CHAPTER 4

Discussion and Conclusion

4.1 Discussion

4.1.1 Screening of total phenolic and flavonoid contents, antioxidant and antiglycation activities of culinary plants

This study involved 26 culinary plants that are regularly consumed in traditional forms of medicines in Thailand. 12 species of spices and condiments, 9 species of vegetables, 2 species of herb and 6 species of young leaves of fruits were studied. After defatting all samples with hexane, samples were sequentially extracted with ethyl acetate (EA) and 80% ethanol (ET), respectively. Total phenolic and flavonoid contents of EA and ethanolic extracts from these plants were measured by spectrophotometry. As is well-known, the glycation of protein is a series of non-enzymatic reactions involving the reaction between carbonyl groups of reducing sugars with amino groups of proteins and then lead to the formation of advanced glycation end-products (AGEs). In addition, the mechanisms of glycation also associate with oxidative stress generated the free radicals (superoxide radical and hydroxyl radical) (Hunt *et al.,* 1993). Therefore, both antiglycation and antioxidant properties of the culinary plants have been considered. Their antioxidant and antiglycation activities were determined using DPPH free radical scavenging method and BSA/glucose model, respectively. The antioxidant assay was based on their free radical scavenging activity due to their potential hydrogen donation property (Sawa *et al*., 1999). Whereas the antiglycaion activity of the plant extracts was evaluated for the inhibition of advanced glycation end-products (AGEs) formation based on the BSA/glucose model. The results showed that the ethnolic extract of the young leaves of *P. granatum* (pomegranate) exhibited the highest amount of total phenolics and flavonoids and also showed antioxidant and antiglycation activities. Alcohol, such as ethanol and methanol, has been deemed suitable as a solvent for phenolic and flavonoid extraction (Harborne,

1998). Previous studies have reported that the leaves of pomegranate contained high amounts of tannin and phenolic compounds as well as possessing antioxidant properties (Cavalcanti *et al.*, 2012; Gil *et al.*, 2000). Moreover, it has been revealed that the phenolics apigenin and luteolin glycosides present in the pomegranate leaves were responsible for the antioxidative properties. Barreto and colleagues (2008) have compared the antioxidant capacity of different parts of *M. indica* (mango). They found that its young leaves showed higher amounts of four major phenolic compounds (mangiferin, gallic acid, methyl gallate, and penta-*O*-galloyl-glucoside) when compared with the other parts of the plant (bark, kernel and peel) and the young leaves were reported to possess an exceptionally strong antioxidant capacity.

Another group of plants that also displayed strong antioxidant and antiglycation properties were the ethanolic extract of certain spices and condiments, including *M. cordifolia*, *O. sanctum*, *P. odoratum* and *P. sarmentosum*. Although they revealed moderate amounts of total phenolic and flavonoid contents when compared with other plants, they, however, have been reported to produce several therapeutic effects in terms of antioxidant and antidiabetic activities (Farnworth and Bunyapraphatsara, 1992; Shavandi *et al.*, 2012). Numerous studies have indicated strong oxidant activity of *P. odoratum* (Vimala *et al*., 2003; Huda-Faujan *et al*., 2009). The phenolic compounds which were identified in *Ocimum sanctum* exhibited antioxidant and ant-inflammatory, including caffeic acid and its derivatives (rosmarinic acid) and flavones (apigenin, luteolin, crismarin, isothymusin and luteolin glycosides) (Devi *et al.,* 1999; Pattanayak *et al.,* 2010).

In this study, there was a strong correlation between the phenolic and flavonoid contents versus the antiglycation and antioxidant activities. This relationship indicated that both the antigylcation and antioxidant activities of these plant extracts were associated with their phenolic and flavonoid contents. This result agreed with those of previous studies which reported that phenolic compounds in various plant extracts are the major constituents that display free radical scavenging properties to donate a hydrogen atom from their phenolic hydroxyl groups (Ardestani and Yazdanparast, 2007; Tang *et al*., 2004; Porkorny *et al*., 2001; Sawa *et al.*, 1999). This is similar to the results presented in Thitilertdecha's research (2008), which suggested that

the antioxidant activities of rambutan extracts were remarkably related to their phenolic contents. Moreover, free radicals are also involved in diabetes leading to a failure of cellular function and several pathologies of diabetic complications (Rolo and Palmeira, 2006). Many published studies have suggested that the phenolic and flavonoid compounds in plant extracts are responsible for the antiglycation activity (Wu *et al.*, 2009; Kim and Kim, 2003; Matsuda *et al.*, 2003; Wu and Yen, 2005; Ardestani and Yazdanparast, 2007). For example, it has been reported that cinnamon bark extract could inhibit the formation of AGEs and this is mainly attributed to its phenolic constituents, such as catechin, epicatechin, and procyanidin B2 (Peng *et al*., 2008).

