CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals and materials	Company
Absolute ethanol	Lab Scan
Acarbose	Sigma-Aldrich
Acetic acid	BDH
Acetonitrile (HPLC)	J.T. Baker
Aminoguanidine	Sigma-Aldrich
Aluminium chloride	Fluka
Apigenin	Sigma-Aldrich
Butylated hydroxytoluene	Sigma
Bovine serum albumin	Sigma-Aldrich
Collagen type I	Sigma-Aldrich
Diethyl ether (AR)	J.T. Baker
Dimethylsulfoxide (AR)	Lab Scan
1,1-Diphenyl-2-picryl hydrazyl	Sigma
Ethyl acetate (AR)	J.T. Baker
Fetal calf thymus histones (type II S)	Sigma-Aldrich
Folin-Ciocalteu reagent	Carlo Erba
Formic acid (AR)	Fisher
Gallic acid	Merck

Chemicals and materials	**Company**
D-Glucose	Fluka
Hexane (AR)	J.T. Baker
Luteolin	Sigma-Aldrich
Methanol (AR)	Lab scan
Methanol (HPLC)	J.T. Baker
Methyl eugenol 99.5% (GC)	J.T. Baker
Methylglyoxal 37%	Sigma-Aldrich
2,3-methylquinoxaline	Fluka
p-nitrophenyl-α-D-glucopyranoside (pNPG)	Sigma-Aldrich
1,2-Phenylenediamine (AR)	Sigma
Potassium dihydrogenphosphate (GR)	Merck
Potassium hydrogenphosphate (GR)	Merck
Quercetin	Fluka
D-Ribose	Fluka
Rosmarinic acid	Sigma-Aldrich
Rat small intestinal α-glucosidse	Sigma-Aldrich
Sodium azide	Fluka
Sodium carbonate (GR)	Fluka
Sodium Chloride (AR)	Fluka
Sodium dodecyl sulfate	Sigma-Aldrich
Sodium hydroxide (GR)	Fluka
Sodium nitrite	Fluka

2.1.2 Materials and instruments

Instruments	**Models**	**Company**
Analytical balance	BA210S	Sartorius
Autoclave	MLS3750	Sanyo
Blender		Moulinex
Centrifuge	JA20-MC	Beckman
HP GC/MS system	6890	Hewlett-Packard
Hot-air oven	-	-

Instruments	**Models**	**Company**
HPLC	HP1100 binary	Agilent Technologies
Incubator	INC.100.310G	Gallenkamp
LC-MS/MS spectrometer	ZQ4000	Hewlett-Packard
Lyophilizer	LY-3-TT	Schneiger
Luminescence	Synergy H4	Biotek
Micro plate reader	ELX808	Biotek
Rotary evaporator	B740	Büchi
Silica 60 (70-230 mesh) for column chromatography	-	Merck
TLC-plate	-	Merck
spectrofluorometer	LS-50B	Perkin Elmer
Ultraviolet lamp	-	-
UV-Vis spectrophotometer	Lambda 25	Perkin Elmer

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

2.2.1 Preparation of sample materials

Plant materials (Table 2.1) were purchased from the multiple cropping center (MCC) market, a pesticide-free vegetable market, in Chiang Mai, Thailand during the period of August, 2010 to January 2011. The plant materials were dried in a hot-air oven at 60°C and then powdered with blender (Moulinex). The water content of all dried sample materials was less than 10% and these dried samples were kept at 4 °C at the Department of Chemistry, Faculty of Science, Chiang Mai University.

2.2.2 Preparation of the crude extracts

The plant extracts were prepared as described by Harborne (1998) with slight modifications. Thirty grams of each sample was pre-extracted with hexane for defatting then the residues were extracted with ethyl acetate (500 mL, x3) over 1 h in a shaker at room temperature. Ethyl acetate (EA) extract was filtered through Whatman's no. 1 filter paper. The dried residue was then successively extracted with 80% (v/v) ethanol (500 mL, x3). After filtration, the ethyl acetate and ethanolic extracts (ET) were filtered and allowed to evaporate and lyophillize.

