. 1. Standard curve of methylene blue

The percentage of tau anti-aggregation increased linearly with methylene blue concentration. At 10 μM (0.032 mg/mL) alone, the inhibition rate reached 91.32 %. The IC_{50} was 1.35 μM which was equal to 0.004 mg/mL. The standard curve between the concentration and the inhibitory rate can be expressed as Equation (1), $y = 21.33x–19.29$ ($R^2= 0.972$) , where x is the concentration of blue methylene and y is the inhibitory rate. The descriptive results of anti-aggregation efficacy of various concentrations of 9 methanol extracts have been displayed in Fig. 2.

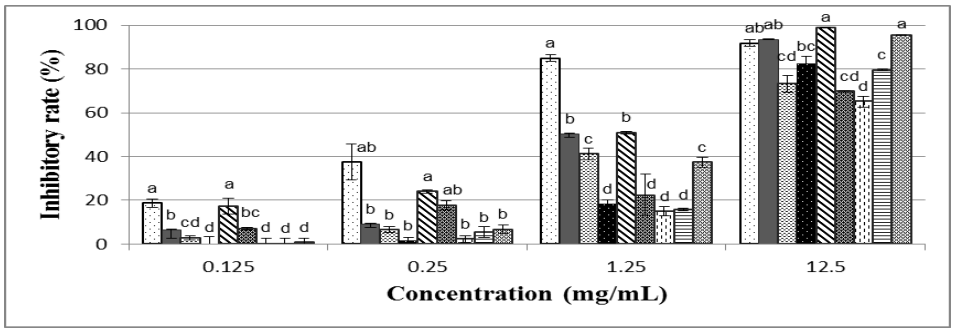

Fig. 2. Tau anti-aggregation effects of plant extracts

Extracts as appear in the chart from left to right: *Psidium guajava* leaf; *Nelumbo nucifera* leaf; wild *Ipomoea aquatic*, *Cleome rutidosperma* aerial parts; *Artocarpus altilis* leaf; Cultivated *Ipomoea aquatic*; *Centella asiatica* leaf; *Mimosa pudica* L. aerial parts; *Nelumbo nucifera* seed pod.

Each value is the mean ± standard deviation (n=3). Significant different (p=0.000) within each concentration are denoted by different letters (i.e., bars with the same letter are not significantly different).

The results shown in Fig. 2 and Table 2 demonstrated that while all concentrations of

Psidium guajava and *Artocarpus altilis* showed consistent tau anti-aggregation efficacy, only higher concentrations of *Nelumbo nucifera* seed pod were found to have tau anti-aggregation properties. Overall, it was observed that the tau anti-aggregation efficacy of all the extracts increased with an increase in the concentration. Comparison of mean efficacy values of blue methylene has been shown in Fig. 1. Albeit extracts from *Psidium guajava* and *Artocarpus altilis* leaves showed some tau anti-aggregation efficacy, their values were not comparable to the positive control since all extracts were diluted in methanol. Extracts of *Nelumbo nucifera* leaf, stem and leaf of wild *Ipomoea aquatic*, *Cleome rutidosperma* aerial parts, stem and leaf of cultivated *Ipomoea aquatic*, *Centella asiatica* leaf and *Mimosa pudica* L. aerial parts revealed no significant effect at 0.125 mg/mL but all extracts were proved to inhibit tau aggregation at 12.5 mg/mL, ranging between 65 % and 98 %. The IC_{50} values of *Psidium guajava* leaves, *Artocarpus altilis* leaves and *Nelumbo nucifera* leaves were determined to be 0.39, 1.05 and 1.24 mg/mL, respectively (Table 2).

Table 2. IC_{50} values of 9 methanol extracts and methylene blue against tau aggregation

Entry	Extract	Parts used	IC_{50} [mg/mL] against tau
1	*Psidium guajava*	Leaf	0.39
2	*Artocarpus altilis*	Leaf	1.05
3	*Nelumbo nucifera*	Leaf	1.24
4	*Nelumbo nucifera*	Seed pod	2.06
5	Wild *Ipomoea aquatic*	Stem and leaf	2.45
6	*Cleome rutidosperma*	Aerial parts	3.90
7	*Mimosa pudica* L.	Aerial parts	4.32
8	Cultivated *Ipomoea aquatic*	Stem and leaf	4.79
9	*Centella asiatica*	Stem and leaf	6.24
10	Blue methylene		1.35 (μM)

The Guava leaf extract was further fractionated by column chromatography and tested for tau aggregation inhibitory activity. The data in Table 3 showed that the ethyl acetate fraction yielded highest inhibition rate against tau aggregation with 48.49 %, followed by fractions 3, 2 and 4 with the inhibitory ranging of 33.70–39.65 %. The hexane fraction showed the lowest effect on tau aggregation inhibitor, at only 4.29 %. From these results, it is suggested that the major components with tau aggregation activities have high polarity.

