RESEARCH ARTICLE

Neuroprotective Effect of *Durio zibithinus* against Beta Amyloid

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Abstract

In searching for a promising candidate for treatment of Alzheimer's disease (AD), the effects of ethanol extract (CE) and defatted ethanol extract (dCE) of *Durio zibithinus* cultivar "Mon Thong" on pathological cascade of AD were investigated by *in vitro* and cell culture models. The results exhibited that both of CE and dCE extracts could inhibit acetylcholinesterase function in bioautography assay. For thioflavin T assay which studies an effect on beta-amyloid (Aβ) aggregation indicated that both CE and dCE at the concentration of 10 mg/mL were able to inhibit Aβ aggregation with inhibitory percentage values of 44.96±3.50 and 36.91±5.50, respectively. From the neuroprotection study in cell culture revealed that both CE and dCE could reduce human neuroblastoma cell (SH-SY5Y) death induced by Aβ. Moreover, the result from Western blotting analysis indicated that CE inhibited Aβ-induced cell death via DR5 inhibition, resulting in inhibiting of cleave-caspase 8, cleave-caspase 3 activation and changing in the phosphorylation level of Akt (protein kinase B). The overall results indicated that the *Durio zibithinus* extract possesses multimode of action involved with AD pathology cascade including anti-Aβ aggregation, acetylcholinesterase inhibition, and neuroprotection against β-amyloid. Thus, the *Durio zibithinus* cultivar "Mon Thong" might be a potential candidate for further developing as a functional food or a drug for Alzheimer's disease.

Keywords: *Durio zibithinus*, Alzheimer, beta amyloid, acetylcholinesterase, neuroprotection

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ฤทธิ์ปกป้องการทำลายเซลล์ประสาทจากการเหนี่ยวนำด้วยเบต้าอะไมลอยด์ของสารสกัดจากทุเรียน

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บทคัดย่อ

การวิจัยครั้งนี้เป็นการศึกษาถึงผลของสารสกัดจากทุเรียนพันธุ์หมอนทอง 2 กลุ่ม ได้แก่ สารสกัดเอทานอลจากผลทุเรียน (CE) และสารสกัดเอทานอลจากผลทุเรียนที่กำจัดไขมันออก (dCE) ต่อฤทธิ์ทางชีวภาพที่เกี่ยวข้องกับพยาธิสภาพของโรคอัลไซเมอร์ โดยทำการศึกษาทั้งในหลอดทดลองและในเซลล์เพาะเลี้ยง ผลการศึกษาพบว่าสารสกัดทุเรียนทั้ง 2 กลุ่ม ได้แก่ สารสกัดเอทานอลจากผลทุเรียน และสารสกัดเอทานอลจากผลทุเรียนที่กำจัดไขมันออกมีฤทธิ์ดีในการยับยั้งการทำงานของเอนไซม์อะซีตีลโคลีนเอสเทอเรส รวมทั้งมีฤทธิ์ดีในการต้านการเกาะกลุ่มของโปรตีนเบต้าอะไมลอยด์เมื่อทดสอบโดยวิธี Thioflavin T assay โดยสารสกัดที่ความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตร สามารถยับยั้งการเกาะกลุ่มกันของเบต้าอะไมลอยด์ได้ร้อยละ 44.96±3.50 และ 36.91±5.50 ตามลำดับ สำหรับการศึกษาฤทธิ์ป้องกันการทำลายเซลล์ประสาทในเซลล์เพาะเลี้ยง พบว่าสารสกัดเอทานอลจากผลทุเรียนและสารสกัดเอทานอลจากผลทุเรียนที่กำจัดไขมันออก มีฤทธิ์ป้องกันการทำลายเซลล์ประสาทจากการถูกเหนี่ยวนำด้วยเบต้าอะไมลอยด์ และผลการศึกษาจาก Western blotting assay ชี้ให้เห็นว่าสารสกัดเอทานอลจากผลทุเรียน มีฤทธิ์ป้องกันการตายของเซลล์ประสาทที่ถูกเหนี่ยวนำด้วยเบต้าอะไมลอยด์โดยผ่านการยับยั้ง DR5 ส่งผลต่อการป้องกันการตัดของ caspase-3 และ caspase-8 นอกจากนี้ยังมีผลป้องกันการเติมฟอสเฟตให้กับ Akt (protein kinase B) ผลจากการศึกษาในครั้งนี้แสดงให้เห็นว่าสารสกัดจากทุเรียนพันธุ์หมอนทองมีฤทธิ์ที่เกี่ยวข้องกับพยาธิสภาพของโรคอัลไซเมอร์ ได้แก่ ฤทธิ์ยับยั้งการทำงานของเอนไซม์อะซีตีลโคลีนเอสเทอเรส ฤทธิ์ต้านการเกาะกลุ่มกันของเบต้าอะไมลอยด์ และการป้องกันการทำลายเซลล์ประสาทจากเบต้าอะไมลอยด์ ดังนั้น ทุเรียนหมอนทองน่าจะมีศักยภาพในการพัฒนาเป็นผลิตภัณฑ์เสริมอาหารหรือเป็นยาเพื่อรักษาโรคอัลไซเมอร์

