การศึกษาเรื่องความสัมพันธ์ระหว่างโครงสร้างและฤทธิ์นั้น การวิจัยนี้สรุปได้ว่าอนุพันธ์เอสเทอร์และเอไมด์ที่สังเคราะห์ขึ้นนั้นมีความสามารถกำจัดอนุมูลอิสระ ยับยั้งปฏิกิริยาออกซิเดชัน กระตุ้นเอนไซม์เฟส 2 ในเซลล์มะเร็งตับชนิด Hep G2 ได้ ซึ่งกลไกเหล่านี้จัดเป็นกลไกพื้นฐานในการป้องกันการเกิดมะเร็งตับได้ ซึ่งจะต้องทำการศึกษาถึงความปลอดภัยของสารแต่ละชนิดว่ามีความปลอดภัยมากน้อยเพียงใดในสัตว์ทดลองต่อไป

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ภาคผนวก

รายละเอียดงานวิจัยที่ส่งเข้าร่วมเสนอผลงานในงาน The 1st SFRR-Thai Meeting and International workshop on advance of Free radicals, oxidative stress and their evaluation methods. December, 2008. Chiang Mai, Thailand

Free radical scavenging and antioxidative properties of Ethyl 1-(3',4'-dihydroxyphenyl) propen amide.

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Abstract

The aim of this study to investigate free radical and antioxidant properties of synthetic Ethyl 1-(3',4'-dihydroxyphenyl)propen amide (EAM) using in vitro assay system .EAM was synthesized using PyBOP as a coupling agent. EAM was investigated for the free radical scavenging on 1,1- diphenyl-2-picrylhydrazyl (DPPH•) radical, superoxide anion ($O_2^{\bullet -}$), nitric oxide radical (•NO) as well as the anti-lipoperoxidative capacity on human erythrocytes haemolysis and ferric reducing ability of plasma (FRAP). α- tocopherol, ascorbic acid and caffeic acid were used as the reference antioxidant compound. The inhibition concentration value (IC 50) of EAM on DPPH•, $O_2^{\bullet -}$, and •NO were 61.32, 44.56, and 45.77 μg/ml respectively. EAM could inhibit human erythrocytes heamolysis 47.53 % at concentration 1 mg/ml. The reducing power increased in a dose response relationship.There results suggested that synthetic EAM might be beneficial as a potent antiradical and antioxidant .

Introduction

Caffeic acid is widely distributed in the plant kingdom and found in coffee beans, olives, properlis, fruits and vegetables. They have attracted much attention and have been studied by many research groups for several properties such as antiviral, anti-inflammatory, antioxidantive, immunostimulatory and neuroprotective properties.

The aim of this study to investigate free radical and antioxidant properties of synthetic EAM using *in vitro* assay system.

Material and Methods

EAM was synthesized by using PyBOP as a coupling agent. The residue was purified using column chromatography and the structure confirmed by FT-IR, H^1-NMR, C^{13}-NMR, GC-MS and spectrophotometer.

1,1- Diphenyl 2- picrylhydrazyl (DPPH) radical scavenging activity was measured according to the method of Blois (1958). Superoxide anion scavenging activity wasmeasured based on the described method by Robak and Gryglewski (1988). Superoxide radicals were generated in nicotinamide adenine dinucleotide, phenazine methosulphate (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue trtrazolium (NBT). Nitric oxide scavenging was measured by generating nitric oxide from sodium nitroprusside and measured by Griess reation. Assess for normal human erythrocyte heamolysis induced by peroxyl radicals was according to the procedures described by Yuan et al (2005) The FRAP assay was measured by the change in absorbance at 593 nm. IC 50 were calculated among percent of inhibition with concentration. α- tocopherol, ascorbic acid and caffeic acid were as the reference standard.

Statistical analysis

All data are arranged of triplicate analysis. The data were recorded as mean± SD.

Results and discussions.

The structure of EAMshown in fig.1.

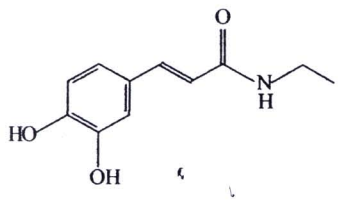

Fig.1. EAM structure

The inhibition concentration values (IC50) of EAM for free radical scavenging are summarized in table 1. The reducing power and free radicals scavenging were increased in a dose response relationship shown in figure 2 and 3. The result clearly indicate EAM exhibits strong antioxidant activity both in scavenging free radicals and antioxidation, as compared to that of the structure-related compound caffeic acid. These *in virto* results also suggest the possible that EAM could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress.