Table 2.1 Culinary plants used in this study

	Common name	Extracted part
1. Spices and condiments (9 species)		
Metha cordifolia Opiz.	Kitchen mint	Leaves
Ocimum sanctum	Holy basil	Leaves
Polygonum odoratum	Vietnamese coriander	Leaves
Piper sarmentosum	Wildbetal	Leaves
Alpinia galanga	Greater galangal	Rhizome
Zingiber officinale Rose.	Ginger	Rhizome
Allium cepa	Onion	Whole bulb
Allium ascalonicum	Shallot	Whole bulb
Allium sativum	Garlic	Whole bulb
2. Vegetables (9 species)		
Leucaena leucocephala	Lead tree	Leaves
Gymnema inodorum	-	Leaves
Coccinia grandis	ivy gourd	Leaves
Gynostemma pentaphyllum	jiaogulan	Leaves
Coriandrum sativum	coriander	Leaves
Apium graveolens	Chinese celery	Leaves
Eryngium foetidum	false coriander	Leaves
Centella asiatica Urban	Asiatic pennywort	Leaves
Clitoria ternatea	blue pea	Flowers
3. Herbs (2 species)		
Cissus quadrangularis	-	Stems
Andrographis paniculata Wallex Nees	king of bitter	Leaves
4. Fruits (6 species)		
Musa sapientum	banana	Flowers
Tamarindus indica	tamarind	Young leaves
Psidium guajava	guava	Young leaves
Mangifera indica	mango	Young leaves
Dimocarpus longan	longan	Young leaves
Punica granatum	pomegranate	Young leaves

2.2.3 Determination of total phenolic content

The total phenolic content of each extract was assessed by the Folin-Ciocalteu method with some modifications (Waterman and Mole, 1994) and gallic acid was used as the standard phenolic compound. The extract which was redissolved in ethanol (100 μL) was transferred to a test tube containing 7.9 mL of distilled water. The samples were mixed with 500 μL of the Folin-Ciocalteu reagent and left to react for 5 min. The reaction mixture was neutralized with the addition of 1.5 mL of 200g/L sodium carbonate (Na_2CO_3), followed by 2 h incubation with constant shaking. The absorbance was then measured at 760 nm. The total phenolic content was expressed as mg gallic acid equivalent (GAE)/g sample.

2.2.4 Determination of total flavonoid content

Total flavonoid content was determined by a colorimetric method *(*Zhishen *et al.*, 1999) with slight modifications and quercetin was used as the standard flavonoid. One half mL of the extract was mixed with 2 mL of distilled water, followed by addition of 0.15 mL of 50 g/L sodium nitrite ($NaNO_2$). After 5 min of reaction, 0.15 mL of 100 g/L aluminium chloride ($AlCl_3$) solution was added. The reaction solution was mixed well and incubated at room temperature for 15 min, and the absorbance at 415 nm was measured. Total flavonoid content was expressed as mg quercetin equivalent (QE)/g sample.

2.2.5 *In vitro* determination of antioxidant activity by using DPPH radical scavenging assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of different sample extracts was determined (Gülçın *et al.*, 2003). One mL of DPPH radical solution (0.1 mM DPPH$^•$ in methanol) was well mixed with 3 mL of the extract and incubated for 30 min at room temperature. The decrease in absorbance caused by the proton donating property of the active compounds was measured at 517 nm. The percent DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH radical scavenging effect (\%)} = [(A_o - A_1)/A_o] \times 100$$

where A_o represented the absorbance of the control solution and A_1 represented the absorbance of the extract solutions.

2.2.6 *In vitro* determination of antiglycation activity in BSA-glucose model

Inhibition of protein glycation method was performed according to Matsuura *et al.* (2002) with some modifications. The reaction mixture (2 mL) contained 800 µg/mL of bovine serum albumin (BSA), 200 mM D-glucose and with/without the extract (1 mg/mL) in phosphate buffer (50 mM, pH 7.4) in the presence of 0.2g/L of sodium azide (NaN_3). The reaction mixture was incubated in incubator at 37 °C for 7 days. The fluorescence intensity was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm with a Perkin Elmer LS-50B spectrofluorometer. Aminoguanidine (AG) (1 mg/mL) was used as a positive control. Results were expressed as percent AGE inhibition calculated using the following equation:

$$\text{Inhibition (\%)} = [(F_0 - F_t)/F_0\ ] \times 100$$

where F_t *and* F_0 represent the fluorescence intensity of the sample and the control mixtures, respectively. Different extract concentrations (50-500 µg/mL) providing 50% AGE inhibition (IC_{50}) were calculated from the graph of inhibition percentage against the extract concentration.

