CHAPTER III

MATERIALS AND METHODS

3.1. Apparatus and chemicals

See in the Appendix A

3.2. Synthesis of caffeic acid derivatives

3.2.1. Synthesis of caffeic acid ester derivatives

Caffeic acid ester derivatives were synthesized by modified method of Lee *et al.*[60]. The scheme of reaction is shown in Fig. 3.1. The hydroxyl groups of CAF were protected by methylation reaction to give the methyloxyphenyl compound. The methyloxyphenyl compound was reacted with ethanol, n- octanol, benzyl alcohol and phenethyl alcohol with 1, 3- dicyclohexyl carbodiimid/ dimethyl aminopyridine (DCC/ DMAP) as a coupling reagent. CAF esters were demethylated by adding boron trichloride. The residue was purified by column chromatography using silica gel G60 and eluted with hexane, hexane/ ethyl acetate (2:1 to 1:1) and ethyl acetate, respectively. The ethyl acetate layer was collected, evaporated and recrystrallized with toluene. The structure of the compounds was confirmed by using Fourier Transform Infrared Spectroscopy (FT-IR), Proton Nuclear Magnetic Resonance (^{1}H-NMR), and Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) and mass spectometry (MS).

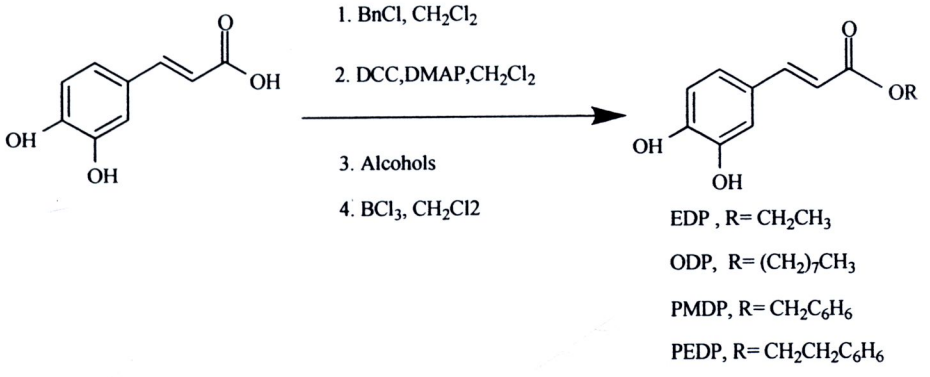

Fig. 3.1 Synthetic procedure of caffeic acid ester derivatives

3.2.2 Synthesis of caffeic acid amide derivatives

Caffeic acid amide derivatives were synthesized by the method of Lee *et al* [60]. The scheme of synthesis is shown in Fig. 3.2. A reaction mixture contained caffeic acid, DCC and amines including ethylamine, n- octylamine, benzylamine and phenethylamine. The reaction was refluxed in tetrahydrofuran (THF) at 50 °C for 7 hours, and then the solvent was removed under vacuum. The residue was purified by column chromatography using silica gel G60 and eluted with hexane, hexane/ ethyl acetate (2:1 to 1:1) and ethyl acetate, respectively. The ethyl acetate layer was evaporated and recrystrallized with toluene. The compound structures were confirmed by using FT-IR, ^{1}H-NMR, ^{13}C- NMR and MS.