Due to the results of their strong antioxidant and antiglycation activities (>90% inhibition), the ethanolic extracts of 5 species of the young leaves of fruits (*T. indica, P. guajava, M. indica, D. longan* and *P. granatum*) and 4 species of spices and condiments (*O. sanctum, M. cordifolia, P. sarmentosum* and *P. odoratum*) were selected for further investigation of their antiglycation activities mediated by glucose and methylglyoxal. We used a glucose-based protein glycation system because glucose-mediated protein glycation may occur under oxidative and non-oxidative conditions to form AGE protein adducts. Unlike protein glycation by glucose, the reactive intermediates (methylglyoxal (MGO) and glyoxal) which are generated during the middle stage of protein glycation, is used for a non-oxidative model of protein glycation (Muthenna *et al*., 2012). The non-oxidative model offered very important advantage over the glucose induced glycation model because some glycation inhibitors that showed a high level of antioxidant properties in the inhibition of the formation of AGEs may not effectively inhibit non-oxidative protein glycation (Pashikanti *et al*., 2010). Our results suggest that *P. odoratum* extract has potential to inhibit AGE formation in the BSA-glucose model through both oxidative and non-oxidative pathways. On the other hand, the ethanolic young leave extracts of *M. indica, P. granatum* and *D. longan* displayed significantly higher inhibitory activity against AGE formation induced by methylglyoxal correlated with their strong MGO trapping abilities. These results imply that their antiglycation activities may be due to the direct trapping of the reactive di-carbonyl compounds, especially MGO. The previous study demonstrated that (-)-epigallocatechin-3-gallate (EGCG), which is the major bioactive polyphenol in young leave of green tea, could efficiently trap reactive di-carbonyl compounds (MGO or GO)

to form mono- and di-MGO or GO adducts (Sang *et al.*, 2007). Several phenolic compounds, such as catechin, epicatechin, and procyanidin B2, and phenol polymer, identified from the subfractions of the aqueous cinnamon extract, displayed significant inhibitory effects on the formation of AGEs (Peng *et al*., 2008). Their antiglycation activities were related to the trapping abilities of the reactive carbonyl species, such as methylglyoxal (MGO), an intermediate reactive carbonyl of AGE formation, of which proanthocyanidins (condensed tannins) were shown to be more effective as a scavenging reactive carbonyl species than other isolated compounds. Wu and Yen (2005) have reported that flavonoids, especially luteolin and rutin, developed a more significant inhibitory effect on methylglyoxal-medicated protein modification. While, rutin, quercetin and kaempferol were reported to be effective at the last stage of protein glycation in the BSA-glucose model.

4.1.2 Antiglycation and antidiabetic activities of Lamiaceae plants species

Although, the group of young leave extracts showed higher contained phenolic compounds and stronger inhibitory activity against AGE formation induced by methylglyoxal when compared with the group of spices extract, it had limitation of sample harvesting. Besides, previous study has reported that the leaf extract of pomegranate (*Punica granatum*) failed to be used in clinical trials due to poor pharmacokinetic profile and safety issue caused by toxicity and side effect (Wang *et al.*, 2010). Based on the results of screening for total phenolic and flavonoid contents, antioxidant and antiglycation activities, the ethanolic extracts of Lamiaceae plants was selected for further investigation because of their high antioxidant and antiglycation activities. Moreover, Lamiaceae plants are the popular culinary plants that are considered medicinal herbs in Thailand. Their leaves have been traditionally used to treat various diseases, such as high blood pressure and have been used to lower cholesterol levels in diabetic patients (Pripdeevech *et al*., 2010). However, the preventive and therapeutic approaches against protein glycation have not been investigated in detail. In this study, five plants from the Lamiaceae family (*Ocimum basilicum*, *O. americanum*, *O. sanctum* (green), *O. sanctum* (purple) and *Metha cordifolia* Opiz.) were primarily investigated for their chemical compositions in terms of total phenolic content and antioxidant activity. The results indicated that the ethanolic