Substance/ radicals	DPPH	$O_2^{\bullet}$	•NO (μg/ml)	red blood cell heamolysis
EAM	61.32	44.56	45.77	1468.77
Caffeic acid	44.49	36.47	19.15	1879.30
α- tocopherol	267.70	26.06	14.16	1861.48
ascorbic acid	382.98	45.17	66.66	2472.8

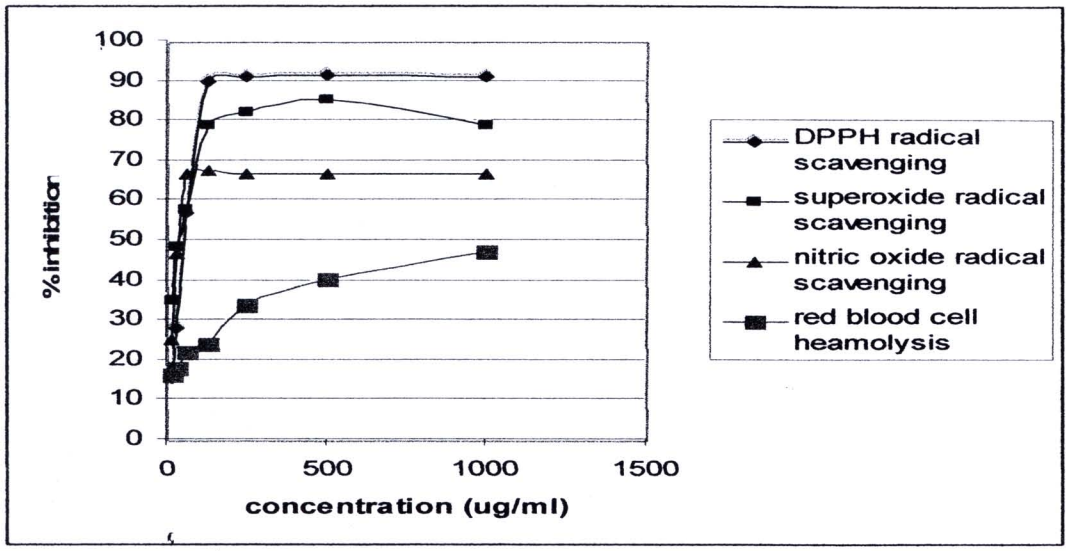

Fig 2. Free radicals scavenging of EAM

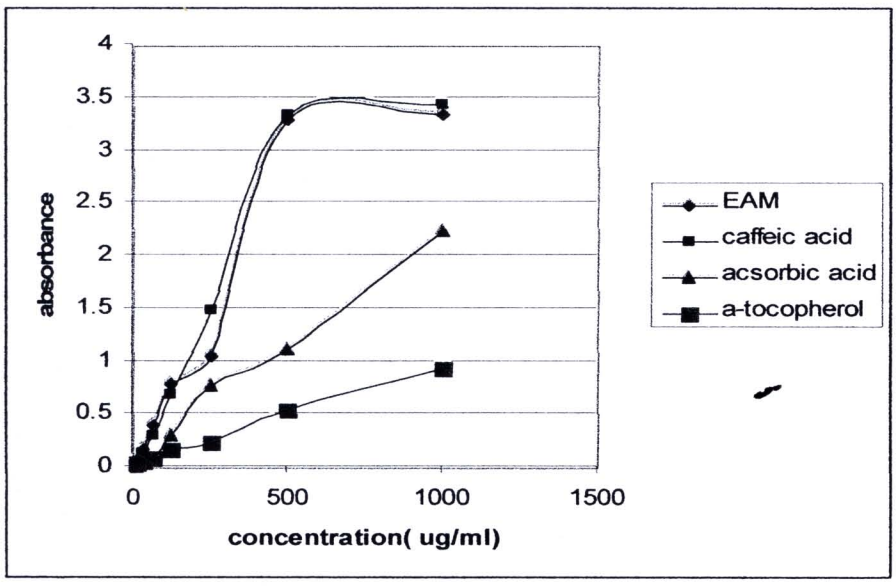

Fig 3. FRAP of EAM and standard references.