คำสำคัญ: ทุเรียนพันธุ์หมอนทอง, โรคอัลไซเมอร์, เบต้าอะไมลอยด์, อะซีตีลโคลีนเอสเทอเรส, การป้องกันการทำลายเซลล์ประสาท

Introduction

Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder characterized by loss of memory, progressive deficits in cognitive functions, and severe behavioral abnormalities. Currently, AD is increasing in elder people over 65 years and affects over 35 million people worldwide. In line with an increase in average life expectancy, the number of affected persons is expected to triple by 2050 if the lack of an efficient treatment persists.[1] Thus, AD is considered one of essentially troublesome diseases for Public Health System. The etiology of AD is unclear, but it has been found to be related with the decrease of acetylcholine (ACh) neurotransmitter, the accumulation of beta amyloid (Aβ) and neurofibrillary triangle and the occurrence of oxidative stress.[2-5] Based on the cholinergic hypothesis of AD, the current strategy of AD intervention is mainly to ameliorate the cognitive symptoms related to ACh depletion, thereby enhancing the central cholinergic neurotransmission by reversing acetylcholinesterase (AChE) inhibition. This hypothesis is based on several findings that cholinergic neurodegeneration can be a major pathologic feature in the brains of patients with AD, and experimental studies suggest ACh plays a vital role in learning and memory.[6]

Aβ aggregation is thought to be responsible for initiating the pathogenic cascade that results in neuronal loss and dementia.[7] Aβ aggregation has been shown to have crucial neurotoxic effects, which significantly supports the amyloid hypothesis.[8,9] Hence, the inhibition of Aβ aggregation and toxicity may carry therapeutic value by hindering the pathogenesis of AD.[10]

A previous study reports that cholinergic degeneration can accelerate Aβ plaque burden *in vivo*.[11] Additionally, it has been reported that AChE inhibitors can inhibit AChE-induced Aβ polymerization.[12] One noncholinergic role of AChE in the pathogenesis of AD is that AChE may play a substantial part in the development of the senile plaques by accelerating Aβ polymerization, which will induce greater neurotoxicity, depending on the amount of AChE bound to the complexes.[13] Dual inhibitors of both Aβ aggregation and AChE activity are considered to be potential therapeutic approaches that can slow or mitigate the progression of AD.[14,15]

Durian (*Durio zibithinus*) is one of the most important seasonal fruits in Thailand and commonly known as the "King of the Fruits". It belongs to the family *Bombacaceae* and the genus Durio. It has been reported that the durian possesses several biological activities including, antidiabetic[16], antihyperlipidemia[17] and antiproliferation.[18] However, the effect of durian fruit on neurodegenerative diseases especially AD have not been reported. The phytochemical investigations revealed that the durian contains a number of bioactive compounds including polyphenols, flavonoids, and anthocyanin. The polyphenols and flavonoids are known to possess several biological activities related AD such as antioxidant, anti-Aβ aggregation, anti-acetylcholinesterase, and neuroprotection. Moreover, from animal and clinical investigation, the data implicate a protective effect of polyphenols and flavonoids from plants against neurodegenerative disease.[19-22] Based on its chemical constituents' activities, the durian might provide a useful therapeutic choice in either the prevention or the treatment of AD. Therefore, the aim of our study was to evaluate the effects of the durian cultivar "Mon Thong" extract on the biological activities related AD pathological cascade, namely AChE activity and Aβ aggregation. In addition, the

neuroprotective effects against Aβ toxicity of the extracts were also investigated in a cell culture model.