2.2.7 *In vitro* determination of antiglycation activity in the BSA-methylglyoxal model

The evaluation for the inhibition of the middle stage of protein was performed according to Peng *et al.* (2008a). Thirty microliters of 500 mM methylglyoxal (MGO) were mixed with 300 µL of 10 mg/mL BSA in the presence of 0.2 g/L of NaN_3. The BSA-methylglyoxal reaction mixture was incubated in incubator at 37°C for 5 days with/without various concentrations (50-500 µg/mL) of the selected plant extracts. Aminoguanidine (AG) (10-100 µg/mL) was used as a positive control. The fluorescence intensity was measured at an excitation wavelength of 370 nm and an emission wavelength of 440 nm with a Perkin Elmer LS-50B. The percentage of the AGE inhibition was calculated using the same equation as in the BSA-glucose model.

2.2.8 Methylglyoxal (MGO) trapping capacity

Direct MGO trapping capacity was tested using the method described by Peng *et al.* (2008b) with a slight modification. MGO (5 mM), 1,2- phenylenediamine (PD; derivatization agent, 20 mM) and 2,3-methylquinoxaline (DQ; internal standard, 5 mM) were freshly prepared in phosphate buffer saline (pH 7.4). Two hundred fifty microliter of 5 mM MGO solution was mixed with 250 µL of PBS (blank), the extracts (concentration of 2 mg/mL) or aminoguanidine (AG) standard solution (concentration of 200 µg/mL). After mixing, the mixtures were incubated in a water bath at 37ºC at 0.5 and 4 h. The samples (125 µL) were taken out and 125 µL of 20 mM PD and 125 µL of 5 mM DQ were added and shaken by vortex for 5 s. After incubation 0.5 h, HPLC analysis was performed on an Agilent 1100 HPLC system (Agilent technologies, Palo Alto, CA). Compound separation was carried out in a Zorbax SB-C_{18} column(4.6×250 mm, 5 µm, Agilent Technologies, Palo Alto, CA). The flow rate was 0.6 mL/min, and the injection volume was 10 µL. Isocratic elution was applied using H_2O/Methanol (50:50, v/v) as the mobile phase. Methylglyoxal-*o*-phenylenediamine adduct, 1-methylquinoxaline, was detected at 315 nm using a UV detector. The peak area of 1-methylquinoxaline in each sample was integrated. The percentage of remaining methylglyoxal was calculated using the following equation:

$$\text{Remaining percentage} = \frac{\text{Peak area at different incubation time}}{\text{Peak area at time zero}} \times 100\%$$

2.3 Antiglycation and antidiabetic activities of Lamiaceae plant species

2.3.1 Preparation of the ethanolic extracts of Lamiaceae plant species

From the screening of antioxidant and antiglycation properties, we found that various ethanolic extracts under Lamiaceae family, such as *O. sanctum* and *M. cordifolia*, exhibited the higher potential activities. Therefore, the ethanolic extract from 5 Lamiaceae plant species was considered for determination of active compounds, particularly phenolic compounds. Five species of plants in Lamiaceae family, which were *O. sanctum* (green), *O. sanctum* (purple), *O. basilicum*, *O. americanum* and *M. cordifolia* Opiz., were used to study (Figure 2.1).

Figure 2.1 Showing 5 plant samples in Lamiaceae Family used in this study: *Ocimum basilicum* (a), *Ocimum americanum* (b), *Metha cordifolia* Opiz.(c), *Ocimum sanctum* (green) (d) and *Ocimum sanctum* (purple) (e)

2.3.2 Chemical compositions of Lamiaceae plant species

2.3.2.1 Total phenolic content

The total phenolic content in the crude extract of Lamiaceae plants was assessed by the Folin-Ciocalteu method as described in the section 2.2.3.

2.3.2.2 Determination of antioxidant activity

The antioxidant activity of the crude extract of Lamiaceae plants was determined by 1,1-diphenyl-2-picryl-hydracyl (DPPH$^{\bullet}$) method as described in the section 2.2.5.