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รายละเอียดงานวิจัยที่ส่งเข้าร่วมเสนอผลงานในงาน The 2 nd International conference on natural products for health and beauty (NATPRO). December 2008. Phayao , Thailand

Free radical scavenging and antioxidative properties of ethyl caffeate.

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Abstract

The aim of this study to investigate free radical and antioxidant properties of synthetic ethyl caffeate using in vitro assay system.Ethylcaffeate was synthesized by esterification among ethanol and caffeic acid. Ethyl caffeate was investigated for the free radical scavenging on 1,1- diphenyl-2-picrylhydrazyl (DPPH●) radical, superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (OH●), nitric oxide radical (●NO) as well as ferric reducing ability of plasma (FRAP). α- tocopherol, ascorbic acid and caffeic acid were used as the reference antioxidant compound.The inhibition concentration value (IC 50) of ethyl caffeate on DPPH●, $O_2^{\bullet -}$, OH●, and ●NO were 44.88, 144.45, 1.42 and 27.59 μg/ml respectively. The reducing power increased in a dose response relationship. There results suggested that synthetic ethyl caffeate might be beneficial as a potent antiradical and antioxidant and can be applied to use in cosmetic and food industry.

Introduction

Caffeic acid and its derivatives such as ethyl caffeate are widely distributed in the plant kingdom and found in coffee beans, olives, properlis, fruits and vegetables. They have attracted much attention and have been studied by many research groups for several properties such as antiviral, anti-inflammatory, antioxidative, immunostimulatory and neuroprotective properties. The aim of this study to investigate free radical and antioxidant properties of synthetic ethyl caffeate by using *in vitro* assay systems.

Material and Methods

Ethyl caffeate was synthesized by esterification among ethanol and caffeic acid. The residue was purified using column chromatography and the structure confirmed by FT-IR, H^1-NMR, C^{13}-NMR, GC-MS and spectrophotometer.

1,1- Diphenyl 2- picrylhydrazyl (DPPH) radical scavenging activity was measured according to the method of Blois (1958). Superoxide anion scavenging activity wasmeasured based on the described method by Robak and Gryglewski (1988). Superoxide radicals were generated in nicotinamide adenine dinucleotide, phenazine methosulphate (PMS-NADH) system by oxidation of NADH and assayed by reduction of nitroblue trtrazolium (NBT). Hydroxyl radical scavenging activity was measured by the compertition between deoxyribose and ethyl caffeate for the hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/ H_2O_2 system. Nitric oxide scavenging was measured by generating nitric oxide from sodium nitroprusside and measured by Griess reation. The reducing power was measured by the method of Oyaizu (1986). IC 50 were calculated among percent of inhibition with concentration. **α**- tocopherol, ascorbic acid and caffeic acid were as the reference standard

Statistical analysis

All data are arranged of triplicate analysis. The data were recorded as mean± SD.

Results and discussions.

The structure of ethyl caffeate shown in fig.1.

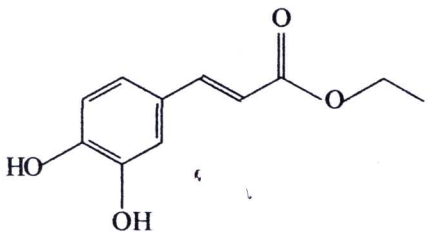

Fig.1. Ethyl caffeate structure

The inhibition concentration values (IC50) of ethyl caffeate for free radical scavenging are summarized in table 1. The reducing power and free radicals scavenging were increased in a dose response relationship shown in figure 2 and 3. The result clearly indicate ethy caffeate exhibits strong antioxidant activity both in scavenging free radicals and antioxidation, as compared to that of the structure-related compound caffeic acid. These *in virto* results also suggest the possible that ethyl caffeate could be effectively employed as an ingredient in health or functional food, to alleviate oxidative stress.

Substance/ radicals	DPPH	$O_2^{\bullet}$	$OH^{\bullet}$	.NO (μg/ml)
Ethyl caffeate	44.88	144.45	1.42	27.59
Caffeic acid	44.49	36.47	59.36	19.15
α- tocopherol	267.70	26.06	65.87	14.16
ascorbic acid	382.98	45.17	51.83	66.66