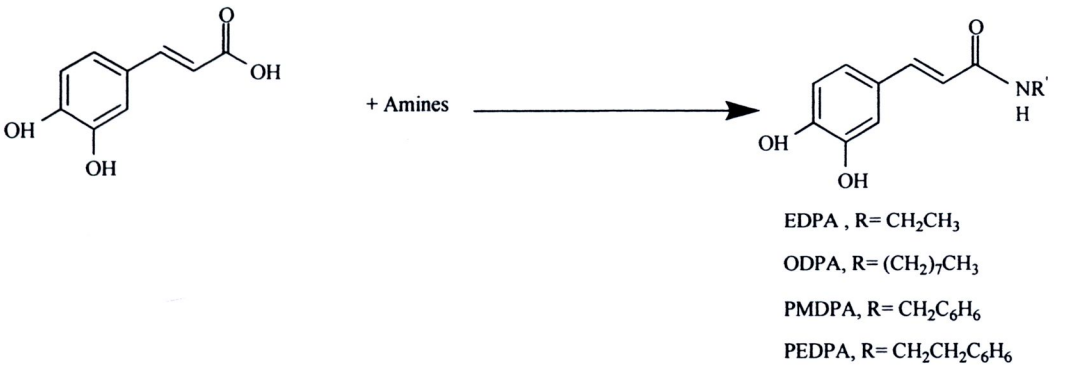

Fig. 3.2 Synthetic procedure of caffeic acid amide derivatives

3.3 Antioxidant properties of caffeic acid and its derivatives

3.3.1. DPPH radical scavenging activity

The antioxidant activities of the caffeic acid and its derivatives were evaluated on the basis of the scavenging activity of the stable 2, 2-diphenyl-2-picrylhydrazyl (DPPH) free radical following the method described by Banerjee *et al.* [61]. Test compound in aqueous vehicle (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percent inhibition was calculated as [(Ao–Ae)/Ao] *100 where Ao is absorbance without test compound and Ae is absorbance with test compounds.

3.3.2. Hydroxyl radical scavenging activity

The assay was based on the competition between deoxyribose and compounds for hydroxyl radical generated by Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) according to the method of Halliwell *et al.*[62]. One millilitre of the

final reaction solution consisted of aliquots of various concentrations of CAF and its derivatives, 1 mM $FeCl_3$, 1mM EDTA, 20mM H_2O_2, 1mM L-ascorbic acid, and 30 mM deoxyribose in potassium phosphate buffer pH 7.4. The reaction mixture was incubated for 1 h at 37°C, and further heated in a boiling water bath for 15 min after addition of 1 ml of 2.8% (w/ v) trichloroacetic acid and 1 ml of 1% (w/ v) 2-thiobarbituric acid. The colour development was measured at 532 nm.

3.3.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Wong *et al.* [63] with slight modification. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol tripyridyltriazine (TPTZ) solution in 40 mmol HCl and 20 mmol iron (III) chloride solution in proportions of 10:1:1 (v/v), respectively. The FRAP reagent was freshly prepared daily and was warmed to 37 °C in a water bath prior to use. The test compound was added to 1.5 ml of the FRAP reagent. The absorbance of the reaction mixture was recorded at 593 nm after 4 min. The standard curve was plotted using iron (II) sulfate solution in the range of 100–2,000 μM. All the measurements were taken in triplicate and the mean values were calculated.

3.3.4 Reducing power

The reducing power was determined by the method of Govindarajan *et al.* [64]. Different concentrations of CAF and its derivatives were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated for 20 min at 50 °C. After incubation, 2.5 ml of 10%

tricholroacetic acid was added to the mixtures, followed by centrifugation at 650g for 10 min. The upper layer was mixed with distilled water and 0.1% ferric chloride and then the absorbance of the solution was measured at 700 nm. Increasing of absorbance of the reaction mixture indicated increasing of reducing power.

3.3.5 Superoxide anion scavenging activities

The influence of the compounds on the generation of superoxide anion was measured according to the method described by the method of Chen *et al.* [65]. Superoxide anion was generated in a non-enzymic system and determined by a spectrophotometric measurement for nitro blue tetrazolium reduction. The reaction mixture contained 1 ml of the compounds in methanol, 1ml of PMS (60 μM) in phosphate buffer (0.1 M, pH 7.4), 1 ml of NADH (468 μM) in phosphate buffer and 1 ml of NBT (150 μM) in phosphate buffer. The reaction mixture was incubated at room temperature for 5 min, and the color was measured at 560 nm against blank samples.