2.3.3 Characterization of phenolic compounds of Lamiaceae plant species (Fecka and Turek, 2008; Hossian, 2010)

The amount of rosmarinic acid, apigenin and luteolin in the 5 ethanolic extracts from Lamiaceae plants were quantified on the basis of the external standard calibration curve using HPLC. Standards concentrations for calibration curve preparation were prepared by dissolving in methanol at various concentrations. The samples were eluted with a gradient elution comprising of 0.2% formic acid in water (solvent A) and 0.2% formic acid in acetonitrile (solvent B). The gradient elution was performed as follows: 0-5 min, 10% B; 5-25 min, 40% B; 26-31 min, 55%; 32-40 min, 65% B; 41-55 min, 75% B. The column was a hypersil ODS, 5µM C_{18} (250 x 4.6 mm ID). The flow rate was set to 0.9 mL/min. The fingerprint profiles were recorded at an optimized wavelength of 280 nm. The injection volume was 10 µL (1 mg/mL). The quantification of each compound was determined based on peak area measurements.

2.3.4 Determination of α-glucosidase (maltase) inhibition of the Lamiaceae plant extracts

The assessment of the rat small intestinal α -glucosidase inhibitory activity was slightly modified according to a method of Adisakwattana *et al*. (2009). Briefly, 100 mg rat intestinal acetone powder (Sigma-Aldrich, Singapore) was homogenized in 3 mL of 0.9% NaCl solution. The solution was centrifuged at 10,000 rpm for 30 min and the supernatant was collected for α-glucosidase assay. A mixture of 10 µL of the crude enzyme solution (the specific activities of maltase were 0.68 units/mg protein) in the presence or absence of 20 µL of plant extracts (1 mg/mL) was incubated at 37°C for 15 min. Then, 70 µL of 36 mM maltose in 0.1 M phosphate buffer pH 6.9 was added and incubated at 37°C for 15 min. The mixtures were suspended in boiling water for 10 min to stop the reaction. The concentration of glucose released from the reaction mixtures was determined by the glucose oxidase method on a microplate reader (BioTex ELX808, UK) at 450 nm.

$$\% \text{ Inhibition} = (Abs_{control} - Abs_{extract}) \times 100 / Abs_{control}$$

where $Abs_{control}$ represented the absorbance of the control solution and $Abs_{extract}$ represented the absorbance of the extract solutions. The IC_{50} value was defined as the concentration of the extract required to inhibit 50% of the enzyme activity.

2.3.5 Determination of antiglycation activities of the Lamiaceae plant extracts

2.3.5.1 Determination of glycation of histone (intracellular) and BSA (extracellular) proteins

The ability of the ethanolic extracts from Lamiaceae plants to inhibit AGEs formation was evaluated using the BSA-MGO assay and histone-MGO assay. As is well-known, MGO, an intermediate of AGE formation, can induce crosslinking of both extracellular and intracellular proteins in body tissue. BSA which is a serum protein was considered to be an extracellular protein. Histone was chosen an intracellular protein because it contains a very rich of arginine and lysine residues which are targeted for glycation. The antigylcation assay in BSA-MGO model as described in section 2.2.7. While the antiglycation assay in the histone-MGO model accorded to a slightly modified method of Gugliucci *et al.* (2009). Fetal calf thymus histone (Types II S) was dissolved in 10 mM PBS buffer, pH 7.4 containing 150 mM NaCl and 0.01% NaN_3 to a final protein concentration of 1 mg/mL. It was incubated with methylglyoxal (MGO) of final concentration of 1 mM. The histone-MGO reaction mixture was incubated with the plant extracts in different concentrations at 37°C for 5 days.

After incubation, the AGE fluorescence was determined at an excitation wavelength of 370 nm and an emission wavelength of 440 nm with a luminescence microplate reader (Synergy H4, Biotek).The percentage of the AGE inhibition was calculated using the same equation as in the BSA-Glucose model as described 2.2.6.