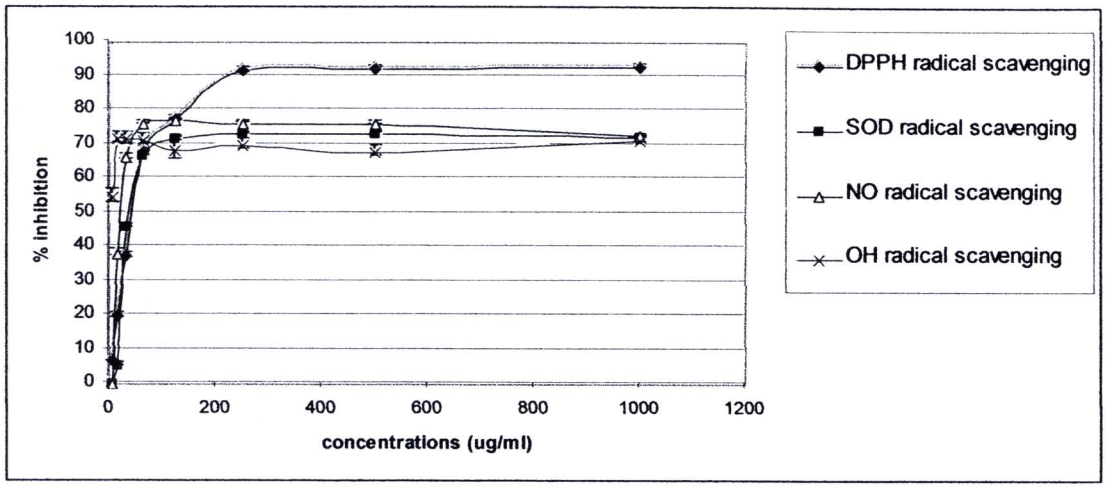

Fig 2. Free radicals scavenging of ethyl caffeate

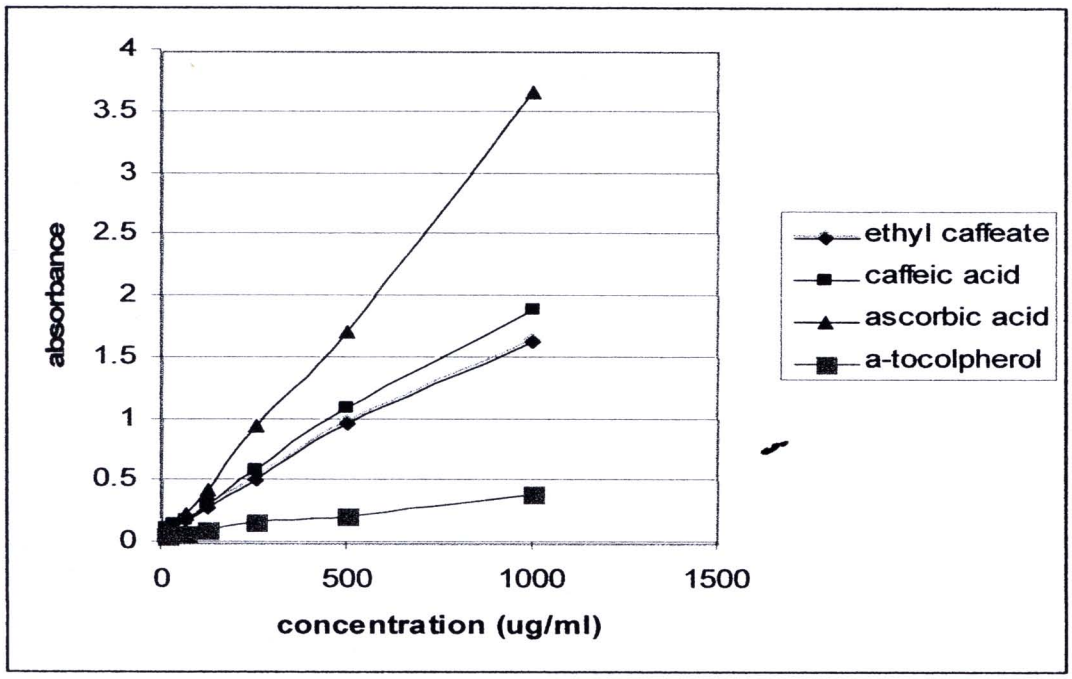

Fig 3. Reducing power of ethyl caffeate and standard references.