Table 3. Inhibitory rate of 5 fractions extracted from Guava leaf (*Psidium guajava* L.)

Fraction	Hexane (%)	Ethyl acetate (%)	Inhibitory rate (%)
1	100	0	4.29±1.36
2	90	10	37.34±2.78
3	70	30	39.65±2.45
4	50	50	33.70±3.38
5	0	100	48.49±0.79
	DMSO		0

CONCLUSION

According to our results, it is possible to assume that the methanolic extracts from leaves of *Psidium guajava*, *Nelumbo nucifera* and *Artocarpus altilis* may contain components that may offer great potentials for the treatment of tauopathy. However, there are needed further research studies to examine their tau protein anti-aggregation properties both *in vitro* and *in vivo*. Besides, these plants can be examined in order to isolate and identify the active ingredients, and this may serve as a foundation to find safer and more effective agent for therapeutic use.

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Khảo sát khả năng kháng kết tập protein tau ở bệnh Alzheimer của cao chiết methanol thảo dược thu hái ở Đồng bằng sông Cửu Long, Việt Nam

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TÓM TẮT

Protein tau ở tế bào thần kinh của người có vai trò cố định và ổn định cấu trúc của vi ống ở sợi trục, giúp đảm bảo sự dẫn truyền thần kinh giữa những neuron kề nhau được diễn ra xuyên suốt. Ở não của bệnh nhân Alzheimer, protein tau bị phosphoryl hóa quá mức và tách khỏi vi ống. Do đó, cấu trúc ở sợi trục của tế bào thần kinh bị phá vỡ là nguyên nhân làm sự dẫn truyền trên tế bào thần kinh bị gián đoạn. Ngoài ra, những protein tau ở dạng tự do sẽ kết tập với nhau tạo thành đám rối tơ ở tế bào thần kinh (neurofibrillary tangle). Những đám rối tơ này đồng thời gây độc cho những tế bào thần kinh khác trong não bộ. Cho đến nay thì y học vẫn chưa tìm ra được loại thuốc chữa trị hiệu quả căn bệnh Alzheimer và nhóm bệnh mất trí (dementia) ở người cao tuổi. Ở Việt Nam, người dân vẫn tin dùng một số loại thực vật để chữa trị những rối loạn của hệ thần kinh và tăng cường trí nhớ. Vì vậy, mục đích của nghiên cứu này là khảo sát khả năng kháng kết tập protein tau của lá ổi (Psidium guajava L.), lá sen (Nelumbo nucifera), rau muống đồng (Ipomoea aquatic), thân và lá màng màng tím (Cleome rutidosperma), lá sa kê (Artocarpus altilis), rau muống trồng (Ipomoea aquatic), rau má (Centella asiatica), thân và lá mắc cở (Mimosa pudica L.), gương Sen (Nelumbo nucifera). Chín loại thảo dược thuộc 7 loài thực vật được chiết xuất bằng dung môi methanol theo phương pháp ngâm dầm. Khả năng kháng kết tập protein tau của các cao chiết methanol được xác định bằng phương pháp đo mật độ huỳnh quang của phản ứng kết tập protein tau với phẩm nhuộm Thioflavin T. Kết quả cho thấy 9 mẫu cao chiết methanol đều có khả năng kháng đông protein tau ở cả 4 nồng độ 12,5 mg/mL; 1,25 mg/mL; 0,25 mg/mL và 0,125 mg/mL. Ở nồng độ 12,5 mg/mL, hiệu quả kháng kết tập của chín mẫu cao chiết dao động từ 65 % đến 98 %. Hiệu quả

kháng kết tập của cao chiết lá ổi (Psidium guajava L.), lá sen (Nelumbo nucifera) và lá sa kê (Artocarpus altilis) là cao nhất với IC_{50} lần lượt là 0,39 mg/mL, 1,24 mg/mL và 1,05 mg/mL so với IC_{50} của đối chứng dương là 1.35 µM (blue methylene). Cao chiết lá ổi (Psidium guajava L.) được tách thành 5 phân đoạn bằng sắc ký cột silica gel (70-230 mesh). Tỉ lệ kháng kết tập protein tau của 5 phân đoạn dao động từ 33.7 % đến 48.49 %, ngoại trừ phân đoạn với 100% η-hexan.

***Từ khóa**: Artocarpus altilis, Bệnh Alzheimer, hiệu quả kháng kết tập protein tau, Nelumbo nucifera, Psidium guajava L, Thioflavin T*

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