Bioactive Compounds, Antioxidant Activity and Inhibition of Key Enzymes Relevant to Alzheimer’s Disease from Sweet Pepper (Capsicum annuum) Extracts

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ABSTRACT: Sweet pepper is a non-pungent chili of the Capsicum annuum species and is an important ingredient in daily diets due to its characteristics such as pungency, aromas, and flavors. Sweet pepper is a rich source of bioactive compounds such as phenols, carotenoids, and flavonoids, which can promote potential health benefits against various non-communicable diseases. However, research focused on anti-Alzheimer’s disease (AD) properties of sweet peppers is limited. Thus, this study aimed to investigate bioactive compounds (flavonoids, phenolic acids, and carotenoids), antioxidant activity and anti-AD properties of four colored sweet peppers (green, red, orange, and yellow) via their abilities to inhibit key enzymes relevant to AD [acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and β-secretase (BACE1)]. Extraction solvents [hexane, ethyl acetate, and 70% (v/v) aqueous ethanol] were also investigated. Results suggested that yellow sweet pepper has the highest content of flavonoids, while green sweet pepper has the highest contents of phenolic acids and red sweet peppers have the highest content of carotenoids. In terms of anti-AD properties, green sweet peppers exhibited the highest antioxidant, anti-BChE, and anti-BACE1 activities; however, yellow sweet pepper extract exhibited the highest amounts of AChE inhibition. Bioactive compounds in sweet red peppers may therefore have anti-AD properties, and may be useful for AD prevention.

Keywords: anti-β-secretase activity, anti-cholinesterase activity, antioxidant activity, bioactive compounds, sweet peppers

INTRODUCTION

Sweet peppers or bell peppers (Capsicum annuum) belong to the Solanaceae Juss family. Sweet peppers are plump and bell-shaped vegetables, consisting of either three or four fruit lobes. The fruit has thick walls with several forms, sizes, and colors (Nazzaro et al., 2009). Most unripe sweet pepper fruits are green; however, color change to red when the peppers ripen. Other colors of unusual sweet pepper cultivars are yellow, orange, purple, brown, or black, depending on the genotype or seasonal period of breeding (Nazzaro et al., 2009). Unlike other peppers, sweet peppers do not have pungent flavors, due to the non-pungent pericarp; however, their placenta tissues are still pungent (Nazzaro et al., 2009). The primary substance that controls heat in peppers is capsaicin, which is found in trace amounts in sweet peppers (Al Othman et al., 2011). Interestingly, different fruit colors can influence taste and flavor of each particular pepper. For examples, red, yellow, and orange colored peppers are sweeter than green peppers, due to development of higher glucose contents during their ripening stage (Howard and Wildman, 2006).

Sweet peppers had been reported to contain many significant bioactive compounds, such as phenolic compounds, carotenoids, ascorbic acid, tocopherols, and capsaicin (Sun et al., 2007; Hallmann and Rembiałkowska, 2012; Marin et al., 2008; Matsufuji et al., 2007; Marin et al., 2004; Materska, 2014; Loizzo et al., 2013; Deepa et al., 2007). Types and quantities of bioactive compounds differ among different colored sweet peppers. For example, chlorophyll is higher in unripe green peppers than in ripe orange, yellow, and red peppers (Blanco-Ríos et al., 2013). It is possible that this chlorophyll may be structurally modified into new pigments, such as red or yellow carotenoids (xanthophyll- and anthocyanin-related pigments) (Mateos et al., 2013). These phytochemicals provide many potential health benefits, especially phenolics (phenolic acids and flavonoids).

The phytochemicals in sweet peppers mainly act as an-
tioxidants, which can prevent the occurrence of some oxid-ative stress related diseases, such as cancer, cardiovas-cular disease, and neurodegenerative diseases (Serrano et al., 2010). Alzheimer’s disease (AD) is a major neuro-degenerative disorder, mostly found in the elderly. The causes of AD are not well understood; however, three recent pathways including induction of oxidative stress, termination of cholinergic synapses, and β-amyloid for-mation are hypothesized to induce AD. Free radicals and reactive oxygen species (ROS) are generated during normal physiological and biochemical processes in organ-isms. The imbalance of radical overproduction can lead to biomolecule damage (such as lipids, proteins, and DNA) via oxidative stress, and may be the cause of many oxidative stress related diseases, including AD (Choi et al., 2014; Min and Min, 2014). In addition, the hypoth-esis that loss of cholinergic synapses induces AD is em-phasized by decreased production of acetylcholine (ACh), a neurotransmitter in the brain responsible for memory, thinking, and decision making (Rao et al., 2007). ACh activity can be terminated by cholinesterases [acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] the enzymes that hydrolyze ACh into the inactive metab-olites choline and acetyl-CoA (Rao et al., 2007). Finally, beta-secretase (BACE1) is the main protease that pro-duces β-amyloid peptides, and is a characterized marker of AD (Anand and Singh, 2013). Thus, antioxidant, anti-AChE, anti-BChE, and anti-BACE1 agents are important bioactive compounds to inhibit AD progression.