Materials and Methods

All chemical used were of molecular biology and analytical grade. Analytical grade reagents were purchased from Sigma-Aldrich (SM Chemical supplies Co., Ltd, Thailand), Merck (Merck, Thailand) and Fluka (SM Chemical supplies Co., Ltd, Thailand) and were used as supplied.

Preparation of sample

The durian (*Durio zibithinus*) cultivar "Mon Thong" was collected from Rayong province, Thailand. Dried and finely powdered fruit pulp of Mon Thong (500 g) was extracted with 2 L of ethanol at room temperature for 7 day. The extract was evaporated by a rotary evaporator yielding 49.70 g of ethanol crude extract (CE). For preparing the defatted ethanol crude extract (dCE), dried and finely powdered fruit pulp of Mon Thong (500 g) was extracted with 2 L of hexane and then extracted with 2 L of ethanol. The extract was evaporated by a rotary evaporator yielding 12.04 g of dCE. Both CE and dCE were kept at -20°C until analysis.

AChE bioautography assay[23]

The basic principle of this method is that the enzyme converts α-naphthyl acetate (substrate) into α-naphthol. α-Naphthol reacts with fast blue B salt (chromogenicagent) to make a purple colored background on the TLC plate. Enzyme inhibitors block the formation of α-naphthol and hence no purple coloration is produced.

The crude extracts of *Durio zibithinus*, 10 μL (0.010 g of dried extract dissolved in 1 mL methanol) were applied to the plate and migrated by proper solvent. After drying, the TLC plates was sprayed with AChE enzyme stock solution.[19] The plate was then incubated at 37°C for 20 min and then sprayed with freshly prepared indicator solution 1-naphthyl acetate and Fast Blue B salt to give a purple color plate after a few minute. A white spot indicates inhibition of AChE by the sample. It is well known that tacrine, an acetylcholinesterase inhibitor used for Alzheimer's disease treatment, possesses anti-amnesic effects by inhibiting AChE in the brain. Therefore, tacrine is used as a positive control in this study.

Aβ aggregation inhibition by thioflavin T assay[24]

Thioflavin-T (ThT) fluorescence assay was used to monitor the aggregation state of $A\beta_{1-42}$. The assay was performed as described by Levine with minor modification. Briefly, twenty-five μM of $A\beta_{1-42}$ in 50 mM phosphate buffer, pH 7.4, was incubated at 37°C with various concentrations of the extract for 48 h. After incubation, the samples were mixed with 50 μM glycine/NaOH buffer (pH 9.2) containing 5 μM ThT. Fluorescence intensities were measured at an excitation wavelength of 446 nm and an emission wavelength of 490 nm. The fluorescence intensities were recorded, and the percentage of inhibition on aggregation was calculated by using the following equation: (1 - IFi/IFc) * 100% in which IFi and IFc were the fluorescence intensities obtained for absorbance in the presence and absence of the test compound, respectively, after subtracting the background fluorescence of

5 µM ThT in the blank buffers. Curcumin, a natural compound possessing strong antioxidant, Aβ aggregation inhibition, and acetylcholinesterase inhibition activities was used as a reference standard.

Effect on Aβ-induced cell damage in neuroblastoma cells[25]

Lyophilized $A\beta_{1-42}$ was reconstituted in PBS at a concentration of 250 µM and keep at -20°C until use. Aliquots were diluted with a culture medium to achieve a final concentration of 25 µM and then incubated at 37°C for 72 h to form aggregated amyloid.

Neuroblastoma cells (SH-SY5Y) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 containing 50 IU/mL penicillin, 50 g/mL streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. Cell cultures were maintained at 37°C in an atmosphere of 95% humidified air and 5% CO_2. For assays, SH-SY5Y cells were sub-cultured into a 96 well plate for 48 h. Then, the cells were incubated with various concentrations of the extract for 24 h and after that incubated with aggregated $A\beta_{1-42}$ (25 µM) for 24 h. Cell viability was determined by staining the cells with water-soluble tetrazolium salt (WST8). The absorption was measured by a well plate reader at 450 nm.