2.3.5.2 Analysis of protein conformation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

After the study of glycaton of histone in section 2.3.5.1, the mixture of histone-MGO reaction with the plant extract at different concentrations at 37°C for 5 days was also analysed for protein conformation changes using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Histone-MGO cross-linked products, electrophoresis was run on 12% polyacrylamide gel. Each lane was loaded with 10 µL of histones-MGO solution (10 mg/mL of histone) mixed with 10 µL dye solution. Gels were stained with Coomassie Brilliant Blue R-250 overnight for visualization**.**

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

2.4.1 Separation and partial purification of *Ocimum sanctum* (purple) extract by silica gel 60 column chromatography

The dried ethanolic powder of *O. sancum* (purple) (10g) was suspended in 30 mL of distilled water and then partitioned with ethyl acetate (EA) (50 mL x 3) to obtain the EA layer and the remaining aqueous layer. Both EA and the aqueous layers were concentrated by the evaporator and then lyophilized. The ethyl acetate fraction (5.0g) from *O. sanctum* (purple) was separated by flash column chromatography over silica gel (Merck 7734, Mesh 70-230, 250 g; glass column diameter 7 cm. x height 35 cm.). Gradient solvent system composed of hexane-ethyl acetate-methanol. The gradient elution was conducted initially with hexane (Hex), gradually enriched with ethyl acetate (Hex: EA; 100:0 to 0:100), followed by increasing amount of methanol in ethyl acetate and finally with 100% methanol (ME) (EA: ME; 95:5 to 0:100). All fractions were monitored by TLC which those showed similar TLC profiles grouped into 17 fractions (F1-F17) (Figure 2.2).

2.4.2 TLC and HPLC analysis of the separated fractions of *Ocimum sanctum* (purple)

For TLC analysis, column chromatographic fractions (F1-F17) of the ethyl acetate extract from *O. sanctum* (purple) were spotted on TLC plate (Silica gel 60 F_{254} precoated alumina sheet, Merck). The chromatogram was developed by using 3 systems of system a: hexane-ethyl acetate (7:3) for fractions F1-F8 and system b: hexane-ethyl acetate (1:9) for F9-F14 and system c: ethyl acetate-methanol (5:5) for F15-F17 as mobile phase. After drying, spots were visualized under UV Light (254 and 365 nm).

For HPLC analysis, all fractions were assayed on a Hewlet-Packard 1100 series liquid chromatograph system as described in the section 2.3.3.