Current anti-AD drugs possess several severe side ef-fects that affect each patient on an individual basis. Nat-ural products that can be consumed daily, such as sweet peppers, may therefore be a suitable choice for AD pre-vention. Sweet peppers have been reported to improve learning and memory impairments in senescence-accel-erated prone mouse (Suganuma et al., 1999); however, the responsible pathway is unknown. The aim of this study was to investigate bioactive compounds (flavo-noids, phenolic acids, and carotenoids) and biochemical properties related to anti-AD properties (antioxidant, anti-AChE, anti-BChE, and anti-BACE1 activities) of four colored sweet pepper extracts extracted with solvents of different polarities [hexane, ethyl acetate, and 70% (v/v) aqueous ethanol].

MATERIALS AND METHODS

Sample preparation and extraction
Sweet peppers (Capsicum annuum) including unripe (green) sweet peppers and ripe (red, orange, and yellow) sweet peppers were purchased from Baan Rai Paovaris, Nakhon Ratnasima, Thailand (harvesting in October and No-vember, 2013). The peppers were cultivated in a green-house under controlled water and fertilizer treatments.

Sweet peppers were cleaned with deionized water, and all seeds were removed. The colors of sweet peppers were analyzed by a HunterLab ColorFlex EZ spectrophotom-eter (HunterLab, Reston, VA, USA) using International Commission on Illumination LAB (L*, a*, and b*) sys-tem, where L* describes darkness (−) to lightness (+), a* describes green (−) to red (+) colors, and b* describes indigo (−) to yellow (+) colors. Green sweet peppers exhibited color values (L*, a*, and b*) of 28.92, −8.56, and 16.22, respectively, while red sweet peppers exhib-it ed color values of 27.52, 34.09, and 19.45, respectively. Similarly, orange sweet peppers exhibited color values of 46.49, 31.41, and 50.19, respectively, and yellow sweet peppers exhibited color values of 52.19, 15.74, and 55.23, respectively. Sweet pepper pericarps were cut into small pieces (about 1 cm×1 cm). Samples were then freeze-dried using a freeze dryer (Heto PowerDry PL9000, Heto Lab Equipment, Allerød, Denmark) and homogenized using a blender (Phillips 600W blender, Philips Electronics Co., Ltd., Jakarta, Indonesia). The sweet pepper powder was then stored in aluminum foil bags at −20°C for fur-ther analysis.

The moisture content of fresh and freeze-dried samples was determined using an infrared moisture balance (FD-720, Kett Electric Laboratory, Tokyo, Japan). Freeze-dried sweet peppers (11 g dry weight) was extracted using sol-vents (400 mL) of different polarities, including non-polar hexane, semi-polar ethyl acetate, and polar 70% (v/v) aqueous ethanol using a Soxhlet extractor. Extraction was continued until all colors were removed from the sweet peppers (approximately 6.5 h). The extract solvent was removed by a rotary vacuum evaporator at 35~40°C (N-1200B series rotary evaporator with OSB-2100 bath, EYELA, Tokyo, Japan). The remaining residues were re-dissolved in a minimum volume of dimethyl sulfoxide, and stored at −40°C for further analysis.

Determination of flavonoids and phenolic acids
Determination of flavonoids and phenolic acids was per-formed according to Merken and Beecher (2000), with modifications as follows. Freeze-dried samples (0.5 g) were extracted with 62.5% (v/v) methanol (40 mL) con-taining 0.5 g/L tert-butylhydroquinone before adding 6 N HCl (10 mL). The extracts were then shaken at 90°C for 2 h. After cooling the extract in an ice bath for 5 min, 1% (v/v) ascorbic acid (100 μL) was added and mixed thoroughly. The extract was made up to a total volume of 50 mL, sonicated in an ultrasonic bath for 5 min, and collected by filtering through 0.2 μm polytetrafluoroeth-ylene (PTFE) syringe filter. The high-performance liquid chromatography (HPLC) analysis (Agilent 1100 series HPLC with a photodiode array detector, Agilent Technol-ogies, Santa Clara, CA, USA) was then performed using
Determination of total phenolic contents (TPCs)

TPCs were determined using Folin-Ciocalteu reagents, following the method by Ainsworth and Gillespie (2007) and Sripum et al. (2017), with adaptations. Samples (25 μL) were mixed with 10% (v/v) Folin-Ciocalteu reagent (50 μL). After 5 min incubation, 7.5% (w/v) saturated NaHCO₃ (200 μL) was added. Mixtures were incubated at room temperature (25°C) in a dark room for 2 h. The TPC was measured at a wavelength of 765 nm using a microplate reader (Synergy™ HT 96-well UV-visible spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Gallic acid (10, 20, 40, 60, 80, 100, and 200 μg/mL) was used as a standard. The TPC was expressed in mg gallic acid equivalents (GAE)/g dry weight.

Determination of antioxidant activity

Antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing antioxidant power (FRAP), and oxygen radical antioxidant activity (ORAC) assays. All methods were performed with some modifications, as indicated in Sripum et al. (2017).

The DPPH assay was performed according to the method of Fukumoto and Mazza (2000) with some modifications as follows. Assays were performed in 96-well flat-bottom microplates. DPPH was used as an indicator of free radical scavenging activity. Samples (22 μL) were mixed with 150 μM DPPH in 95% (v/v) aqueous ethanol (200 μL) and incubated in the dark at room temperature (25°C) for 30 min. Absorbance was measured at 520 nm using a microplate reader. The radical scavenging activity was calculated as a percentage of DPPH discoloration using the equation:

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\text{% Radical scavenging activity} = 100 \times \left(1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right)
\]

where \(\text{Abs}_{\text{sample}}\) is the absorbance at 520 nm of the sample and DPPH reagent, while \(\text{Abs}_{\text{control}}\) is the absorbance at 520 nm of 95% (v/v) aqueous ethanol and DPPH reagent. Trolox solution (0.01, 0.02, 0.04, 0.08, 0.16, 0.32, and 0.64 mM) was used as a standard.