extract of *O. sanctum* (purple) showed the highest content of phenolic compounds and strong antioxidant activity. Previous reports have shown that rosmarinic acid, luteolin, apigenin and luteolin glycosides were generally found in Lamiaceae plants (Fecka and Turek, 2007; Gupta *et al*., 2007). This study evaluated the characteristics of phenolic compounds in the selected Lamiaceae plant species by comparing the peak area of the individual constituent to the standard calibration of each compound using HPLC. Rosmarinic acid was found in all samples, of which the ethanolic extract of *M. cordifolia* Opiz. contained the highest amount of rosmarinic acid, while luteolin and apigenin were only found in *Ocimum* species (*O. sanctum* (purple) and *O. sanctum* (green)). These results were supported by the previous studies that reported that luteolin and apigenin are the minor flavone constituents of the *Ocimum* species (Grayer *et al*., 1996; Hiltunen and Holm, 2006), whereas rosmarinic acid was identified as a major component in the stems and leaves of *Ocimum sanctum* L. Moreover, it also exhibited antioxidant and anti-inflammatory activities (Jaggi *et al.*, 2003; Rahman *et al*., 2011).

The ethanolic extract of *O. sanctum* (purple) also showed the strongest inhibitory effects against AGE formation in both extracellular (BSA-MGO) and intracellular (histone-MGO) model proteins as well as α-glucosidase (maltase) activity. In 2011, Ma *et al.* reported that rosmarinic acid, a dimer of caffeic acid isolated from *Salvia miltiorrhiza* Bge. had strong inhibitory effects against AGE formation and α-glucosidase (maltase) activity. Moreover, Miroliaei *et al*. (2011) demonstrated that the presence of rosmarinic acid in *Melissa officinalis* L. extract could prevent the structural changes of BSA induced by D-glucose close to its native polar conformation. In addition, luteolin and apigenin, the flavone aglycones, which are the main components of *Chrysanthenum indicum* L., showed an inhibition of AGE accumulation (Tsuji-Naito *et al*., 2009). Wu and Yen (2005) have evaluated the antiglycation activities of flavonoid compounds. They found that luteolin showed a more significant inhibitory effect on MGO-mediated protein modification than the other flavonoids. The ethanolic extract of *O. sanctum* (purple) not only showed effective inhibition of AGE formation in both extracellular and intracellular model proteins, but they also exhibited the suppression of cross-linking histones induced by methylglyoxal. The cross-linking of proteins induced by di-carbonyl compounds (methylglyoxal or glyoxal) is mentioned as the secondary phase of the glycation reaction leading to causes of diabetic

complications. It is expected that the active compounds, especially the phenolic compounds in *O. sanctum* (purple), might be responsible for their antiglycation activities and α-glucosidase (maltase) inhibition. Therefore, the ethanolic crude extract of *O. sanctum* (purple) was chosen for further investigation.

