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Anti-cholinesterase inhibitory activities of different varieties of chili peppers extracts

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Abstract

Alzheimer's disease (AD) is one of the leading neurological disorders that degrade learning, memory and cognitive functions of a nervous system, eventually leaving a person with an inability to perform any function on his/her own. One of several AD causes involves loss of presynaptic markers of a cholinergic system due to cholinesterase enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which degrade neurotransmitters, acetylcholine (ACh). Currently, the investigation in natural products that can act as functional foods is of an interesting matter to promote health benefits. Chili pepper is previously reported to contain various bioactive compounds that can promote several health benefits. Nevertheless, limited information regarding the control of AD through inhibition of AChE and BChE are available. Therefore, the aim of this experiment was to investigate the cholinesterase inhibitory activities of Thai local chili peppers including Yellow pepper, Bird pepper, Green pepper, Cayenne Pepper, Jinda-green pepper, Jinda-red pepper, Young pepper, Chili Spur pepper and Sweet pepper using a colorimetric high throughput screening methodology. As results, all chili peppers (in exception of Yellow pepper) exhibited AChE inhibitory activities under the range of 5-26% inhibition with Young pepper extract exhibiting the highest inhibition. Interestingly, all chili peppers (in exception of Young pepper) exhibited the BChE inhibitory activities under the range of 2-24% inhibition with Jinda-green pepper extract exhibiting the highest inhibition. Both AChE and BChE inhibitory activities were in dose-dependent manners. These results possibly that the bioactive compounds such as capsaicin, myricetin, quercetin and luteolin in chili pepper might function as anti-cholinesterase agents, since these compounds have been previously reported to be capable of effectively inhibiting cholinesterase enzymes in vitro. The information received from this study would support further investigation on potential natural bioactive compounds from chili pepper with anti-AD property through inhibition of cholinesterase enzymes.

Introduction

Alzheimer's disease (AD), the most common type of dementia, is an age-related neurodegenerative disorder. The causes of AD could possibly come from brain cells damaging/injury and complicate with neuron interaction, which induce brain cells to be inoperable, dysfunctional and eventually death. In the early stage, AD patients are incapable of controlling their performances and might be partially unconscious during daily activity. In the long term, they may lose cognitive abilities necessary for maintaining an independent living style.

As of present, there is no certain pathway for treatment of AD. However, inhibition of cholinesterase has so far provided the most promising results in terms of AD treatment (Luke and Wildman, 2006; Al Othman *et al*., 2011). There are currently two cholinesterase enzymes in focus; acetylcholinesterase (AChE), an enzyme that degenerates neurotransmitter acetylcholine (ACh) as its specific substrate (Vengaiah *et al*., 2007), and butyrylcholinesterase (BChE), an enzyme that degenerates neurotransmitters; butyrylcholine (BCh) and ACh (Park *et al*., 2012). Degeneration of the neurotransmitters results in decline of these neurons. It has been found that the cholinergic markers, the enzymes responsible for ACh synthesis and degradation, were significantly impaired in AD patients in comparison to elderly control subjects (Sun *et al.*, 2007). As well, inhibition of AChE resulted in the increase of non-plaque forming amyloids in the cortex of rat brain (Deepa *et al*., 2007).

The β–amyloid plaque formation along with plaque deposit is one of the main hypotheses or histological associated AD. The β-amyloid plaques

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are aggregated protein fragments, which are degraded from amyloid precursor protein (APP) in brain cell membrane. Hydrolysis of APP by β- and γ-secretases can lead to development of amyloid plaques (amyloidogenic pathway). These β-amyloid plaques or senial plaques can disturb brain cell communication and stimulate innate immune response, leading to cell inflammatory, cell abnormality, and eventually cell death (Checler, 1995).