FRAP assays were carried out according to the method of Benzie and Strain (1996), with some modifications. FRAP reagent containing 300 mM acetate buffer pH 3.6, 10 mM 2,4,6-tri(2-pyridyl)-S-triazine solution in 40 mM HCl and 20 mM FeCl₂·6H₂O solution in a ratio of 10:1:1 was warmed at 37°C before use. The assay was performed in 96-well flat-bottom microplates. Samples (20 μL) were mixed with FRAP reagent (150 μL) and incubated at room temperature (25°C) for 8 min. The reaction was monitored using the plate reader at a wavelength of 595 nm. Trolox solution (7.8125, 15.625, 31.25, 62.5, 125, and 250 μM) was used as a standard.

ORAC assays were carried out according to the method of Ou et al. (2001), with some modifications. Assays were performed in 96-well black microplates for fluorescence measurements. Sodium fluorescein was used as a fluorescent probe. Loss of fluorescence was used as an indication of the extent of damage from the reaction with the peroxyl radical, as induced by 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH). Samples (25 μL) were...
mixed with 40 nM fluorescein solution (150 μL) and incubated for 30 min at 37°C. After incubation, 153 nM AAPH (25 μL), a peroxyl radical generator, was rapidly added to the reaction mixture to initiate the reaction. The fluorescence intensity was monitored for 90 min using microplate readers, with an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Trolox solution (3.125, 6.25, 12.5, 25, 50, and 100 μM) was used as a standard. Results were calculated based on the difference in areas under the sodium fluorescein decay curve (AUC) and expressed in μmol Trolox equivalents (TE)/100 g dry weight. The AUC was calculated as:

$$AUC = (0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + ... + f_i/f_0) \times CT$$

where $f_0$ is the initial fluorescence reading at 0 min, $f_i$ is the fluorescence reading at time $i$ min, and CT is cycle time in min.

Determination of cholinesterase inhibitory activities

Analysis of AChE and BChE activities were adapted from the method of Jung et al. (2009), with modifications indicated in Kukreja et al. (2018). The AChE assay mixture contained 20 ng AChE (100 μL), 0.8 mM acetylthiocholine (100 μL), 16 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (10 μL), and the extract (50 μL). Enzyme and substrate were prepared in assay buffer (50 mM KPB, pH 7.0) (50 μL), while DTNB was prepared in methanol. Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well microplate reader. Inhibitory activities of pepper extracts were calculated as percentage of inhibition using the equation:

$$\% \text{ inhibition} = 1 - \frac{B - b}{A - a} \times 100$$

where $A$ is an initial velocity of the reaction with enzyme, $a$ is an initial velocity of the reaction without enzyme, $B$ is an initial velocity of the enzyme reaction with extract, and $b$ is an initial velocity of the reaction with extract but without enzyme.

The BChE assay mixture contained 100 ng BChE in 50 mM KPB, pH 7.0; 1 mM MgCl$_2$ (100 μL); 1 mM butyrylthiocholine in 50 mM KPB, pH 7.0 (40 μL); 16 mM DTNB in 50 mM KPB, pH 7.0 (10 μL); and extract (50 μL). Enzyme inhibitory activity was spectrophotometrically measured at a wavelength of 412 nm using the 96-well microplate reader. Inhibitory activities of pepper extracts were calculated as percentage of inhibition as above.

Since inhibitory activities of AChE and BChE were determined utilizing enzyme kinetics, interferences from sample colors can be unconcerned. Inhibitory activities were determined using the rate of yellow color development during the enzyme assay (which becomes more yellow with time). Therefore, even though the samples had strong colors (yellow, green, red, and orange), the yellow color will only develop in the assay with enzyme. Thus, the yellow color measured in the assay only results from the reaction between the substrate and the enzyme. Erine, a reversible anti-cholinesterase drug, was used as a control inhibitor for both AChE and BChE assays.

Determination of anti-BACE1 activity

BACE1 inhibitory activity was measured using a BACE1 activity detection kit (fluorescent) (Sigma-Aldrich Co.). The assay kit consisted of 30 unit/μL BACE1 enzyme solution (2 μL), 50 mM BACE1 substrate solution (20 μL), fluorescent assay buffer (58 μL), and extract (20 μL). All reactions were performed using a 96-well microplate reader and monitored at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. The inhibitory activity was reported as percentage of inhibition, as above.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as mean±standard deviation (SD). All analyses for enzyme reactions were carried out using GraphPad Prism software version 5.1 (GraphPad Software Inc., La Jolla, CA, USA). To determine significant differences between values ($P<0.05$), one way analysis of variance (ANOVA) followed by Tukey’s b multiple comparison test was carried out using the SPSS 16.0 statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Flavonoids and phenolic acids