4.1.3 Partial purification and identification of phenolic compounds from *Ocimum sanctum* (purple) extract

For the preliminary separation by solvent partitioning extraction, the ethanolic crude extract of *O. sanctum* (purple) was partitioned in ethyl acetate (EA) and water (aqueous) fractions. The partition between water and organic solvents, such as ethyl acetate can lead to the separation of glycosides from aglycones and to the separation of polar from non-polar aglycones (Harborne *et al*., 1998). The antiglycation effects of EA and the aqueous fraction of *O. sanctum* (purple) against MGO-induced glycation in both extracellular and intracellular model proteins were evaluated. Our results exhibited that the EA fraction of *O. sanctum* (purple) showed a stronger suppressive effect than its aqueous fraction against AGE formation in both extracellular and intracellular model proteins. The anti-diabetic property of both fractions was also examined through the inhibition of α-glucosidase (maltase) enzyme *in vitro*. The results showed that the EA fraction of *O. sanctum* (purple) had the highest α-glucosidase (maltase) inhibitory activity. The EA fraction of *O. sanctum* (purple) exhibited stronger antiglycation activities and α-glucosidase (maltase) inhibition than its aqueous fraction, which may be due to the high amounts of total phenolic compounds. A similar finding was reported by Hussain *et al.*, (2010) who revealed that the ethyl acetate fraction of *Plomis bracteasa* has stronger antiglycation effect than the other fractions. Due to the efficiency in suppressing the AGE formation and α-glucosidase activity, its EA fraction was selected for the partial purification and identification of its phenolic compounds that might be responsible for AGE formation. The flash silica gel 60 column chromatography was used for the separation of the active compounds in the EA fraction of *O. sanctum* (purple). This technique has been commonly used for the separation of phenolic compounds (aglycones) due to its different selectivity for the separation of the compounds, as well as its faster separation and irreversible adsorption. All separated fractions from the column were monitored by TLC and HPLC analyses. The EA

fraction of *O. sanctum* (purple) was separated to afford 17 fractions (F1-F17), according to the occurrence under wavelengths of 254 and 365 nm on TLC. All separated fractions were analyzed for phenolic compounds using the HPLC technique. The HPLC chromatogram of fractions F1-F17 illustrated the peaks corresponding to their spot on their TLC chromatogram. Among these separated fractions of *O. sanctum* (purple), the HPLC chromatogram of fraction F1-F4 and F16-F17 showed a single peak, whereas the HPLC chromatogram of fraction F10-F11 exhibited multiple peaks. All separated fractions were also examined for their antiglycation activity and α-glucosidase (maltase) inhibition. It was found that four separated fractions (F10, F11, F16 and F17) of *O. sanctum* (purple) showed stronger inhibitory effects than the other fractions against AGE formation and α-glucosidase (maltase) inhibition. From the chromatogram and inhibitory properties, these fractions, therefore, were selected to be identified by chromatographic and spectroscopic analyses. The single compound in the oil fractions F1 to F4 was identified by GC-MS. This technique has generally been used in many studies to identify volatile compounds and phenolic compounds from the plant samples. The identification of these fractions was analyzed by comparing the mass spectrum of each constituent with the NISTO5 mass spectral library. From the mass spectral data, the oil fractions F1 to F4 were identified as methyl eugenol. Eugenol and borneol were found as minor constituents in these fractions.

HPLC chromatograms of the separated fractions F16 and F17 from *O. sanctum* (purple) which form the single peak, were identified by LC-MS analysis compared with the mass spectrum of the reference standards. From the LC-MS spectral data, both fractions of F16 and F17 were identified as rosmarinic acid. In the case of fraction F10, its mass spectra matched with the mass spectrum patterns of apigenin and lueolin. The spectral data of the separated fractions F1 and F10 from *O. sanctum* (purple) were confirmed by LCMS/MS technique. The HRMS (ESI) spectrum of fractions F1 and F10 revealed a molecular ion peak corresponding to the mass spectral patterns of methyl eugenol and luteolin, respectively. The molecular formula of the structure obtained from the calculated spectral data for methyl eugenol and luteolin were $C_{11}H_{14}O_2Na$ and $C_{15}H_{11}O_6$, respectively.

The quantitative determination of individual methyl eugenol, rosmarinic acid, luteolin and apigenin in the ethyl acetate (EA) and aqueous fractions of *Ocimum sanctum* (purple) was determined by comparing the peak area of the individual constituents to the standard calibration of each compound using HPLC. Rosmarinic acid and methyl eugenol were found to be the major components, while luteolin and apigenin were the minor components.