Many medical treatments for AD have been investigated, in which 6 major groups of anti-AD drugs, including AChE inhibitors (AChE-I), N-methyl-D-aspartate (NMDA), receptor antagonists, monoamine oxidase (MAO) inhibitors, antioxidants, metal chelators and anti-inflammatory drugs, have been developed. Originally, anti-AD drug was discovered as AChE inhibitors, which cause an increase in acetylcholine levels, leading to reduced AD symptoms such as memory loss, abnormality of thinking and language. These drugs have been approved by Food and Drug Administration (FDA), including donepezil, galantamine, rivastigmine and tacrine. However, these drugs have side effects such as usually diarrhea, tiredness, dizziness, confusion, headache, vomiting, nausea, fatigue, insomnia, heart attack and stroke (Kannappan *et al.*, 2011). Thus, prevention and treatment of AD from natural products such as fruits and vegetables that can be consumed daily are of interest due to no/less side effect or toxicity to be concerned.

Chili pepper, a significant ingredient in many traditional cuisines in various countries, has been widely investigated regarding their biological properties toward health benefits (Pakaski *et al.*, 2009). Chili pepper is an important source of bioactive compounds with antioxidant activities such as vitamin C, vitamin E, carotenoids, phenolic compounds and alkaloids (Kulisic-Bilusic *et al.*, 2008; Stefano, 2013). Interestingly, some of these antioxidants exhibit biological functions against AChE and BChE (multi-functional compounds), suggesting the relationship among these biological functions. Even through chili pepper has been reported as a rich source of antioxidants, its function against AD is limited. Therefore, the aim of this research was to investigate the biochemical properties of customly consumed chili peppers against some key enzymes (beta-site APP-cleaving enzyme 1 (BACE-1) and cholinesterases) that control AD. This research will be useful for promoting chili pepper consumption for health benefits and developing functional food, nutraceutical or dietary supplement with biological property against AD.

Table 1. The general information of chili peppers used in this study

Species	Common name	Fruit color	Maturity
Capsicum annuum	Green pepper	Green	Mature
	Young pepper	Green	Pre-mature
	Chili Spur pepper	Red	Mature
	Sweet pepper	Red	Mature
Capsicum frutescens	Jinda-green pepper	Green	Mature
	Yellow pepper	Yellow	Mature
	Bird chili	Orange	Mature
	Cayenne Pepper	Red	Mature
	Jinda-red pepper	Red	Mature

Materials and Methods

Plant material and extraction

Chili peppers (Yellow pepper, Bird pepper, Green pepper, Cayenne Pepper, Jinda-green pepper, Jinda-red pepper, Young pepper, Chili Spur pepper and Sweet pepper) (Table 1) were purchased during June, 2014 from local market in Nakhon Pathom province, Thailand. The samples were clean with deionized water and cut into small piece (approx. 0.5 x 0.5 cm). The samples were then freeze-dried (Heto Power Dry PL9000, Thermo Fisher Scientific, Waltham, MA, USA) before being ground into a fine powder by a cyclotec sample mill (series 1903 with 200–240V and 50/60 Hz; FOSS, Höganäs, Sweden). The moisture content after freeze drying was determined using Association of Official Analytical Chemists (AOAC) method 930.15 (AOAC, 2005). All samples were kept in vacuum bags and stored at –20°C.

Dry chili powder (0.2 g dry weight) was extracted with 70% (v/v) aqueous ethanol (8 mL). The mixture was vortexed for 5 min and then sonicated in a water bath sonicator (model B1510, 40 KHz; Bransonic Bransonic® Ultrasonic, Danbury, CT, USA) for 10 min before shaking in a water bath shaker (Memmert GmbH, Wisconsin, USA) at 60ºC for 1 hour. The mixture was then centrifuged at 1190 x g for 10 min, and the supernatant was collected for further analysis.