The four different colored sweet peppers contained different contents of flavonoids, including quercetin (71.71–102.33 μg/g dry weight) and luteolin (56.34–95.89 μg/g dry weight) (Table 1). Yellow sweet peppers contained significantly higher concentrations of quercetin (102.33 μg/g dry weight) than the other colored peppers. Similarly, yellow pepper contained higher contents of luteolin (95.89 μg/g dry weight), followed by red sweet peppers, green sweet peppers, and orange sweet peppers. Therefore, the highest total flavonoids contents were detected in yellow sweet peppers, followed by red sweet peppers, orange sweet peppers, and green sweet peppers. The quantity of the flavonoids reported in this study differed from that previously reported, in which red sweet peppers were shown to contain the highest quantity of flavonoids, followed by yellow sweet peppers (Sun et al., 2007). In this study, red sweet peppers were shown to contain 9-fold higher quercetin levels vs a previous study.
Flavonoids and phenolic acids Green sweet pepper Red sweet pepper Orange sweet pepper Yellow sweet pepper

| Flavonoids and phenolic acids | Green sweet pepper | Red sweet pepper | Orange sweet pepper | Yellow sweet pepper |
|------------------------------|-------------------|------------------|---------------------|--------------------|
| Flavonoids                   |                   |                  |                     |                    |
| Quercetin                    | 71.71±1.57<sup>c</sup> | 91.98±2.05<sup>b</sup> | 92.00±0.64<sup>b</sup> | 102.33±1.95<sup>a</sup> |
| Luteolin                     | 62.31±5.02<sup>a</sup> | 68.43±0.98<sup>b</sup> | 56.34±0.65<sup>d</sup> | 95.89±2.19<sup>a</sup> |
| Total flavonoids             | 137.02±6.59<sup>d</sup> | 160.41±3.03<sup>a</sup> | 148.33±1.29<sup>d</sup> | 198.22±4.14<sup>a</sup> |
| Phenolic acids               |                   |                  |                     |                    |
| p-Coumaric acid              | 19.62±0.68<sup>a</sup> | 9.96±0.08<sup>b</sup> | 9.53±0.25<sup>b</sup> | 10.67±0.20<sup>c</sup> |
| Ferulic acid                 | 23.59±0.01<sup>b</sup> | 27.67±0.13<sup>a</sup> | 13.45±0.05<sup>a</sup> | 24.75±0.15<sup>c</sup> |
| Total phenolic acids         | 43.21±0.69<sup>a</sup> | 37.63±0.21<sup>b</sup> | 22.98±0.30<sup>d</sup> | 35.42±0.35<sup>c</sup> |

All data were expressed as mean±standard deviation.
The different letters (a-d) within same type of flavonoids and phenolic acids in each colored sweet pepper are significantly different at P<0.05 using one-way ANOVA followed by Tukey’s post hoc test.

during with 70% (v/v) aqueous methanol was used as the extractant (Blanco-Ríos et al., 2013). In this previous study, but not the present study, myricetin was also detected (Blanco-Ríos et al., 2013). Differences in quantity may be due to the solvent extraction. For example, red sweet pepper extracted with 50% (v/v) aqueous methanol in this study exhibited 9-fold higher levels of quercetin than the previous study that used 70% (v/v) aqueous methanol. This may be because quercetin dissolves in solvents with higher polarity indexes. In this study, myricetin may have been dissolved in the solvent with a lower polarity index.

Sweet peppers also contained different contents of phenolic acids, p-coumaric acid (9.53~19.62 µg/g dry weight) and ferulic acid (13.45~27.67 µg/g dry weight), dependent on the colors of the sweet peppers (Table 1). Green sweet peppers were found to possess the highest content of p-coumaric acid (19.62 µg/g dry weight), followed by yellow sweet peppers, red sweet peppers, and orange sweet peppers. On the contrary, red sweet peppers was found to possess the highest quantities of ferulic acid (27.67 µg/g dry weight), followed by yellow sweet peppers, green sweet peppers, and orange sweet peppers. These results could partially lead to the different quantities of phenolic acids being detected in the different colored sweet peppers. These results suggested that green sweet peppers possess the highest amounts of total phenolic acids, followed by red sweet peppers, yellow sweet peppers, and orange sweet peppers. Our results were similar to those reported in Capsicum annuum cv. Padrón (Estrada et al., 2000). The content of p-coumaric acid has previously shown to be decreased in red peppers, whereas ferulic acid has been shown to only be found in red peppers (Estrada et al., 2000). In addition, caffeic acids (38~109 µg/g dry weight) and chlorogenic acids (117~290 µg/g dry weight) have been previously identified in four colored sweet peppers (Orion, Mazurca, Simpaty, and Taranto) from northwest Mexico (Blanco-Ríos et al., 2013); however, these compounds were not detected in our study. Differences in the type and level of flavonoids and phenolic acids in sweet peppers between studies may depend on both internal and environmental factors. Examples of internal factors include the cultivar and maturity stage of the sweet peppers. In addition, examples of environmental factors are growth and analytical conditions, such as extraction and HPLC conditions.

**Carotenoids**

The carotenoids, capsanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, trans-β-carotene, and cis-β-carotene, were recorded in four of the colored sweet peppers (Table 2). We showed that only capsanthin (16.13~178.20 µg/g dry weight), trans-β-carotene (8.32~41.72 µg/g dry weight), and cis-β-carotene (6.81~34.28 µg/g dry weight) were found in all the four sweet peppers. Lutein (45.16~115.16 µg/g dry weight), zeaxanthin (70.71~191.76 µg/g dry weight), β-cryptoxanthin (7.55~40.49 µg/g dry weight), and α-carotene (3.56~9.02 µg/g dry weight) were only detected in certain colored sweet peppers.