3.3.6 Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined by method of Kumaran *et al.* [66]. Briefly, sodium nitroprusside (10 mM), in phosphate buffer pH 7.4, was mixed with different concentrations of CAF and its derivatives and incubated at room temperature for 150 min. Then, Griess reagent composing of 1% sulfanilamide, 2% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride was added. The absorbance of the chromophore was measured at 546 nm. Ascorbic acid and α-tocopherol were used as positive control.

3.3.7. Antioxidant activity in linoleic acid emulsion system

An inhibitory effect on lipid peroxidation was determined according to the method of Somparn *et al.*[67]. The linoleic acid emulsion was prepared by adding 0.25 ml linoleic acid into 5 ml of 0.05 M borate buffer, pH 9. The volume was adjusted to 50 ml with borate buffer. Thirty microliters of the 16 mM linoleic acid dispersion was added to a test tube containing 2.81 ml of 0.05 M phosphate buffer, pH 7.4 and CAF or its derivatives. The oxidation reaction was initiated at 37°C by adding 1.50 ml of 40mM AAPH solution. Inhibition of conjugated diene formation was measured at 234 nm. Ascorbic acid and α-tocopherol were used as positive control.

3.4. Determination of cytochrome P450 catalytic activity

3.4.1 Microsomal preparation

Human liver samples (n=9) were obtained from donors who mostly died from traffic accident and medication history are unknown. Details of each donors are shown in Table 3.1. The research protocol was approved by Ethical Committee for Human Research, Faculty of Medicine, Chiang Mai University. Liver microsomes were prepared by different centrifugation using a previously described method of Kusirisin *et al.*[68]. The microsome pellets were kept in Tris buffer pH 7.4 and stored at -80 °C until use. Protein contents were determined by bicinchonic acid (BCA) protein assay kit.

Table 3.1 Details of the donors whose liver samples were used

Code	Age (years)	Sex	Cause of death	Medication
08-0156	42	Male	MCA	Not known
08-0157	42	Male	MCA	Not known
08-0159	24	Male	MCA	Not known
08-0163	22	Male	MAC	Not known
08-0170	31	Male	Electrocute	Not known
08-0171	23	Male	MCA	Not known
08-0173	62	Male	MCA	Not known
08-0174	25	Male	Gunshot	No medication
08-0175	30	Male	MCA	Not known
08-0176	45	Male	Gunshot	Not known

*MCA= motorcycle accident

3.4.2 Assay of CYP1A2 activity

Phenacetin *O*-deethylation to acetaminophen was used to assess CYP1A2 activity. Incubation mixture of 1 ml contained 0.1 mg microsomal protein, 100 mM potassium phosphate pH7.4, 0.1mM EDTA, 0.4 mM $MgCl_2$, NADPH generating system (0.5 mM $NADP^+$, 20 mM NADPH, 2 mM glucose-6-phosphate, 1 IU/ml glucose -6-phosphate dehydrogenase), phenacetin (28-1,400μM) and caffeic acid amide derivatives (0.025-0.25 μg/ml). Reaction was stopped after 60 min by the addition of 200μl cooled methanol and 50 μl of caffeine 100μg/ml was added as an internal standard. The mixtures were then centrifuged at 13,000 RPM for 5 min and the supernatant was injected into the analytical high performance liquid chromatography (HPLC). HPLC system consisted of an Agilent 1100 Series high-performance liquid chromatography, Agilent 1100 series diode- array detector. The HPLC pumps, autosampler, column oven, and diode- array system were monitored and controlled using the HP chemstation computer program (Agilent). A wavelength used for the identification of the diode array detector was at 253 nm. Acetaminophen was separated by Water C18 column (250×4 mm, 5 μm), and column oven was set up at 25°C. Isocratic elution was employed with mobile phase consisting of water: methanol: acetonitrile: tetrahydrofuran (55:20:20:5) by volume with flow rate 0.7 ml/ min and the injection volume was 5 μl. The activity of CYP1A2 was calculated in μmol / min/ mg protein.