Cholinesterase inhibitory activity

The enzymatic assay for cholinesterase activity was performed utilizing a well established protocol (Jung *et al.*, 2009) with some changes as follows. The inhibitory enzymatic assay consisted of cholinesterase (5–20 ng Electrophorus electricus AChE (1,000 units/mg) or 10–50 ng equine serum BChE (≥10 units/mg protein)), thiocholine (0.08 mM acetylthiocholine (ATCh) or 0.1 mM butyrylthiocholine (BTCh)), 5,5'–dithiobis(2–nitro benzoic acid) (DTNB, 0.8

mM) and chili extracts (5 mg/mL) in a 96–well plate. All chemicals were received from Sigma–Aldrich (St. Louis, MO, USA). Enzyme inhibitory activity was measured at a wavelength of 412 nm using a 96-well microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) with a Gen5 data analysis software. The initial rate was fitted by the Michaelis–Menten equation with least squares fit parameter using a GraphPad Prism software version 5.00 (GraphPad Software, Inc., La Jolla, CA, USA). The enzymatic inhibitory activity was calculated as % inhibition using the following equation;

$$\% \text{ inhibition} = 100 \times (1 - ((B-b)/(A-a))),$$

where *A* was an initial velocity of the control reaction (without plant extract) with the enzyme, *a* was an initial velocity of the control reaction without enzyme, *B* was an initial velocity of the enzyme reaction with chili extract and *b* was an initial velocity of the reaction with chilli extract but without enzyme. Eserine (Sigma–Aldrich, St. Louis, MO), a reversible anti–cholinesterase drug, was used as a standard inhibitor for both AChE and BChE assays.

Beta-secretase inhibitory activity

Beta-secretase (BACE1) inhibitory activity was analyzed using β-secretase (BACE1) FRET (fluorescence resonance energy transfer) Assay Kit (Sigma–Aldrich, St. Louis, MO, USA). The assay consisted of BACE1 enzyme (0.006 U/μL), BACE1 substrate (Rh–EVNLDAEFK–Quencher in 50 mM ammonium bicarbonate), fluorescent assay buffer (50 nM sodium acetate) and chili extract (5 mg/mL) in the 96–well plate. The reactionwas mixed and incubated at 37°C for 2 hours. The reaction was monitored at an excitation wavelength of 545 nm and an emission wavelength of 585 nm using the 96-well microplate reader. The inhibitory activity will be reported as percentage of inhibition as above.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of triplicate assays. One way analysis of variance (ANOVA) and Duncan test were performed to determine the significant differences between values with $p<0.05$. All statistical analyses were performed using IBM SPSS Statistics version 19.0 (IBM Corp, Armonk, NY).

Results and Discussion

Scientific researches proposed four main hypotheses of AD development, including a loss of presynaptic markers of a cholinergic system, accumulation of β-amyloid plaque in the brain, neurofibrillary tangles or abnormal tau protein and oxidative stress induction (Jung *et al*., 2009). Most researches have been focused on cholinergic hypothesis and β-amyloid formation, which are two important platforms for development of anti-AD drugs. Nevertheless, the adverse effects of these synthesized drugs were recently reported, and green medicines have been in the center of current interest.

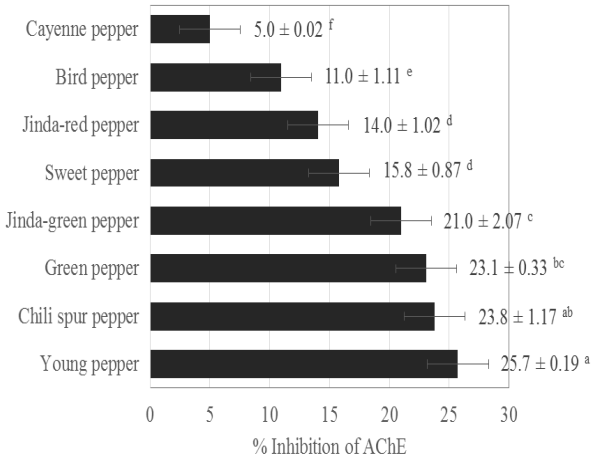

Figure 1. The AChE inhibitory activity from various types of chili peppers (5 mg/mL) including Young pepper, Chili spur pepper, Green pepper, Jinda-green pepper, Sweet pepper, Jinda-red pepper, Bird pepper and Cayenne pepper. The different letters showed statistically significant difference at p value < 0.05using one-way ANOVA and Duncan's Multiple Range Test