The contents of capsanthin, trans-β-carotene, and cis-β-carotene in different colored sweet peppers showed a similar trend, in which red sweet peppers contained the highest concentrations of these carotenoids (178.20 µg/g dry weight of capsanthin, 41.72 µg/g dry weight of trans-β-carotene, and 34.28 µg/g dry weight of cis-β-carotene). Significantly lower amounts of capsanthin were observed in orange sweet peppers, yellow sweet peppers, and red sweet peppers, respectively, and the lowest amounts of trans-β-carotene and cis-β-carotene were detected in yellow sweet peppers. Similarly, the highest quantities of β-cryptoxanthin (40.49 µg/g dry weight) were detected in red sweet peppers, followed by orange sweet peppers and yellow sweet peppers; however, β-cryptoxanthin was not detected in green sweet peppers. On the contrary, yellow sweet peppers contained the highest amounts of lutein (115.16 µg/g dry weight), followed by green sweet peppers and orange sweet peppers; lutein was not detected in red sweet peppers. Moreover, zeaxanthin was only detected in orange sweet pepper (191.76 µg/g dry weight) and red sweet pepper (70.71 µg/g dry weight), and only...
trace amounts of α-carotene (3.56–9.02 μg/g dry weight) were detected in orange, yellow, and green sweet peppers; α-carotene was not detected in red sweet peppers. These results suggest that red sweet peppers contain the highest contents of total carotenoids (365.40 μg/g dry weight), followed by those in orange sweet peppers, yellow sweet peppers, and green sweet peppers.

**TPC and antioxidant activity**

Extract TPCs were determined using the Folin-Ciocalteu method, and antioxidant activity were measured using DPPH radical scavenging, FRAP, and ORAC assays. TPCs and antioxidant activities of all four colored sweet peppers extracted with ethanol were approximately 2–8 and 30–80 folds higher than those extracted with ethyl acetate and hexane, respectively (Table 3). Thus, ethanol or polar solvent have appropriate polarity indexes for extraction of phenolics from sweet peppers. These results correspond with those from a previous report (Blanco-Ríos et al., 2013), in which higher antioxidant activities were found in the phenolic fractions [70% (v/v) aqueous methanol] than the oily fractions [chloroform : methanol (1:1 v/v)], as detected using 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid) and DPPH radical scavenging assays. In addition, the antioxidant activity of sweet pepper extracted with polar solvents (phosphate buffer, pH 7.8) was previously reported to be 10-fold higher than those in lipophilic fractions (ethyl acetate) (Serrano et al., 2010). The high antioxidant activity detected in polar fractions may be due to flavonoid and phenolic acid an-

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**Table 2. Carotenoid contents of four colored sweet peppers (unit: μg/g dry weight)**

| Carotenoids       | Green sweet pepper | Red sweet pepper | Orange sweet pepper | Yellow sweet pepper |
|-------------------|--------------------|------------------|---------------------|---------------------|
| Capsanthin        | 16.13±1.30         | 178.20±5.25      | 45.48±0.01          | 22.46±1.04          |
| Lutein            | 60.04±5.63         | ND               | 45.16±0.58          | 115.16±8.57         |
| Zeaxanthin        | ND                 | 70.71±0.94       | 191.76±1.24         | ND                  |
| β-Cryptoxanthin   | ND                 | 40.49±1.66       | 19.45±0.19          | ND                  |
| α-Carotene        | 3.56±0.23          | ND               | 9.02±0.07           | 4.22±0.23           |
| trans-β-Carotene  | 13.09±1.27         | 41.72±2.17       | 17.74±1.86          | 8.32±0.41           |
| cis-β-Carotene    | 9.64±0.73          | 34.28±1.77       | 8.32±0.03           | 6.81±0.28           |
| Total carotenoids | 102.48±9.17        | 365.40±11.78     | 336.95±0.98         | 164.53±10.83        |

All data were expressed by mean±standard deviation. The different letters (a-d) within same type of carotenoids in each colored sweet pepper are significantly different at P<0.05 using independent t-tests and one-way ANOVA followed by Tukey’s b post hoc test.

ND, not detected.

**Table 3. TPC and antioxidant activities of four colored sweet peppers extracted with hexane, ethyl acetate, and 70% (v/v) aqueous ethanol**

| Sweet pepper extracts | TPC (mg GAE/100 g dry weight) | Antioxidant activity (μmol TE/100 g dry weight) |
|-----------------------|--------------------------------|-----------------------------------------------|
|                       |                                | DPPH radical scavenging assay | FRAP assay | ORAC assay          |
| Hexane extracts       |                                |                                |            |                    |
| Green                 | 6.1±0.5b                       | 32.9±2.7c                      | 51.6±5.3b  | 131.5±10.1a         |
| Red                   | 7.3±0.5a                       | 69.5±6.1a                      | 69.8±6.1a  | 142.4±7.4a          |
| Orange                | 7.5±0.1a                       | 64.2±3.8b                      | 49.8±1.1b  | 140.2±6.1a          |
| Yellow                | 5.1±0.3c                       | 27.4±2.1d                      | 49.6±3.3c  | 124.7±7.5b          |
| Ethyl acetate extracts|                                |                                |            |                    |
| Green                 | 120.7±0.9a                     | 589.4±36.5a                    | 886.6±23.9a| 2,510.4±142.0a      |
| Red                   | 122.5±4.0a                     | 565.5±19.5a                    | 874.6±38.9a| 2,271.5±47.6b       |
| Orange                | 62.2±5.3a                      | 305.9±15.3a                    | 465.6±30.9a| 1,549.9±122.5a      |
| Yellow                | 54.5±5.3c                      | 232.9±12.8b                    | 410.3±36.3c| 1,310.9±60.7b       |
| 70% (v/v) aqueous ethanol extracts | |                                |            |                    |
| Green                 | 339.4±2.2a                     | 2,515.1±103.7a                 | 3,015.3±32.0a| 6,196.5±167.2a      |
| Red                   | 321.1±22.4a                    | 2,379.1±180.3a                 | 2,812.2±26.9a| 5,482.1±78.0b       |
| Orange                | 306.3±10.1b                    | 2,220.9±97.3b                  | 2,520.6±45.8b| 5,112.3±254.6b      |
| Yellow                | 273.5±6.3c                     | 1,863.2±48.2c                  | 1,987.3±44.6d| 5,165.1±154.1e      |