Submitted manuscript ส่งไปเพื่อพิจารณาลงตีพิมพ์วารสาร Medicinal Chemistry (Impact factor 2009, 1.642)

Inhibitory effects of caffeic acid ester analogues on free radicals and human liver microsome CYP1A2 activities

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ABSTRACT

Ethyl caffeate (EC), octyl caffeate(OC), benzyl caffeate(BC) and phenethyl caffeate(PC) were synthesized and evaluated for scavenging of superoxide anion, nitric oxide radical and 1,1-diphenyl-1- picrylhydrazyl radical(DPPH). Antioxidant activity was investigated with reducing power method. Pooled human liver microsome was used for investigating of effects on cytochrome P450 1A2 (CYP1A2) catalytic activities by using phenacetin as a substrate. Dixon and Cornish-Bowden plots were used for enzyme kinetic analysis. The EC, OC, BC and PC potentially inhibited superoxide anion, nitric oxide and DPPH radicals. IC 50 values of superoxide anion scavenging of EC, OC, BC and PC were 16.42, 79.83, 123.69, and 123.69 μg/ ml respectively. EC was more potent than OC and BC in term of nitric oxide radical scavenger: IC 50 values of EC, OC and BC were 24.16, 37.34 and 52.64 μg/ml, respectively. In addition, the IC 50 values of EC, OC, BC and PC on DPPH radical scavenging were 70.00, 184.56, 285.34 and 866.54 μg/ ml, respectively. The IC 50 values of EC, OC, BC and PC on phenacetin *O*-deethylation were 124.98, 111.86, 156.68 and 31.05 μg/ ml, respectively. Enzyme kinetic showed that type of inhibition mechanism was mixed-type. The result of this study shows that caffeic acid ester analogues potentially scavenge free radicals and inhibit catalytic activity of CYP1A2. This may lead to important implication in prevention of CYP1A2-mediated chemical carcinogenesis.

Keywords: caffeic acid ester analogues, cancer chemoprevention, cytochrome P4501A2, enzyme inhibition, ethyl caffeate, free radical scavenging

INTRODUCTION

Cancer is one of the most frequently found illness and that leading to death. Successful intervention strategies await experimentation. The majority of cancers result from exposure to carcinogenic agents from the environment such as chemicals, radiations and viruses. The elimination or avoiding exposure of environmental carcinogens is a basis of primary cancer prevention [1]. Most of the chemicals are not reactive substances, therefore activation by drug-metabolizing enzymes is required[2]. Cytochrome P450 (CYP) is the major enzymes that can activate procarcinogenic chemicals [3,4]. CYP1A2 is a drug-metabolizing enzyme found in human liver that can activate procarcinogen to carcinogen such as benzo[a]pyrine, heterocyclic amines and aflatoxin B1[5]. Activation of CYPA1A2 activity may increase the carcinogenicity from procarcinogenic compound [6]. In various type of cancer chemopreventive agents including natural, synthetic or biological chemical agents, suppression of the expression of procarcinogen activating enzyme leading to inhibition of the initiation stage of chemically- induced carcinogenesis. Many naturally agents have shown chemopreventive and chemotherapeutic properties in variety of bioassay and animal models systems [7,8].

Reactive oxygen species (ROS) and free radicals are important factors in cellular injury and aging process. Under pathological conditions, ROS is over produced and results in oxidative stress. Imbalance between ROS and antioxidant defense mechanisms lead to oxidative modification in cellular membrane or intracellular molecule[9,10]. There are many reports regarding inhibitory effects of phenolic compounds on chemical mutagenesis and carcinogenesis processes [11-13].

Caffeic acid (3, 4-dihydroxycinnamic acid) and its analogues are a naturally phenolic compound, which found in many fruits, vegetables, honeybee and herbs. It has

been reported that caffeic acid and its ester analogues such as caffeic acid phenethyl ester, ethyl cafeate are antimitogenic, anticarcinogenic, anti imflammatory , tumor- suppressive properties and chemoprotection [14-17]. However, information about the effects of caffeic acid esters on chemoprevention is limited. The aim of this work was to characterize the effect of caffeic acid ester analogues on free radical scavenging and catalytic activity of cytochrome P4501A2 which are the mechanisms for preventing cancer initiation.

MATERIALS AND METHODS

Materials

Caffeic acid, nitroblue tetrazolium salt(NBT), sulphanilamide and phosphoric acid were purchased from MERCK. Ethanol, n-octanol, benzyl alcohol, phenethyl alcohol, DCC, boron trichloride, phenazine methosulphate (PMS), sodium nitroprusside, naphthyl ethyldiamine dihydrochloride, potassium ferriccyanide, trichloro acetic acid, ferric chloride, DPPH, NADPH, NADP, NADH, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company (St. Louis, MO,USA). Phenacetin and acetaminophen were obtained from Toxicology Section, Chiang Mai Medical Science Center, Chiang Mai, Thailand. All chemicals were analytical grade.