4.1.4 Inhibitory effects of phenolic compounds in *ocimum sanctum* (purple) on α-glucosidase activity and the formation of advanced glycation end-products (AGEs)

It is well understood that the advanced glycation end-products (AGEs) are formed between proteins and by reducing sugars through oxidative and non-oxidative pathways. Generally, all reducing sugars can participate in protein glycation reactions (Wei *et al*., 2012). Our results showed that the MGO inducer was the most potential AGE formation in all model proteins, followed by D-ribose, whereas D-glucose required a longer incubation time (15 days) The finding indicated that these reducing sugars (D-ribose and D-glucose) and its intermediate (MGO) can markedly induce AGE formation in both extracellular proteins (BSA and collagen) and intracellular proteins (histone). Pashikanti *et al*., (2010) have reported that glucose is a weak glycation inducer and the chemical reaction with protein under physiological conditions occurs under oxidative conditions over months to years. Moreover, glucose can form AGEs under oxidative and non-oxidative conditions. In contrast to the protein glycation of glucose, reactive carbonyl compounds (methylglyoxal and glyoxal) are both extracellular and intracellular glycating agents, which are involved in *in vivo* non-oxidative pathways (Brownlee, 1995). Pentoses, such as arabinose and ribose, can also induce AGE formation via non-oxidative pathways (Litchfield *et al*., 1999). The inhibitory effects of EA and the aqueous fractions from *O. sanctum* (purple) on the glycation of BSA induced by different reducing sugars (glucose, ribose and MGO) were investigated and compared with the selected standard phenolic compounds (luteolin, apigenin, romarinic acid and methyl eugenol). The results showed that the EA fraction from *O. sanctum* (purple) had potential against the glycation of BSA induced by MGO, glucose and ribose and exhibited weak

inhibitory effects in 3 different inducer models. In the case of the selected phenolic standards, including luteolin, apigenin and rosmarinic acid, these standard compounds also demonstrated strong AGE suppressive capabilities induced by different inducers. However, methyl eugenol showed no antiglycation activities with all the different inducers. Similarly to the BSA model, the EA fraction of *O. sanctum* (purple) was more effective in suppressing the AGE formation in the histone and collagen models. In 2011, Miroliaei *et al.* has demonstrated the effect of rosmarinic acid on the structural changes of BSA induced by glucose. They stated that the presence of rosmarinic acid in *Melissa officinalis* L. extract has potential to keep the protein molecule close to its native polar conformation by arresting changes in the α-conformers by concealing the glycation sites and lowering the extent of the solvent – acesssible surface area, thereby producing barriers for cross β-structure formation. It was suggested that some of phenolic compounds (luteolin, apigenin and rosmarinic acid) in the EA fraction of *Ocimum sanctum* (purple) might be responsible for the suppression of AGE formation in both extracellular (BSA and collagen) and intracellular (histone) target proteins. These findings indicate the potential of the bioactive compounds in *Ocimum sanctum* (purple) to prevent and/or inhibit protein glycation and the prospects for controlling AGE-mediated diabetic complications *in vivo*.

Another therapeutic approach for the prevention of diabetic complications is to decrease postprandial hyperglycemia through α-glucosidase inhibition. Previous investigations have only reported on the inhibitory activity of the phytochemicals in terms of percent of inhibition and/or IC_{50} values, rather than giving deep information of the inhibitive modes of these inhibitors against the α-glucosidase enzyme activity. In this study, we have investigated types of inhibition mode of the active fractions (EA and aqueous) from *O. sanctum* (purple) by kinetic method compared with the standard phenolic compounds. The results of *Ki* and IC_{50} values for the α-glucosidase inhibitory activity of the EA and aqueous fractions from *O. sanctum* (purple) were compared with the selected standard phenolic compounds and acarbose. The EA fraction from *O. sanctum* (purple) showed stronger inhibitory activity than its aqueous fraction. The high α-glucosidase inhibitory activity of the EA fraction corresponded to their strong antiglycation activities and their high content of phenolic compounds. In the case of the

selected phenolic compounds, luteolin and apigenin showed strong inhibitory activities. On the other hand, rosmarinic acid and methyl eugenol showed a weak level of inhibition against α-glucosidase. This suggests that some of the phenolic compounds (luteolin and apigenin flavones) in the EA fraction from *O. sanctum* (purple) might be the major contributors for α-glucosidase inhibition, especially flavonoids. These results were in agreement with previous studies; they suggested that flavonoids, such as anthocyanins, isoflavonoes, flavonols and flavones exhibited very strong α-glucosidase inhibitory capacities (You *et al.*, 2012; Tadera *et al.*, 2006).