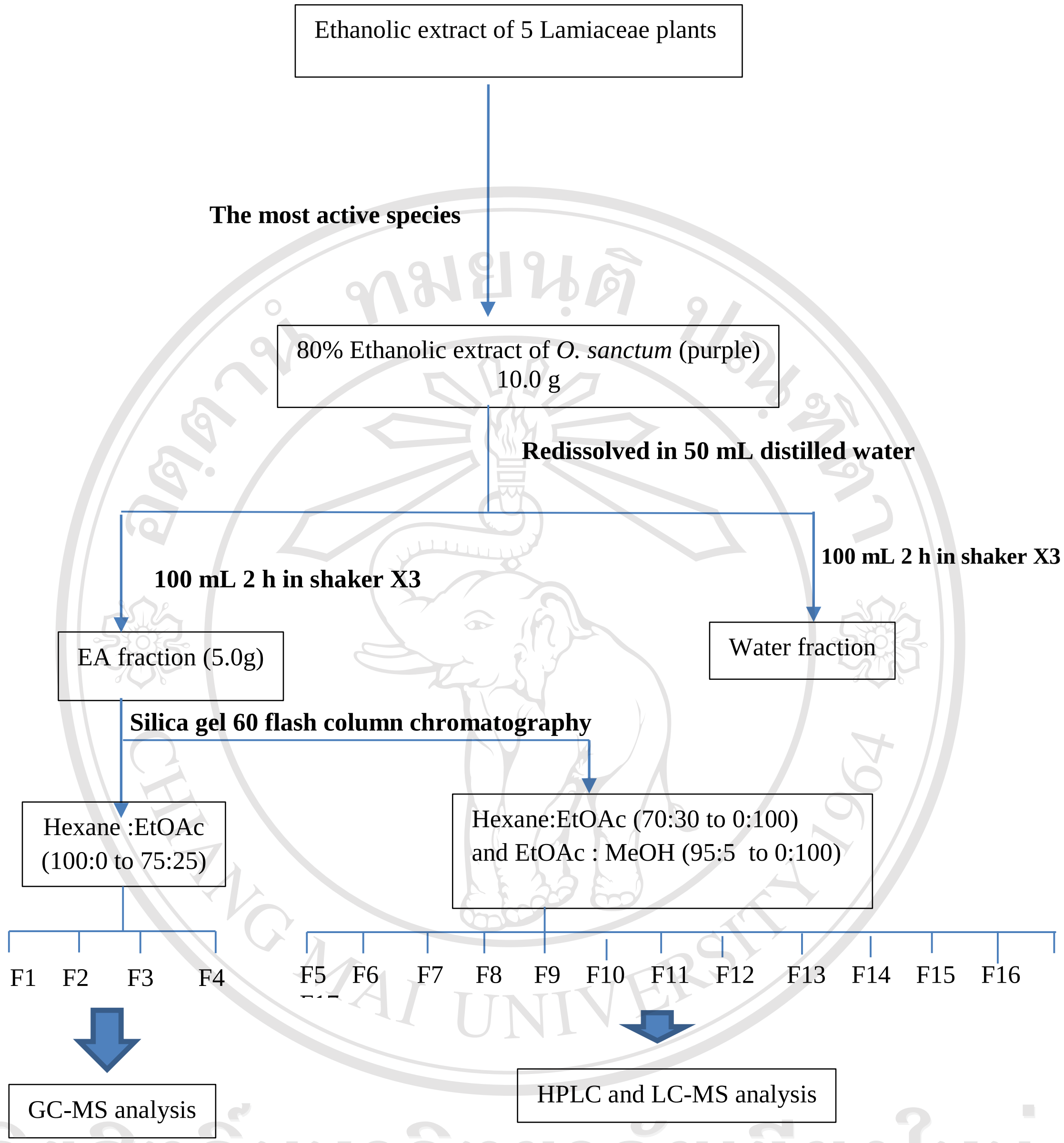

Figure 2.2 A schematic diagram of separation and of *Ocimum sanctum* (purple)

2.4.3 Determination of antiglycation activity of the separated fractions of *Ocimum sanctum* (purple)

All separated fractions of *O. sanctum* (purple), which were separated by silica gel column chromatography, were determined their antigylcation activities in BSA-MGO model as described in section 2.2.7**.**

2.4.4 Determination of α-glucosidase inhibition of the fractions of *Ocimum sanctum* (purple)

For the α-glucosidase inhibitory activity, all separated fractions were evaluated using spectrophotometry measurement as described in section 2.3.4.

2.4.5 Identification of the active fractions from the ethyl acetate fraction of *Ocimum sanctum* (purple)

2.4.5.1 Gas chromatography-Mass spectrometry (GC-MS) analysis of the oil fractions

The oil fractions (F1-F4) of *O. sanctum* (purple), which were separated by silica gel column chromatography, were analyzed by using a Gas Chromatography (GC) 6850 Agilent Technologies/MSD 5973 Hewlett Packard, equipped with a MS detector and HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane 30 m × 0.25 µm, film thickness 0.25 µm). The injector and detector temperatures were set at 270 and 330 ˚C, respectively. The oven temperature was held at 80 ˚C for 2 min, 120 ˚C for 4 min and 155 ˚C for 4 min, then programmed to 270 ˚C at a rate of 10 ˚C /min. The total running time was 60 min. Helium was used as a carrier gas, at a flow rate of 1 mL/min. 1 µL of sample (1 mg/mL of concentration) was injected in the splitless mode. GC-MS detection of an electron ionization system with ionization energy of 70 eV was used. Injector and MS transfer line temperature were set at 270 and 330 ˚C, respectively. The components were identified based on the comparison of their relative retention times and mass spectra with those of the established standards (NIST05 library data of the GC-MS system and previous literature data).