All data were expressed as mean±standard deviation. The different letters (a-d) for each solvent extraction condition are significantly different at P<0.05 using one-way ANOVA followed by Tukey’s b post hoc test.

TPC, total phenolic content; TE, Trolox equivalent; GAE, gallic acid equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical antioxidant activity.
ti-oxidative agents, which strongly dissolve in polar solvents. A previous study suggested that flavonoids and TPCs in peppers were identified in semi-polar and polar solvents, namely ethyl acetate, acetone, methanol, and 80% (v/v) aqueous methanol; however, these compounds were absent in hexane (lipophilic) extractions (Bae et al., 2012). The correlation (r) between DPPH radical scavenging results and TPCs, and between DPPH radical scavenging results and total flavonoids, yielded positive Pearson's correlation coefficients (r=0.66 and r=0.85, respectively) (De Martino et al., 2012). It is therefore highly possible that these phenolics act as antioxidants in peppers, a hypothesis that was confirmed by several previous studies (Sun et al., 2007; De Martino et al., 2012; and Perucka, 2005). Moreover, flavonoids mainly consist of hydroxyl groups, 2-3 double bonds, and 4-oxo functions, which can promote antioxidant activities (Blanco-Ríos et al., 2013). In addition, ascorbic acid has been reported in high amounts in Capsicum species, when compared to other fruits and vegetables (Wahyuni et al., 2013; Yahia et al., 2001).

The colors of sweet pepper also influenced TPCs and antioxidant activities (Table 3). Orange and red sweet peppers extracted with hexane exhibited the highest TPCs and antioxidant activities, followed by green and yellow sweet peppers. The high TPCs and antioxidant activities of orange and red sweet peppers may be influenced by carotenoid activities, since compounds were mainly extracted by non-polar solvents (Bae et al., 2012). In addition, a strong positive correlation was shown between lipophilic total antioxidant activities of sweet peppers and total carotenoids by linear regression (r²=0.81 ~0.97) (Serrano et al., 2010); carotenoids also exhibit antioxidant activity (Serrano et al., 2010; Molnár et al., 2005; Asnin and Park, 2015). This report was associated with our carotenoids analysis, in which orange and red sweet peppers were shown to contain the highest amounts of carotenoids, specifically capsanthin, zeaxanthin, β-cryptoxanthin, and trans- and cis-β-carotene. Therefore, high TPCs and antioxidant activities in red and orange sweet peppers may come from the presence of these compounds. In addition, C. annuum, such as sweet peppers, are good sources of vitamin E and provide high antioxidant activities compared to other fruits and vegetables (Wahyuni et al., 2013). α-Tocopherol may therefore be the compound yielding high reductions of free radicals in red sweet peppers extracted by hexane.

Following extraction with ethyl acetate and 70% (v/v) aqueous ethanol, TPCs and antioxidant activities were significantly higher in green and red sweet pepper, followed by orange and yellow sweet pepper extracts, respectively. Similar results were observed in sweet pepper extracted with 70% (v/v) aqueous methanol (Blanco-Ríos et al., 2013). High TPCs and antioxidant activities detected in solvents with high polarity indexes may be due to the presence of phenolics. This hypothesis was confirmed by higher contents of the phenolic acids p-coumaric and ferulic acid in green and red sweet peppers, respectively. In addition, it has been previously reported that green and red sweet peppers contain the highest ascorbic acid contents of the four colored sweet peppers (Blanco-Ríos et al., 2013).

Cholinesterase inhibitory activities
Our results suggest that the four colored sweet pepper extracted with all three solvents [hexane, ethyl acetate, and 70% (v/v) aqueous ethanol] at the final concentration of 30.56 g dry weight/L could inhibit AChE activity (range of inhibition: 12.21 ~91.69%) (Table 4). Ethyl acetate (semi-polar) extracts showed significantly higher amounts of AChE inhibitory activity (51.58 ~91.69% inhibition), followed by hexane (14.06 ~84.84% inhibition) and ethanolic extracts (12.21 ~57.52% inhibition).