Synthesis of caffeic acid analogues

Synthesis of caffeic acid ester was modified using method of Lee *et al* [18]. Briefly, caffeic acid was protected selectively at the hydroxyl groups of the aromatic ring by methylation to give the methyloxyphenyl compound. The methyl-protected caffeic acid was transformed to the methyl caffeic acid ester by reaction with ethanol; n-octanol, benzyl alcohol and phenethyl alcohol followed by esterification with DCC/, method. Methyl-

caffeic acid esters were demethylated by BCl_3 cleanly to give caffeic acid ester compounds. Structures of compounds were confirmed using MS, IR, H-NMR and C13-NMR. The structure of caffeic acid ester analogues are shown in Fig.1. Details of the structure of each caffeic acid ester analogues are shown below.

1. Ethyl 1-(3',4'-dihydroxyphenyl) propenate, ethyl caffeate, EC

   Yield: 60%. Mp: 149-151 °C (colourless powder)

   FT-IR ν_{max} (KBr): 3468.75, 3375.05, 2953.12, 2890.62, 1679.69, 1640.62, 1589.06;

   H^1-NMR(400 MHz, acetone –d6); δ: 8.21 (2H, br s), 7.46 (1H,d, *J*=16.1 Hz), 7.11 (1H,d, *J*=2.0 Hz) 7.03 (1H,dd, *J*=2.0, 8.3 Hz), 6.87 (1H, d, *J*=7.8 Hz), 6.27 (1H,d, *J*=16.1 Hz), 4.17 (2H,q, *J*=7.3 Hz), 1.25 (3H,t, *J*=7.3 Hz); C13-NMR; 126.2, 114.6, 144.1 146.6, 114.3, 121.1, 144.25, 114.4, 166.4, 59.3, 13.5 MS *m/z*: 208 (M^+), 180(100%), 163, 134, 89, 77

2. Octyl 1-(3',4'-dihydroxyphenyl)propenate, n-octyl caffeate, OC

   Yield: 45%, mp: 110-111°C (colourless powder)

   FT-IR ν_{max} (KBr): 3487.62, 3328.14, 2917.95, 2832.52, 1682.66, 1599.07, 1523.22

   H^1-NMR(400 MHz, acetone –d6); 8.41 (1H,s), 8.13 (1H,s), 7.54 (1H,d, *J*=16.1 Hz), 7.15 (1H,d, *J*=2.0 Hz), 7.03 (1H, dd, *J*=2.0, 8.1 Hz), 6.86 (1H,d, *J*=8.1 Hz , 6.28 (1H,d, *J*=16.0 Hz), 4.13(2H,t, *J*=6.7 Hz), 1.66 (2H,quint, *J*=6.7 Hz), 1.45-1.14 (10H,m), 0.86 (3H,t, *J*=6.9 Hz)

   C13- NMR; 167.3, 146.7, 145, 144.24, 126.4, 121.2, 114.7, 114.6, 113.4, 63.8, 32, 31.3, 25.6, 24.7, 24, 22.2, 13.5 MS *m/z*: 292 (M^+), 193, 180 (100%), 163, 117

3. Phenylmethyl 1-(3',4'-dihydroxyphenyl) propenate , benzyl caffeate, BC

   Yield: 40% mp 153-155°C colourless powder)

   FT-IR ν_{max} (KBr): 3406.25, 3335.92, 1687.50, 1640.62, 1593.75, 1515.63

   H^1-NMR(400 MHz, acetone –d6); d: 8.31 (2H, br s), 7.58 (1H, d, J = 15.8 Hz), 7.45–7.28 (5H, m), 7.17 (1H, d, J = 2.1 Hz), 7.07, 7.06(1H, dd, J = 2.1, 8.1 Hz), 6.86 (1H, d, J = 8.1 Hz), 6.34 (1H, d,J = 16.0 Hz), 5.21 (2H, s).

   C13-NMR; 167.2, 166.1, 146.8, 146.7, 144.2, 135.8, 128, 126.11, 125.99, 114.6 MS *m/z*: 270 (M+, 100%), 179,163, 136, 117.