Western blotting analysis[26]

SH-SY5Y cells were cultured in 6-well plates, after 48 h incubation the cells treated with various concentrations of CE for 30 min. After removing the unabsorbed CE, the cells were treated with $A\beta_{1-42}$ (25 µM) for 4 h. The treated cells were washed with PBS and then lysed in ice cold lysis buffer for 30 min. The lysate was centrifuged at 12000 rpm for 15 min at 4°C and the supernatant was collected. The total protein concentration was determined by using the bicinchoninic acid (BCA) assay. Proteins were separated using SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membranes. The membrane was treated with Block Ace (Bio-Rad Laboratories, Hercules, CA, USA) and probed with primary antibodies including DR5, caspase-3, caspase-8 and Akt. The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-goat and visualized with the enhanced chemi-luminescence system (Amersham Biosciences, Waltham, MA, USA).

Statistical analyses

The results are expressed as mean±SD (n = 4-6). Statistical significance was determined by one way analysis of variance (ANOVA). For all statistical analysis, significance levels were set at p value < 0.05.

Results

AChE bioautography assay

The durian extracts showed AChE inhibitory activity on the TLC plates by autography assay (Figure 1). In order to establish detection limits of the new bioautographic assay, CE and dCE were applied at various amounts onto the TLC plate, and the lowest amount with the observable light yellow spot was determined. The amount of CE and dCE inhibiting the enzyme was as low as 100 µg, while the positive control, tacrine, revealed with inhibitory concentration value of 1 ng/mL.

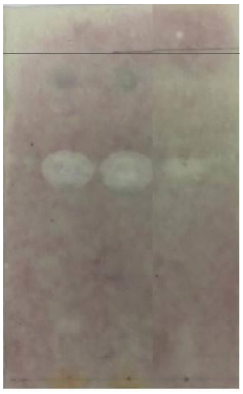

Figure 1. TLC qualitative acetylcholinesterase inhibition assay.
PC: positive control, tacrine (1 ng); CE: ethanol crude extract of durian (100 µg); dCE: defatted ethanol crude extract of durian (100 µg). TLC elution system: methanol:dichloromethane (1:9 v/v)**.**

Aβ aggregation inhibition by thioflavin T assay

In the present study, we examined the effects of CE and dCE on the inhibition of Aβ aggregation using the ThT fluorescence assay (Figure 2). The CE was found to inhibit the aggregation of $A\beta_{1-42}$ with %Inhibition of 27.99±9.4 and 44.96±3.5 at the concentration of 1 and 10 mg/mL, respectively. The dCE were found to inhibit the aggregation of $A\beta_{1-42}$ with %Inhibition of 16.87±6.6 and 36.91±8.5 at the concentration of 1 and 10 mg/mL, respectively.

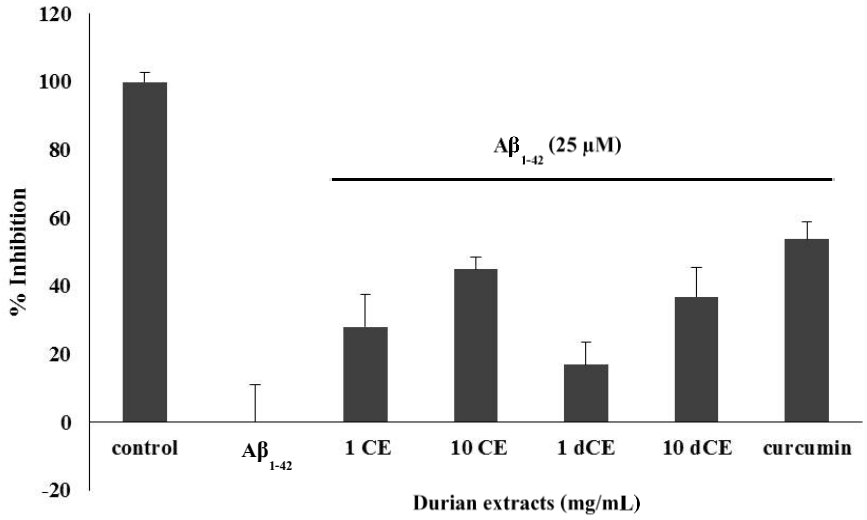

Figure 2. Effect of the ethanol extract (CE) and defatted ethanol extract (dCE) of durian (1 and 10 mg/mL) on the inhibition of $A\beta_{1-42}$ aggregation. Data are means±SD (n=4).