2.4.5.2 Liquid Chromatography-Mass spectrometry (LC-MS) analysis of F10 and F16 of *Ocimum sanctum* (purple) (Sun, 2007)

The phenolic compounds in the fractions (F10 and F16) of *O. sanctum* (purple), which were separated by silica gel column chromatography, were identified using LC-MS analysis. The LC-MS system involved the Agilent 1100 series system, which consisted of degasser, a quaternary pump, an autosampler, a heating exchanger and diode array detector, as well as a MS detector, with Zorbax SB-C_{18} column (250x4.6 mm, particle size of 5 µM). The negative ion mode for mass spectra was adopted to analyze the phenolic compounds based on their ionization tendency. The phenolic compounds were eluted with a gradient mobile phase composed of 1% acetic acid in water (solvent A) and 1% acetic acid in methanol (Solvent B) at the flow rate of 0.3 ml/min. The gradient elution was performed as follows: 0-5 min, 10-22%B; 0-25 min, 22-50% B; 26-45 min, 50-95%B; 46-55 min, 95% B; 56-60 min and maintained at 95-10% B; 61-66min. The wavelength of the UV-Vis detector was set at 280 nm. Ions were scanned from 200 to 800*m/z*. The mass spectrum was compared with the mass spectrum of standards and has been described in previously published data (Hossian, 2010).

2.4.5.3 Liquid chromatography- tandem mass spectrometry (LC MS/MS)

High resolution-mass spectrometry (electrospray) of HRMS (ESI) spectrum was recorded on a Micromass Instrument type QTOF 2 spectrometer.

2.4.6 Quantification of the identified phenolic compounds (methyl eugenol, rosmarinic acid, luteolin and apigenin) in the ethyl acetate and aqueous extracts of *Ocimum sanctum* (purple)

The amount of four identified compounds—methyl eugenol, rosmarinic acid, luteolin and apigenin— were quantified on the basis of the quantity calculated from the external standard calibration curve using HPLC. Four standard phenolic compounds were dissolved in solvent to establish five concentrations (0.010, 0.020, 0.050, 0.10, 0.200 mg/mL). The ethyl acetate and aqueous extracts of *Ocimum sanctum* (purple) (1 mg/mL) was dissolved in methanol and then filtered through 0.45 µm nylon membrane prior to HPLC analysis as previously described in 2.3.3.

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

The active fractions of *O. sanctum* (purple) were determined the AGEs inhibition in different models and the types of α-glucosidase inhibition being compared with the selected standard phenolic compounds.

2.5.1 Glycation of bovine serum albumin (BSA)

The glycation of BSA (10 mg/mL) is described in section 2.2.7. Different reducing sugars (D-glucose, D-ribose and MGO) at various concentrations (1, 5 and 10 mM) were incubated at 37°C for 1-15 days. The antiglycation activities in BSA was incubated with 5 mM of D-glucose, D-ribose and MGO in the presence of the active fractions from *O. sanctum* (purple) in different concentrations (50-800 µg/mL) were assessed being compared with the selected phenolic compounds (methyl eugenol, rosmarinic acid, luteolin and apigenin) in various concentrations (5-100 µg/mL). The AGE fluorescent formation was determined at an excitation wavelength of 370 nm and an emission wavelength of 440 nm with a luminescence microplate reader (Synergy H4, Biotek).The percentage of the AGE inhibition was calculated using the same equation as in the BSA-glucose model as described 2.2.6.

2.5.2 Glycation of histone (Gugliucci, 2009)

The same glycation protocol was described in 2.5.1. Histone was dissolved with 50 mM phosphate buffer pH 7.4 containing 0.02%NaN_3 to a final protein concentration of 1 mg/mL. Histone (1mg/mL) was incubated with different reducing sugars (D-glucose, D-ribose and MGO) at various concentrations (1, 5 and 10 mM) at 37°C for 1-15 days. The antiglycation assay in histone-MGO model, the reaction mixture containing 1 mg/mL histone and 5 mM MGO was incubated with the active fractions from *O. sanctum* (purple) in different concentrations (50-800 µg/mL) being compared with the selected phenolic compounds (methyl eugenol, rosmarinic acid, luteolin and apigenin). The AGE fluorescent formation was determined as described in 2.2.6.

2.5.3 Glycation of collagen

The protein model of collagen (1 mg/mL) was dissolved with 50 mM phosphate buffer pH 7.4 containing 0.02%NaN_3. It was incubated with different reducing sugars (D-glucose, D-ribose and MGO) at various concentrations (1, 5 and 10 mM) at 37°C for 1-15 days. The antiglycation assay in collagen-MGO model was also determined by modified of Hsieh *et al.* (2007). Collagen (1mg/mL) was incubated with 5 mM of methylglyoxal (MGO) at 37°C in the presence the extract (100 and 200 µg/mL) for 14 days of incubation time. The AGE fluorescent formation was determined as described in 2.2.6.