4. Phenylethyl 1-(3',4'-dihydroxyphenyl)propenate ,phenethyl caffeate,PC

   Yield: 47% mp 153-155°C colourless powder

   FT-IR ν_{max} (KBr): 3439.0, 3317.19, 2909.37, 2851.56, 1632.81, 1601.56, 1531.25, 1054.68 H^1-NMR(400 MHz, acetone –d6); 7.58 (1H,d, J = 15.8 Hz), 7.26-7.40 (5H, m), 7.06 (1H,d, J = 2.1 Hz), 6.93 (1H,dd, J = 2.1, 8.1 Hz), 6.79 (1H,d, J = 8.1 Hz), 6.30 (1H,d, J = 15.8 Hz) ,5.19 (2H,s), 5.04 (2H,broad)

Superoxide radicals scavenging

Measurment of superoxide anion scavenging activity of CA and its analogues was measured following the method of GÜlcin [19]. Superoxide radicals were generated in PMS-NADH system by oxidation of NADH and assayed by reduction of NBT. The superoxide radicals were generated in 3 ml of Tris-HCl buffer (16mM, pH 8.0) containing 1 ml of NBT (50μM) solution, 1 ml NADH (78 μM) solution and various concentration of CA and its derivatives 100 μl. The reaction was started by adding 1 ml of PMS solution

(10μ M) to the mixture. The reaction mixture was incubated at 25 °C for 15 min and absorbance was measured at 560 nm. The percentage of inhibition of superoxide anion generation was calculated.

Nitric oxide radicals scavenging

Nitric oxide scavenging activity was measured following the method of Govindarajan *et al* [20]. Briefly, the assay mixture contained 10 mM sodium nitroprusside in phosphate buffer and CA and its analogues in different concentrations were incubated at 25 °C for 150 min. Then 1.5 ml of the incubation solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequence coupling with napthylethylene diamine was measured at 546 nm.

Reducing power

The reducing power of extracts was determined by the method of Shon *et al* [21]. Different concentrations of extracts 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% trichloroacetic acid was added to the mixtures, followed by centrifugation at 650 × *g* for 10 min. The upper layer was mixed with distilled water and 0.1% ferric chloride, and then the absorbance of the resultant solution was

measured at 700 nm. Increasing of absorbance of the reaction mixture indicates increasing of reducing capacity.

DPPH radicals scavenging

The stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used for determination of free radical scavenging activity of caffeic acid ester analogues following the method of Banerjee *et al* [22]. Aqueous dilutions of caffeic acid ester analogues were added to 3 ml of 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage of inhibition was calculated.

Preparation of Human liver microsome

Human liver samples (n=9) were obtained from donors who died from traffic accident within 4 hours. In accordance with Thai law, 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.*[23]. 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.

Determination of cytochrome P450 1A2 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 analogues (0.025-0.25 μg/ml). Reaction was stopped after 60 min by the addition of 200μl cooled methanol and 50 μl of caffeine100μg/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), column oven was set up at 25°C. Isocratic elution was employed with mobile phase consisting of water: methanol: acetronitrile: tetrahydrofuran (55:20:20:5), with flow rate 0.7 ml/ min and the injection volume was 5 μl.

Study of pharmacokinetic

Different concentrations of phenacetin, ranging from 0.28 to 2.80 mM, were used in kinetic experiments for phenacetin *O*-deethylation by using human liver microsome as source of CYP1A2 enzyme. The type of the enzyme inhibition was analyzed by Dixon and Cornish-Bowden plots.

Statistic analysis

Data was expressed as mean ± S.D. of three values and analysed by one way ANOVA followed by Duncan. *P* values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Superoxide anion radical scavenging

Superoxide is the one electron reduced from the oxygen molecule which produced by activated phagocytes. It is a precursor of hydrogen peroxide, hydroxyl radical and singlet oxygen that have the potential to react with macromolecules and induce tissue damages[24]. In the PMS-NADPH- NBT system, superoxide anion produced from dissolved oxygen from the coupling reaction of PMS-NADPH that reduced NBT. The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture[25]. Figure 2 shows the ability of caffeic acid ester analogues to inhibit superoxide radical generation when using caffeic acid, ascorbic acid and α-tocopherol as standards. The EC, OC, BC and PC demonstrated a concentration- dependent scavenging ability by neutralizing superoxide radicals with IC50 values of 16.42, 79.83, 123.69 and 123.69 μg/ ml, respectively. These results clearly indicate that caffeic acid ester analogues are potent scavenger of superoxide radicals in a dose-dependent manner.