Effect on Aβ-induced cell damage in neuroblastoma cells

The neuroprotective effects of the durian extracts were determined against $A\beta_{1-42}$ peptide induced toxicity in SH-SY5Y neuroblastoma cells. Both of the CE and dCE showed a similar neuroprotective effect against $A\beta_{1-42}$ peptide induced neurotoxicity (Figure 3). Treatment of SH-SY5Y cells with the CE at concentrations of 1-100 µg/mL significantly reduced the cell viability loss evoked by $A\beta_{1-42}$ peptide. For the dCE at concentrations of 10-100 µg/mL significantly reduced the cell viability loss evoked by $A\beta_{1-42}$ peptide. The result obtained from anti-aggregation assay indicated that our extract could inhibit Aβ aggregation. Thus, the protective action against the $A\beta_{1-42}$ peptide of the CE and dCE might be enhanced by the anti-Aβ aggregation action.

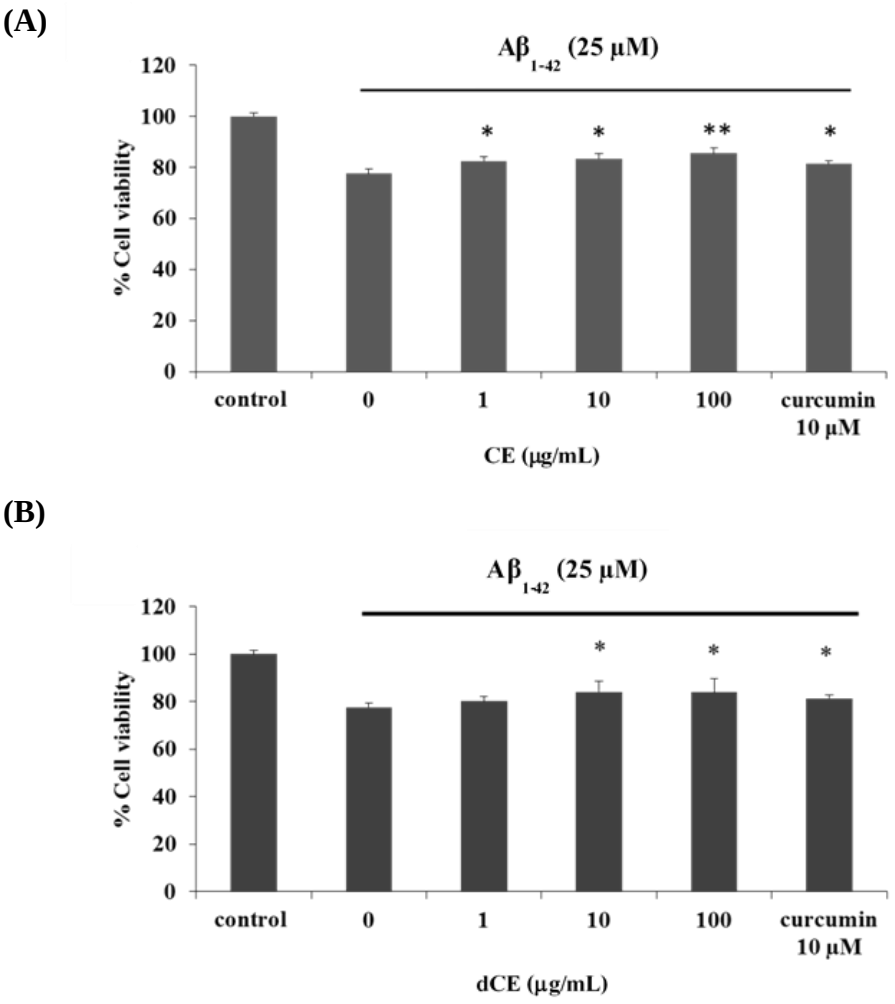

Figure 3. Effect of the durian extracts on Aβ-induced cell damage in SH-SY5Y cells: (A) ethanol extract (CE); (B) defatted ethanol extract (dCE). Data are means±SD (n=3) and *p<0.05, **p < 0.01 compared to the H_2O_2-treated group.

Durian ethanol extract inhibited Aβ-induced neuron cell death by suppressing DR5 expression

Since DR5 has been characterized in human brain derived from patients with AD, making DR5 receptor is a possible candidate as a target for Alzheimer's treatment. The aim of our study was to investigate the effect of durian ethanol extract (CE) on DR5-mediated apoptosis signaling pathway. The result was shown in Figure 4. Comparing with control, Aβ induced DR5 level correlated with increasing of Akt phosphorylation and activation of caspase-8 and caspase-3. CE at the concentration of 10 and 100 µg/mL and positive control (curcumin 10 µM) strongly inhibited Aβ-induced DR5 expression, phosphorylation of Akt and activation of caspase-8 and caspase-3, indicating that CE protected Aβ induced DR5-mediated apoptosis signaling pathway.