These results were in agreement with previous results of Capsicum chinense Jacq. cv Habanero (Menichini et al., 2009), in which red peppers in lipophilic fractions (hexane) exhibited high AChE inhibitory activities [half maximal inhibitory concentration (IC₅₀) of 0.73 g/L]; however, only trace AChE inhibitory activities were in ethanolic extracts (IC₅₀>1.00 g/L). It is possible that anti-AChE agents were dissolved in solvents with low polarity indexes. The bioactive compounds of sweet peppers that likely dissolved in low polar solvents, such as carotenoids and α-tocopherol (vitamin E) (Blanco-Ríos et al., 2013), may influence anti-AD properties. It was previously shown that serum carotenoids are related to cognitive performance in healthy elderly people (Min and Min, 2014; Akbaraly et al., 2007), in which the participants those with lowest cognitive scores also had low levels of plasma carotenoids. Moreover, vitamin E and carotenoid deficiencies could lead to memory and learning impairments (Menichini et al., 2009). For ethyl acetate fractions, which contain the highest AChE inhibitory activities, it has been reported that most carotenoids are soluble in organic solvents such as chloroform acetone, alcohol, ethyl ether, and ethyl acetate, and that flavonoid aglycones are also soluble in organic solvents such as alcohol, acetone, ethyl acetate, and ethyl ether (Ferreira and Pinho, 2012). It is therefore possible that since most bioactive compounds (carotenoids and flavonoids) can be dissolved in ethyl acetate, this fraction exhibits highest AChE inhibition.

Each colored sweet pepper had different levels of AChE inhibitory activity. Yellow sweet peppers extracted with all three solvents were most effective in inhibiting AChE activity (57.52 ~91.69% inhibition with an IC₅₀ of 5.17 ~13.38 g dry weight/L). This result corresponded to the flavonoids content; yellow sweet peppers contained the
Table 4. AChE, BChE, and BACE1 inhibitory activities of four colored sweet peppers extracted using hexane, ethyl acetate, and 70% (v/v) aqueous ethanol

| Sweet pepper extracts | AChE % Inhibition | IC50 (g/L) | BChE % Inhibition | IC50 (g/L) | BACE1 % Inhibition |
|-----------------------|-------------------|-----------|-------------------|-----------|-------------------|
| Hexane extracts       |                   |           |                   |           |                   |
| Green                 | 43.55±3.62b       | 52.90±5.07a | 10.22±1.22a       | ND        | 88.30±1.82a       |
| Red                   | 25.38±0.15c       | 106.40±5.09b | 6.35±0.66b        | ND        | 72.37±4.15b       |
| Orange                | 14.06±1.04d       | 116.70±3.69a | 6.25±0.83d        | ND        | 56.33±4.42c       |
| Yellow                | 84.84±3.19a       | 5.17±0.26d | 6.79±0.78b        | ND        | 66.99±2.81b       |
| Ethyl acetate extracts|                   |           |                   |           |                   |
| Green                 | 51.58±2.22b       | 37.69±2.62b | 22.25±1.54a       | 85.34±1.17 | 74.83±4.08a       |
| Red                   | 24.75±1.04c       | 77.63±0.99c | 10.91±0.80b       | ND        | 70.88±4.68a       |
| Orange                | 22.25±1.64d       | 117.95±0.64d | 7.45±0.63c        | ND        | 60.18±6.61b       |
| Yellow                | 91.69±4.71a       | 5.86±0.27a | 10.69±0.78b       | ND        | 69.58±3.84a       |
| 70% (v/v) aqueous ethanol extracts | | | | | |
| Green                 | 39.00±3.75b       | 59.35±2.62b | 32.25±2.55a       | 62.64±2.13c | 58.73±3.72a       |
| Red                   | 17.21±1.29c       | ND        | 29.79±2.53ab      | 69.95±3.90bc | 41.52±4.48b       |
| Orange                | 12.21±0.83d       | ND        | 24.50±1.99b       | 81.53±3.20c | 39.18±0.79b       |
| Yellow                | 57.52±3.02d       | 13.38±0.42a | 28.63±2.31b       | 79.48±2.03a | 43.13±2.77b       |

All data are expressed as mean±standard deviation. The different letters (a-d) for each sweet pepper extracted using the same solvent and the same enzyme assay are significantly different at P<0.05 using one-way ANOVA followed by Tukey’s b post hoc test. Final concentrations of all sweet pepper extracts (30.56 g dry weight/L) were used to determined IC50 values. AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BACE1, β-secretase; IC50, the half maximal inhibitory concentration. ND, not detected.

highest contents of quercetin and luteolin and the highest content of total flavonoids. Previous research has suggested that flavonoids may inhibit AChE reactions (Xie et al., 2014; Katalinić et al., 2010; Szwajgier, 2014). Quercetin and luteolin strongly inhibit AChE comparing to other flavonoids (IC50 values of 0.0047 and 0.0050 g/L in quercetin and luteolin, respectively) (Xie et al., 2014). Interestingly, potent anti-AChE agents (luteolin, quercetin, and myricetin) contain hydroxyl groups on position 3’ of their B rings, whereas other flavonoids, such as apigenin and kaempferol, do not contain any (Xie et al., 2014; Katalinić et al., 2010). We may therefore hypothesize that the AChE inhibitory activity of flavonoids may depend on their structures. From molecular docking studies, flavonoid hydroxyl moieties have been shown to form hydrogen bonds with active site residues of AChE (Xie et al., 2014). In addition, these flavonoids may occupy AChE active sites, blocking ACh substrates from entering and inhibiting AChE catalytic activity (Xie et al., 2014). Furthermore, high concentration of ascorbic acid in sweet peppers may also inhibit AChE activity in the brains of mice (Dhingra et al., 2006).