3.4.5. Assay of CYP2E1 activity

Activity of CYP2E1 was determined by using *p*-nitrophenol as substrate according to Kusirisin *et al.* [68]. The reaction mixture contained 0.5 mg microsomal protein in phosphate buffer pH 7.4, *p*-nitrophenol (0–20 μM) and caffeic acid and its derivatives in various concentrations (62.5-500 µg/ ml). The reaction was initiated by the addition of NADPH solution containing 0.5 mM $NADP^+$, 20 mM NADPH, 2 mM glucose-6-phosphate and 1 IU/ml glucose -6-phosphate dehydrogenase. After 1 hour, the reaction was terminated by the addition of trichloroacetic acid (0.6 M; 0.5 ml). The precipitated protein was then removed by centrifugation (1500 ***g***, 10 min) and 0.1 ml 10 M sodium hydroxide was added to the supernatant. The yellow color was developed and then absorbance was measured at 546 nm. The CYP2E1 activity was calculated in pmol/ min/ mg protein.

3.4.6. Assay of CYP3A4 activity

CYP3A4 activity was determined by using metabolism of diazepam to oxazepam. Incubation mixture of 1 ml contained 0.1 mg microsomal protein, 100 mM potassium phosphate pH7.4, 0.1mM EDTA, 0.4 mM $MgCl_2$, NADPH generating system (0.5 mM $NADP^+$, 20 mM NADPH, 2 mM glucose-6-phosphate, 1 IU/ml glucose -6-phosphate dehydrogenase), diazepam (0.35 – 3.47 µM) and caffeic acid and its derivatives (0.025-0.25 µg/ml). Reaction was stopped after 60 min by the addition of 200µl cooled methanol. The mixtures were then centrifuged at 13,000 RPM for 5 min and the supernatant was injected into the analytical high performance liquid chromatography (HPLC). HPLC system consisted of an Agilent 1100 Series

high- performance liquid chromatography, Agilent 1100 series diode- array detector. The HPLC pumps, autosampler, column oven, and diode- array system were monitored and controlled using the HP chemstation computer program (Agilent). A wavelength used for the identification of the diode array detector was at 235 nm. Diazepam and oxazepam were separated by Water C18 column (250×4 mm, 5 μm), column oven was set up at 25°C. Isocratic elution was employed with mobile phase consisting of 0.125% isopropylamine in water: acetonitrile: methanol (70:15:15) by volume with flow rate 0.7 ml/ min and the injection volume was 5 μl. The activity of CYP3A4 was calculated in μmol/ min/ mg protein.

3.4.7. Enzyme kinetic of cytochrome P450s

Six different concentrations of phenacetin, ranging from 0.28 to 2.80 mM, were used in kinetic experiments for phenacetin O-deethylation using Human liver microsomes. Michaelis-Menten parameters for phenacetin O-deethylation were estimated by two enzyme kinetic approach. The results of the inhibition study were analyzed by Dixon and Cornish-Bowden plots to determine type of inhibition and value of the inhibitory constant (Ki).

3.5. Cytotoxicity test on Hep G2 cell line

3.5.1 Cell culture and treatment

Human hepatoma cell lines, Hep G2 (ATCC HB8065) was obtained from Asst. Prof. Dr. Suchart Kotan, Department of Associated Medical Science, Faculty of Associated Medical Science, Chiang Mai University. Hep G2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ ml penicillin G, 100 μg/ ml streptomycin and 0.25 μg/ ml amphotericin B (GIBCO BRL Gaithersburg, MD, USA), in a 37 °C humidified incubator under an atmosphere of 5% CO_2.

3.5.2 Treatment of Hep G2 with caffeic acid and its derivatives

Caffeic acid and its derivatives were dissolved in DMSO and stored at -20 °C until used. The required caffeic acid and its derivatives were freshly prepared before use by diluting the stock solution in DMEM medium. A various CAF and its derivatives concentrations were added to Hep G2 cells for evaluation of cytotoxicity. The plates were moved to the CO_2 incubator at 37 °C for 24 hour. For all the assays in the present study, Hep G2 cells incubated with DMEM without phenol red or FBS served as controls.