2.5.4 Determination of the inhibition mode (K_i and IC_{50} values) of α-glucosidase inhibitory activity of the fractions from *Ocimum sanctum* (purple)

2.5.4.1 Preparation of α-glucosidase (AGH) solution from rat intestinal acetone powder

AGH solution was prepared using the procedure of Oki *et al.* (1999). Firstly, 100 mg of intestinal acetone powder (Sigma Aldrich Singapore) was mixed with 3 mL of 0.9% (w/v) sodium chloride solution and the solution was homogenized with a vortex for 30 s. The enzyme mixture was centrifuged at 10,000 rpm for 30 min at 4 °C. Finally, the AGH supernatant was kept in an ice bath and the α-glucosidase inhibitory activity assay was recorded.

2.5.4.2 Assay of α-glucosidase inhibitory activity

The α-glucosidase inhibitory assay was performed using the slightly modified method of You *et al.* (2012). Para-nitrophenyl-α-D-glucopyranoside (pNPG) was used as the substrate**.** Seventy microlitre of AGH supernatant (0.6 units/mL) was pre-incubated with 30 µL of the plant extract at various concentrations in a 96-well plate for 15 min at 37°C. After incubation, 100 µL of 0.5 mM pNPG solution in 100 mM phosphate buffer pH 6.8 were combined and incubated for 15 min at 37°C under shaking conditions. The concentration of pNPG was hydrolysed by α-glucosidase to release *p*-nitrophenol (yellow reagent) that could be measured at 405 nm every 10 min for 60 min by a micro plate reader (BioTek ELX808, UK). The kinetic inhibition study of α-glucosidase of the EA and aqueous fraction from *O. sanctum* (purple) at different concentrations was conducted. The ranges of final pNPG substrate concentration were 0.5, 1.0, 2.0 and 3.0 0 mM for α-glucosidase inhibition assay. The types of inhibition, *Km* and *Vmax* values, were determined graphically from double-reciprocal Lineweaver-Burk plots *1/s* versus *1/v*. The K_m is the Michaelis constant and the V_{max} is the maximam rate of the enzymatic reaction. Meanwhile, the K_i value of the sample was determined based on the competitive and mixed non-competitive inhibition types, for which the formula is listed below. Acarbose and the standard phenolic compounds of methyl eugenol, rosmarinic acid, luteolin and apigenin were used as standard inhibitors to be compared with the plant extracts.

The Lineweaver-Burke equation for competitive inhibition type is:

$$1/V = \frac{K_m\ [I]}{V_{max}K_i[S]} + 1/V_{max}\left(1+\frac{K_m}{[S]}\right)$$

The Lineweaver-Burke equation for mixed non-competitive inhibition type is (Segal, 1993; Garret and Charles, 2005):

$$1/V = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]}\left(1+\frac{[I]}{Ki}\right) + 1/V_{max}\left(1+\frac{[I]}{\alpha Ki}\right)$$

αK_i was obtained from :

$$\text{y-intercept} = \frac{1}{Vmax.\ \alpha Ki} \cdot \frac{1}{[I]} + \frac{1}{Vmax}$$

IC_{50} value was obtained from:

$$IC_{50} = K_i(1+[S]/\ K_m)$$

Where the K_m is the Michaelis constant and the K_i is the dissociation constant. The V_{max} is the maximam rate of the enzymatic reaction. The [S] represents the concentration of the pNPG substrate and [*I*] is the concentration of the inhibitor (the plant extracts or the standard phenolic compounds). The IC_{50} value is the concentration of the inhibitor providing 50% inhibitory activity.

2.6 Statistical analysis

All triplicated experimental results were presented as means ± SD. One way analysis of variance (ANOVA) was applied for comparison of the mean values. The Pearson correlation coefficient (*R*) was used to determine two variables. P value < 0.05 was regarded as significant. All statistical analyses were performed using SPSS software (SPSS 17.0 for windows; SPSS Inc., Chicago).