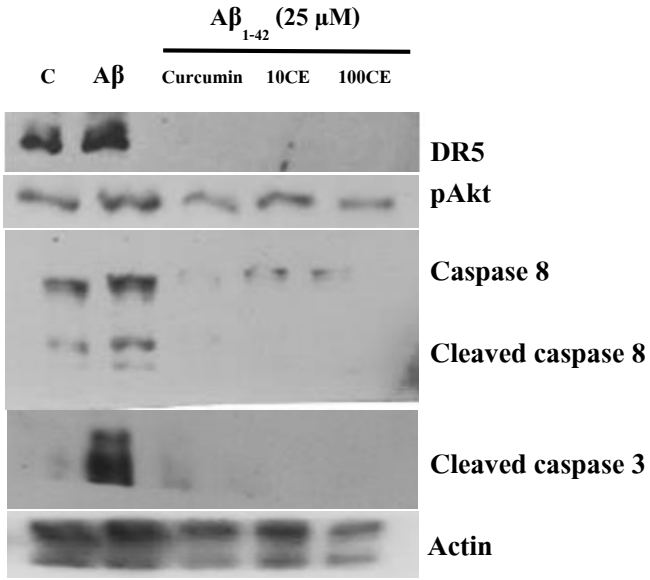

Figure 4. Effect of the ethanol extract of durian on DR5-mediated cell death signaling pathway induced by Aβ. The SH-SY5Y cells were treated with CE (10 and 100 µg/mL) for 30 min, then treated with Aβ. The proteins were separated and detected by Western blot analysis.

Discussion

Due to the multi-pathogenesis of AD, the agents acting at multiple sites of pathologic cascade seem to be a potential drug for AD treatment. The durian, a rich source of biological and chemical diversity, may be a potential multi-target drug for treatment of AD. There are a variety of durian cultivars, including Mon Thong, Chani, Kan Yao, Pung Manee and Kradum. Among them, Mon Thong had the highest phenolic content.[27] The polyphenolic compounds from plants are known to possess several bioactivities including antioxidant, antithrombotic, anti-Aβ aggregation,

antiacetylcholinesterase, anticancer, and antidiabetic activities.[19-22] Therefore, the durian cultivar "Mon Thong" was chosen to evaluate its neuroprotection for AD. Due to the high content of lipid which may be harmful to health, the durian extract that removed lipid was also prepared for activities evaluation. Therefore, the purpose of the present study was to investigate the effect of two durian extracts including the ethanol extract (CE) and defatted ethanol extract (dCE) on the biological activities related AD pathological cascade, namely AChE activity and Aβ aggregation. In addition, the neuroprotective effect against Aβ toxicity of the extracts was also determined in a cell culture model.

AD has been found to be associated with a cholinergic deficit in the post-mortem brain characterized by a significant decrease in acetylcholine amount. The main role of AChE is to rapidly hydrolyze acetylcholine at the cholinergic synapses, ending the transmission of nerve impulses.[28] Therefore, inhibition of AChE function appears to be a useful therapeutic path to reduce, at least temporarily, the cognitive deficit in AD. In this experiment, the effect of CE and dCE on AChE function was evaluated by the TLC qualitative AChE inhibition assay (bioautographic assay). It is well known that the anti-amnesic effects of tacrine are due to AChE inhibition in brain. Tacrine was used as a positive control. The result exhibited that both durian extracts, CE and dCE, at the amount of 100 µg showed an ability to inhibit acetyl-cholinesterase function on the TLC plates.