Moreover, the types of α-glucosidase inhibition of the EA and aqueous fractions of *O. sanctum* (purple) were also determined by being compared with the selected standard phenolic compounds. The Lineweaver-Burk plot of their inhibition is displayed Both the EA and aqueous fractions of *O. sanctum* (purple) were the mixed non-competitive types. The mixed non-competitive inhibition of these fractions indicated that their inhibition resulted from the binding of both the free enzymes or in the enzyme-substrate complex. In the case of the standard phenolic compounds, both luteolin and apigenin also showed their inhibition types as being of the mixed non-competitive inhibitor type. While, rosmarinic acid and methyl eugenol displayed patterns of competitive inhibition, indicating that both of these compounds compete with the substrate in binding to the active site of the enzyme. Similar results were reported by Tadera *et al.* (2006) and Lin *et al.* (2011), who demonstrated that the inhibition of luteolin on yeast α-glucosidase was done by a mixed-typed inhibitor, while that of rosmarinic acid was done by a competitive inhibitor. These results may also suggest that these phenolics (rosmarinic acid, methyl eugenol, luteolin and apigenin) might be the bioactive compounds in the EA fraction of *O. sanctum* (purple) which contributed to the α-glucosidase inhibitory activity. These results are very encouraging and could lead to the development of therapeutic approaches used to treat diabetic patients.

4.2 Conclusion

The present study shows an evaluation of total phenolic and flavonoid content for the antioxidant and antiglycation activities of 26 culinary plants. We found a correlation between phytochemical compositions and their antiglycation and antioxidant activities.

Among these extracts, the ethanolic extracts of the young leaves (*T. indica*, *P. guajava*, *M. indica*, *D. longan* and P. *granatum*) and spices (*P. odoratum*, *O. sanctum*, *M. cordifolia* Opiz. and *P. sarmentosum*) showed strong antiglycation activities (>90%inhibition).

Based on the results of screening for total phenolic and flavonoids contents antiglaction activties, the ethanolic extract of Lamiaceae plants was selected for investigation. This study investigated the inhibitory effects of five Lamiaceae plants on the formation of advanced glycation end-products (AGEs) in both extracellular and intracellular model proteins. The most active extract was found in the ethanolic extract of *O. sanctum* (purple).

After *O. sanctum* (purple) was partially purified by the partitioning method, its ethyl acetate fraction, which showed the strongest inhibitory activities against AGE formation, was subjected to silica gel 60 column chromatography for isolation and partial purification of phenolic compounds to afford 17 fractions (F1-F17). The separated fractions F10, F11, F16 and F17 of the *O. sanctum* (purple) exhibited stronger inhibitory effects than the other fractions against AGE formation in extra- and intra-cellular model proteins and α-glucosidase inhibition. These fractions were identified by LC-MS analysis compared with the mass spectrum of the reference standards. From the LC-MS spectral data, the single peak in F16 and F17 was rosmarinic acid, whereas the major peaks in F10 were identified as luteolin and apigenin. Moreover, the oil fractions (F1-F4) of the *O. sanctum* (purple), which showed a single peak, were identified by GC-MS technique as methyl eugenol. The molecular formulas of methyl eugenol and luteolin, which were confirmed by LC-MS/MS technique, were $C_{11}H_{14}O_2Na$ and $C_{15}H_{11}O_6$, respectively.

The EA and aqueous fractions of *O. sanctum* (purple) were quantified for the individual identified phenolic compounds by HPLC techniques. The results showed that rosmarinic acid and methyl eugenol were found as major components, while luteolin and apigenin were the minor components.

The inhibitory effects of the phenolic compounds in *O. sanctum* (purple) were examined for the AGE formation mediated by different inducers on model proteins. The

results exhibited that the ethyl acetate fraction of *O. sanctum* (purple) had the strongest inhibitory activities against AGE formation induced by different inducers. Moreover, its ethyl acetate fraction also displayed high α-glucosidase inhibition based on the mixed non-competitive inhibition mode. The results implied that the strong AGE inhibition and α-glucosidase inhibition of the EA fraction of *O. sanctum* (purple) might be ascribed to some of the phenolic compounds (luteolin, apigenin and rosmarinic acid). On the basis of these results, these bioactive compounds might potentially be alternative resources for therapeutic adjuncts for the prevention and inhibition of diabetic complications.