In the cholinergic hypothesis, cholinesterase enzymes are predicted to inhibit AChE and BChE. We showed that sweet peppers extracted with all three solvents (concentration of 30.56 g dry weight/L) could inhibit BChE activity (Table 4). Sweet peppers extracted with 70% (v/v) aqueous ethanol possessed the highest anti-BChE activities (24.50~32.25% inhibition), followed by peppers extracted with ethyl acetate and hexane. These results were corresponded to those obtained from Capsicum chinense Jacq. cv Habanero extracts (Menichini et al., 2009), in which both unripe green and ripe red peppers in ethanolic extracts were able to inhibit BChE activity (IC50 of 0.562 and 0.806 g/L by green and red pepper extracts, respectively) to a greater extent than when lipophilic fractions (hexane fraction with the IC50>1.0 g/L). It is therefore likely that anti-BChE agents may be soluble in polar solvents but not non-polar solvents.

Color of the sweet peppers also affected BChE inhibition. Green sweet peppers extracted by all three solvents exhibited the highest BChE inhibitory activities (10.22~32.25% inhibition). A similar result was previously reported for Capsicum chinense Jacq. cv Habanero extracts (Menichini et al., 2009), suggesting that green peppers extracted with ethanol possess higher inhibitory activities than ripe red peppers (IC50 of 0.562 and 0.806 g/L in unripe green and ripe red pepper, respectively). These data are in agreement with our results showing high TPC, antioxidant activity, and p-coumaric acid content of green sweet peppers extracted in 70% (v/v) aqueous ethanol and ethyl acetate. The high inhibitory activity of green stage sweet peppers may therefore result from the inhibitory abilities of these compounds.

BACE1 inhibitory activity
Sweet pepper at concentrations of 30.56 g dry weight/L could inhibit BACE1 by up to ~90% (Table 4). These results were similar to those of anti-AChE reactions, in which sweet peppers extracted with hexane and ethyl ac-
etate showed higher BACE inhibitory activities (56.33 ~ 88.30% inhibition) than those extracted with 70% (v/v) aqueous ethanol. These results are in disagreement with the previous study of chili peppers (C. annuum), which indicated that methanolic extracts (0.25 g/L) could inhibit BACE1 activity (55% inhibition) more strongly than hexane and ethyl acetate extracts (25 and 5% inhibition, respectively) (Murata et al., 2015). The availability of the bioactive compounds, which depends on growth environments, may account for these differences.

Of the colored sweet pepper, green, and orange peppers showed the highest and lowest anti-BACE1 activities, respectively. The BACE1 inhibitors may be flavonoids with AChE and/or BChE inhibitory activities. Quercetin and luteolin have been previously shown to possess anti-BACE1 activities, showing IC50 of 0.96 and 0.56 mg/L, respectively (Choi et al., 2014). In addition, quercetin possesses relatively high cell-free BACE1 inhibitory activity (IC50 of 0.85 mg/L) when compared to other flavonoids such as kaempferol and apigenin (Shimmyo et al., 2008). Quercetin at concentrations of 6 mg/L could also reduce neural BACE1 activity in primary neuronal cultures (cell-based assays) (Shimmyo et al., 2008). In addition, quercetin inhibited formation and extension of amyloid fibrils (Szwajgier, 2014), and induced the highest reduction of β-amyloid levels during ELISA analysis (Shimmyo et al., 2008). Through molecular docking studies (Anand and Singh, 2013; Shimmyo et al., 2008), flavonoids have been shown to inhibit BACE1 through forming of hydrogen bonds in the catalytic core. Quercetin may form hydrogen bonds between C3-OH on the C ring to Asp32 (the BACE1 active catalytic residue). This C3-OH is absent in apigenin; therefore, interactions with Asp32 residues are not formed, which results in lower activity. Moreover, anti-BACE1 activities may result from hydroxyl moieties on the B ring since flavonoids with more B ring hydroxyl moieties exhibit higher BACE1 inhibitory activities (Anand and Singh, 2013; Shimmyo et al., 2008).

However, the potential for AD inhibitors to be used in the human brain depends on their abilities to pass the blood brain barrier (BBB), a layer between the central nervous system and the blood circulation which only allows passing of small molecules (solutes) (Rasool et al., 2014). The compounds suitable for crossing the BBB should be highly lipophilic, such as alkylated flavonoids (Anand and Singh, 2013). Therapeutic drugs preferably have molecular weights of less than 600 Da. Flavonoids may be suitable drug candidates due to their small molecular weights (<400 Da), resulting in higher viabilities than general inhibitors that are therapeutic peptide-based drugs (Anand and Singh, 2013; Shimmyo et al., 2008). Thus, both AChE and BACE1 inhibitors from sweet pepper extracts might contain hydrophobic moieties, and may be able to cross the BBB to present with a high viability. There is limited literature available describing the bioavailability of the main bioactive compounds in sweet peppers (luteolin, quercetin, ferulic acid, and p-coumaric acid) to brain tissue; these flavonols have been reported to reach the brain or a target site, and thus may have potential to reduce risk of AD in vivo (Sawmiller et al., 2014; Wang et al., 2016; Yu et al., 2015; Sabogal-Guáqueta et al., 2015; Guven et al., 2015; Nabavi et al., 2015). However, the further cell culture and animal studies are required to explore this further.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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