Another key hallmark of AD pathogenesis is the formation of toxic Aβ plaques in the brain of AD patients. Therefore, preventing or reducing the aggregation of Aβ has been the primary goal of a number of therapeutic strategies under development or in clinical trials. $A\beta_{1-42}$ is a major component of amyloid plaques in AD brain. Several studies have indicated that $A\beta_{1-42}$ is an important factor in the etiology of AD.[29,30] In addition, the generation of $A\beta_{1-42}$ was found to increase in familial AD patient.[31-33] *In vitro* and transgenic mice studies have shown that $A\beta_{1-42}$ could aggregate to be amyloid plaque faster than $A\beta_{1-40}$. We therefore employed $A\beta_{1-42}$ to examine the Aβ aggregation and the Aβ-induced cytotoxicity in SH-SY5Y neuronal cells throughout our study. In the present study, we examined the effects of durian extracts on the inhibition of Aβ aggregation using the ThT fluorescence assay. The results showed that CE could inhibit the aggregation of $A\beta_{1-42}$ more potent than dCE. However, dCE still had the activity to inhibit Aβ aggregation. Therefore, dCE might be useful for the AD patient with high cholesterol by not upgrade the amount of cholesterol in patients.

Aβ has also been reported to be toxic to neurons through various mechanisms including ROS generation, mitochondrial dysfunction and apoptosis.[34,35] The present study has investigated the cytotoxic effect of $A\beta_{1-42}$ on SH-SY5Y cells using WST-8 assay. Our results demonstrated that $A\beta_{1-42}$ could induce neuronal cell death. These results were consistent with previous studies, indicating that $A\beta_{1-42}$ at µM concentration levels caused neuronal death in culture cells.[36]

From animal and clinical investigation, the data implicate a protective effect of polyphenols and flavonoids from plants against neurodegenerative disease.[37-39] Therefore, the pulp of durian that contains abundant flavonoids and polyphenols should be the good candidate for further study to explore the molecular mechanisms of neuroprotective effect. In previous study, durian pulp extracts has been found to have active compounds including campherol, quercetin campherol, quercetin, apigenin,

caffeic acid and β-carotein which showed antioxidant, AChE inhibitory and neuro-degeneration protective effects.[40] In addition, the present study also showed that the extracts of durian pulp possess Aβ aggregation inhibitory action. It was therefore postulated that CE and dCE could prevent the neurotoxicity induced by Aβ. Our data reported herein confirm the protective effect of CE and dCE against Aβ-induced neurotoxicity. These data implicate that the protective effect of CE and dCE against $A\beta_{1-42}$-induced neurotoxicity might be, at least in part, due to its antioxidant and anti-Aβ aggregation activities.

The marker of apoptosis is caspase activation which resulting in neuronal cell death. The caspases enzyme including caspase-8 and caspase-3 are activated by various stimuli such as chemical agents, oxidative stress and cytokines. The pathways of apoptosis can be divided into two pathways including mitochondrial pathway and death receptors-mediated pathway. In AD brain, levels of caspase-3 and caspase-3-degraded product were increased.[41] Also, there was an increase in caspase-mediated cleavage product of APP colocalized with Aβ in senile plaques that might ultimately induce neuronal death in AD brain.[42] Several studies have reported that the activated caspase-3 was involved mainly in Aβ-induced apoptotic events.[43,44] The present study showed the increase of caspase-3 activity in SH-SY5Y cells treated with $A\beta_{1-42}$, indicating that cell death mechanism was through apoptosis. Moreover, preincubation with CE successfully prevented caspase-3 activation. These data were in accordance with the findings on protective effect of CE on cell viability. In death receptor pathway, after the death ligand such as fas, trail and chemical agents bind to death receptor, the death signal will activate caspase-8 and caspase-3 which induced apoptosis of cells. The serine–threonine kinase Akt, also called protein kinase B, has been known as a survival kinase. However, the previous study showed that strong Akt activation increases oxidative stress and induces cell death by reactive oxygen species (ROS). The death receptor 5 (DR5) has been reported to play an important role in Aβ-induced neuronal cell death. The increasing of expression of DR5 receptor after Aβ treatment correlated with AKT phosphorylation, caspase-8 and caspase-3 activation consequences to neuronal cell death. The Aβ induced DR-5 expression directly or induced through ROS generation. This study demonstrated that the durian ethanol extract suppression Aβ-induced neuron cell death through DR-5-mediated AKT, caspase-8 and caspase-3 signaling pathway.

Conclusion

The results of the present study indicate that the durian extracts possess multimode of action involved with AD pathology cascade including anti-aggregation of Aβ, AChE inhibition and neuroprotection against Aβ toxicity. Thus, the *Durio zibithinus* cultivar "Mon Thong" might be a potential candidate for further developing as a functional food or a drug for Alzheimer's disease.

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