Chili with different degree of spicy favor is an important ingredient that is customly consumed in various quantities in many dishes around the world. Being involved in many traditional cuisines, it is of interest to investigate the effect of different types of chili regarding anti-AD properties via inhibition of some key enzymes that control the disease. Chili, a plant in the *Capsicum* genus and Solanaceae family, can be divided into five domesticated species, including *C. annuum, C. frutescens, C. chinense, C. baccatum* and *C. pubescensa.* In Thailand, only *C. annuum* (Green pepper, Young pepper, Chili Spur pepper and Sweet pepper) and *C. fretescens* (Yellow pepper, Bird pepper, Jinda-red pepper, Jinda-green pepper and Cayenne Pepper) are agriculturally and commercially available. Thus, variation between these species that affects anti-AD properties is of interest for health maintaining purpose.

As a result, all chili peppers, in exceptionof Yellow pepper, exhibited AChE inhibitory activities under the range of 5-26% inhibition with Young pepper extract exhibiting the highest AChE inhibitory activity (Figure 1). Young pepper *(C. annuum)* that

was used in this experiment is a hot pepper in its young-green (premature) stage. It was previously found that hot pepper fruits in *C. annuum* L. var. *acuminatum* species contain different bioactive compounds, depending on ripening stage (Conforti *et al*., 2007; Menichini *et al.*, 2009). The first stage of maturation (small green premature stage) exhibited the highest antioxidant activity (IC_{50} of 129 µg/mL) as being detected by DPPH (1,1–diphenyl–2–picrylhydrazyl)–radical scavenging assay and total phenolic contents (76 mg/g) as being detected by Folin–Ciocalteu method (Conforti *et al*., 2007). Besides, the investigation on the effect of ripening stage of methanolic extract of *C. annuum* L. var. *acuminatum* regarding its AChE inhibitory activity was suggested that the half maximal inhibitory concentration (IC_{50}) of the premature green pepper extract exhibited the highest AChE inhibitory activity (IC_{50} of 84.30 µg/mL) (Loizzo *et al*., 2008). This inhibitory activity was decreased during ripening stage of mature green and red peppers (IC_{50} of 96.69 and 130.03 µg/mL, respectively). However, when comparing to the IC_{50} of physostigmine (0.07 µg/mL), the commercial anti-cholinesterase drug, it was suggested that the chili peppers under this investigated extraction condition might be insufficient for treatment of AD. Nevertheless, when comparing to other fruits and vegetables such as ginkgo (Mitra *et al*., 2013), pomegranate (Choi *et al*., 2011), mulberry (Shih *et al.*, 2010), lemon juice (Girones-Vilaplana *et al.*, 2012), black chokeberry (Girones-Vilaplana *et al*., 2012), turmeric (Kannappan *et al*., 2011), garlic (Kannappan *et al*., 2011), black pepper (Kannappan *et al*., 2011), ginger (Kannappan *et al*., 2011), and cinnamon (Kannappan *et al*., 2011), these chili peppers might be the potential food source for prevention of AD.

The chemical composition analysis of *C. annuum* L. var. *acuminatum* suggested that some polyphenolic compounds with antioxidant properties such as luteolin, myricetin and sterol might be responsible for high antioxidant and anti-AChE activities as being observed in the experiments (Helmja *et al*., 2007). It was found that premature green pepper exhibited the highest quantity of luteolin (76.0 mg/g), followed by mature green pepper (73.8 mg/g) and matures red pepper (43.2 mg/g), respectively (Conforti *et al*., 2007). Luteolin possessed the DPPH radical-scavenging activity (IC_{50}) of 2.051 µg/mL (Conforti *et al*., 2007) and AChE inhibitory activity with reversible inhibition constant (K_i) of 65.8 µM (Katalinic *et al*., 2010). Likewise, it was found that green pepper exhibited the highest quantity of myricetin (658.2 µg/g), comparing to other chili