3.5.3. MTT assay

The MTT method was described by Mark *et al.* [69]. Briefly, following incubation with the range of caffeic acid derivatives concentrations (0–200 μM) for 48 hours, the caffeic acid derivatives-containing medium was removed and replaced with 1 mg/ml of sterilized MTT solution. The MTT solution was freshly prepared in distilled water. The plates with added MTT solution were then wrapped in aluminum foil and placed in the 5% CO_2 incubator for 1 h at 37 °C. The MTT solution was then removed and 100 μl of DMSO was added to each well to dissolve the blue formazan crystals. The optical density was measured at 570 and 650 nm wavelengths using the ELISA spectrophotometer. Each assay involved 16 wells per condition and was performed in triplicate.

3.6. Phase II enzymes assay

3.6.1. Hep G2 cells treatment

Hep G2 cells were seeded in 24 well plates at cell concentration 1×10^5 cell/ well. After incubating for 24 hours, the media was changed then the test compounds were added to each well at different concentrations. The cells were incubated at various times for 6, 12, 24, 48 and 72 hours. The cell culture medium was decanted and trypsinized with trypsin EDTA reagent. The Hep G2 cells were collected into microcentrifuge tube, then cells were washed with PBS for 3 times. The cells were lysed by using lysis solution (0.8% digitonin and 2 mM EDTA). The solution was centrifuged at 15,000 g for 10 mins to separate cell debris. The supernatant was stored at -80°C until use for phase 2 enzyme tests.

3.6.2. UDP-glucuronosyl transferase assay

UDP-glucuronosyl transferase activity was determined using the method of Viollon *et al.* [70]. The reaction contained lysis cell solution, 100 mM Tris-HCl buffer pH 7.8, 1mM *p*-nitrophenol and 5 mM UDPGA. After incubation for 60 minutes at 37°C, 0.1 M sodium hydroxide solution was added to stop the reaction. Then, *p*-nitrophenol glucuronide was quantified by measuring the decrease of absorbance at 405 nm.

3.6.3. Glutathione-S- transferase assay

Glutathione S-transferase activity was determined using the standard substrate 1-chloro-2, 4- dinitrobenzene (CDNB), following the procedure of Chen *et al.* [71]. The reaction mixture contained 1 mM glutathione in 0.1 mM K_3PO_4 buffer with 1 mM EDTA. After addition of 1 mM CDNB to the sample cuvette, the absorbance of the mixture was measured at 340 nm for the baseline, with methanol as a reference. Cytosolic protein in 0.1 mM K_3PO_4 buffer, pH 6.5, was added to the mixture to start the reaction for 1 min. The rate of reaction was monitored by measuring the absorbance increased at 340 nm.

3.6.4. Heme oxygenase assay

Heme oxygenase 1(HO-1) activity was measured using the method of Jaikang and Chaiyasut [72]. Briefly, 50 µl of microsomes from harvested cells were added to 250 µl of reaction mixture containing 0.1 mM NADPH, 1 mM NADP, 1mM glucose-6-phosphate and 5 mU of glucose -6- phosphate dehydrogenase, 2 mg rat livers cytosol as a source of bilirubin reductase, 100 mM potassium phosphate buffer (pH 7.4) and 1 mg/ml hemin. The reaction was conducted at 37°C in the dark for 1 h. The samples were left in an ice bath to terminate the reaction and 1 ml of chloroform was added. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε = 40 mM^{-1} cm^{-1}). The HO activity was expressed as micromoles of bilirubin per milligram of protein per hour.

3.7. Statistical analysis

Experimental data were expressed as mean± standard deviation (SD). The difference between groups were analyzed by one-way analysis of variance (ANOVA) followed by Duncan. *p* values of less than 0.05 were considered statistically significant.