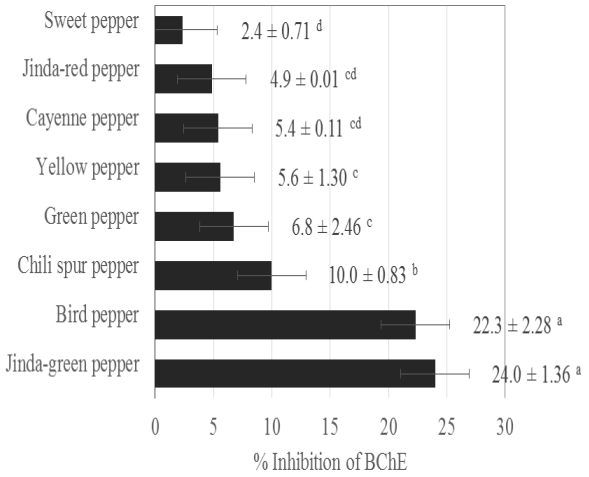

Figure 2. The BChE inhibitory activity from various types of chili peppers (5 mg/mL) including Jinda-green pepper, Bird pepper, Chili spur pepper, Green pepper, Yellow pepper, Cayenne pepper, Jinda-red pepper and Sweet pepper.The different letters showed statistically significant difference at p value < 0.05using one-way ANOVA and Duncan's Multiple Range Test

pepper fruits in other ripening stages (Conforti *et al.*, 2007). Myricetin is a strong antioxidant (DPPH value of 16.2 µM) (Khanduja and Bhardwaj, 2003) with anti-AChE activity (K_i of 37.8 µM) (Katalinic *et al*., 2010). Similarly, sterol content was found to be decreased during ripening stage of *C. annuum* L. var. acuminatum (Conforti *et al.*, 2007). Some sterols were proven to be AChE inhibitors (Hopia and Heinonen, 1999). However, when comparing to eserine (K_i of 0.144 µM as being measured in our laboratory), a reversible anti–AChE drug, it was found that these compounds might not be the effective anti-AChE agents for AD treatment but might be useful for chemoprevention of AD.

On the other hand, all chili peppers, in exception of Young pepper, exhibited the BChE inhibitory activities under the range of 2-24% inhibition with Jinda-green pepper and Bird pepper extracts exhibiting the highest BChE inhibitory activities (Figure 2). Jinda-green pepper and Bird red-pepper *(C. frutescens)* that were used in this experiment are hot peppers in their mature stage. This species *(C. frutescens)* is commonly used to make flavoring in food due to their hot spicy taste (hotter than chili peppers in *C. annuum* species). Among all five domesticated species, *C. frutescens* chili peppers possess the highest quantity of capsaicinoid (1560 mg/100 g), a phenolic compound that causes chili to have spicy and pungent favors (Orhana *et al*., 2007). Capsaicin (8-methyl-N-vanillyl-6-nonenamide), a main capsaicinoid in chili peppers, under the concentration of 1 mg/mL was significantly inhibited BChE reaction with 75.3% inhibition (Orhana *et al*.,

2007). Comparing to galantamine, an anti-AD drug for mild to moderate AD patients, under the same concentration with 80.3% BChE inhibition, capsaicin could be a future potential anti-AD agent (Katalinic *et al*., 2010). Other bioactive compounds, including myricetin, quercetin and luteolin could also reversibly inhibit human plasma BChE with K_i of 71.0, 68.0 and 166.1 μM, respectively (Orhana *et al*., 2007; Katalinic *et al*., 2010). However, when comparing to eserine (K_i of 0.305 μM as being measured in our laboratory), it was found that these compounds might not be the effective anti-BChE agents for AD patients but might be useful for AD prevention.

Interestingly, it was previously reported that capsaicin could promote amyloidogenic route of brain amyloid precursor protein processing (Pakaski *et al*., 2009). However, no inhibitory activity in β-secretase assay was observed in all chili peppers extracted under investigated conditions (final concentration of extracts in the assay was 5 mg/mL). It was possible that low concentration of the extract could not inactivate β-secretase. The experiment was then repeated using Sweet pepper with higher concentration (30 mg/mL) as a study case and was found that the extract could inhibit β-secretase with 31% inhibition. Thus, it could be concluded that chili peppers at the concentration of 5 mg/mL could effectively inactivate cholinesterase reactions but not β-secretase. Since effective inhibitors are normally designed to mimic natural substrate of individual enzyme, these results might be correlated to the particular characteristics of the substrates (such as structure, size, and interaction(s) between enzyme and substrate) for each enzyme. Cholinesterase hydrolyzes small organic compounds, ACh and BCh, while β-secretase hydrolyzes APP, the integral membrane protein. The inhibitors for β-secretase can be divided into two main groups, pseudopeptide β-secretase inhibitors and non-peptidomimetic β-secretase inhibitors (Ghosh *et al*., 2012; Ghosh and Osswald, 2014). The former are generally substrate analogues, while the later are organic compounds i.e., macrocyclic inhibitors, hydroxyethylamine-based inhibitors and carbinamine-based inhibitors (Ghosh *et al*., 2012; Ghosh and Osswald, 2014). These non-peptidomimetic β-secretase inhibitors possess large molecular size with unique functional groups in their structures, which can form specific interactions with β-secretase. Thus, smaller anti-cholinesterase agents found in chili peppers might not be able to form proper interaction(s) or fit properly in the catalytic pocket of β-secretase, leading to no inhibitory activity that was observed at low concentration of chili extracts.

From these experimental results, it could be suggested that (1) consumption of chili peppers might possibly prevent AD occurrence through inhibition of some key enzymes that control the disease, (2) some antioxidants could exhibit multi-functional properties, (3) AD hypotheses could be related to each other, and (4) different types of chili peppers could influent different degree of anti-AD properties. The first suggestion was also depended on bioavailability and absorption of bioactive compounds in chili peppers. These bioactive compounds were also found to possess multi-biological functions i.e. being antioxidants and anti-cholinesterase agents, thus leading to correlation between AD hypotheses (i.e. cholinergic hypothesis and oxidative stress induction).

Interestingly, different types of chili peppers could interact with AChE and BChE with different degrees of inhibition. It has been previously found that BChE has less substrate specificity than AChE and has a wider range of substrates acting on both ACh and BCh substrates (Orhan *et al*., 2007). The acyl (functional moiety on the substrate) binding region of AChE and BChE are different in terms of catalytic residues. AChE consists of larger Phe residues in the active site, leading preferable small substrates such as ACh. This smaller substrate would fit better in the catalytic pocket than the larger molecule. On the other hand, the active site of BChE consists of Leu and Val residues, which are smaller amino acids, allowing it to accept larger substrates such as BCh (Orhan *et al.*, 2007). Hence, AChE is able to hydrolyze ACh efficiently but has low ability in cleaving esters with bulkier acyl moieties such as BCh. BChE, on the other hand, is an esterase with less substrate specificity due to its structure that allows cleavage of various esters including ones with bulkier acyl moieties (Katalinic *et al*., 2010). Not only that these two enzymes are also found to work differently, but also may contribute to the reason as to why BChE inhibitory activity was higher in comparison to AChE. BChE acts even when the substrate is in excess, but under the same condition, feed-back inhibition is observed for AChE (Tougu, 2001). In this experiment, all chili peppers, in exception of Jinda-green pepper, Bird pepper and Yellow pepper, exhibited higher AChE inhibitory activities than BChE inhibitions, which possibly suggested that anti-cholinesterase agents in these peppers might be more specific towards AChE active site than that of BChE. On the other hand, anti-cholinesterase agents in Jinda-green pepper, Bird pepper and Yellow pepper might be less specific to cholinesterases. For example, these peppers might contain various types of large molecules that could fit into the active site of BChE but could not enter

the catalytic pocket of AChE, thus resulting in higher BChE inhibition than AChE inhibition.

Conclusion

The cholinesterase inhibitory activities from different varieties of chili peppers showed that chili peppers extracts could effectively inactivate the key enzymes that control AD occurrence. The results from these experiments could possibly be used for explaining (1) fundamental knowledge of functional food for health benefit regarding AD prevention from chili peppers, (2) potential development of food supplement and nutraceutical, and (3) future drug design for AD treatment based on enzyme-drug interactions.

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