Nitric oxide scavenging

Recent studies have shown that reactive nitrogen intermediates, nitric oxide (NO), play an important role in inflammatory process and carcinogenic process [26]. Figure 3 presents the NO-scavenging effects on tested ester analogues of caffeic acid. All of the derivatives had NO-scavenging activity with dose- response curve in range 15.625 to 125 μg/ml. The IC50 values for NO- scavenging ability were calculated from the linear range to compare the potency of NO-scavenging. The order of potency considering from the IC50 values was CAF>EC>OC>BC>PC by which the IC 50 value of EC, OC and BC were

24.16, 37.34 and 52.64 μg/ml respectively. IC 50 value of PC was more than 1,000 μg/ ml because caffeic acid esters were not stable molecules [14, 17]. The results show that the small molecules of ester analogues of caffeic acid have high potential NO-scavenging due to its hydrophobicity properties.

Reducing Power

The Fe^{2+}/Fe^{3+} transformation was investigated for measurement of the reducing ability. The reducing capacity of compound is significant indicator of its potential antioxidant activity[27]. Figure 4 shows the reductive capability of ester analogues of caffeic acid compared with ascorbic acid and α-tocopherol. Increasing of absorbance indicates the reducing power of the compounds. As shown in Fig4. The reducing potential of CAF ester analogues increased in a dose-response relationship and exhibited in the following order: ascorbic acid> EC> BC> CAF> OC> tocopherol> PC. It is reported that the antioxidant action is based on the breaking of the free radical chain by donating hydrogen atom or reacting with certain precursors of peroxide to prevent peroxide formation. The data presented here indicate that the marked reducing power of compounds seem to be the result of their antioxidant activity. It is presumed that ester analogues of CAF may act by donating hydrogen atom [14]and reacting with free radicals to convert them to more stable products and terminating the free radical chain reaction.

Scavenging of DPPH radical

Determination of stable nitrogen radical, DPPH, is a simple method for evaluating free radical scavenging activity. DPPH radical can donate an electron or hydrogen radical and then transform to stable molecule. The maximum absorbance of stable DPPH in

ethanol was at 517 nm. Decreasing of the absorbance of DPPH radical causes from antioxidant activity, which results in the radical scavenging by hydrogen donation [28]. Figure 5 shows ability of Caffeic acid ester analogues to scavenge DPPH radical when compared with caffeic acid, ascorbic acid and α-tocopherol as standards. The potency of the scavenging activity of caffeic acid ester analogues on the DPPH radical are as followed: EC> OC >BC> PC with IC 50 values 70.00, 184.56, 285.34 and 866.54 μg/ml respectively. The mechanism of DPPH radical scavenging may occur from 2, 3 double bond of caffeic acid which is the radical-targeting site [29]. This study found that the longer side chain or aromatic ring in side chain are less potent scavenger for DPPH radical than short side chain.

Effects of caffeic acid ester derivatives on CYP1A2 activity

The inhibition of CYP1A2 activity by ester analogues of caffeic acid was determined using phenacetin as a substrate. The amount of acetaminophen, the reaction product, was measured as the area under the curve by using HPLC. Caffeic acid ester analogues significantly inhibited the phenacetin *O* deethylation reaction. The IC50 values of EC, OC, BC and PC were 124.98, 111.86, 156.68 and 31.05 μg/ml and the maximal percent of inhibition of phenacetin *O*-deethylation were 77.97%, 43.31%, 55.48% and 78.77% respectively. The PC was the most potent inhibitor of CYP1A2 activity. Normally, CYP1A2 substrates contain planar ring that can fit the narrow and planar active site of the enzyme[5]. The PC structure consists of two benzene rings which can disturb active site of CYP1A2 enzyme. Beltrán-Ramírez and colleagues also reported that PC could decrease the enzyme activity of CYP1A1 and CYP1A2 and reduced carcinogen bioactivation.[30]

Dixon and Cornish-Bowden plots were chosen to study type of kinetic inhibition of each compound. Dixon and Cornish-Bowden plots were obtained by using different phenacetin

concentrations. According to Dixon plot (Fig.6) and Cornish-Bowden plot (Fig. 7), EC, OC, BC and PC showed mixed-inhibition on CYP1A2-mediated phenacetin O-deethylation activity.

Cytochrome P450s are the most important enzyme in the metabolism process of xenobiotics substances. The CYPs are significant source of reactive oxygen species such as superoxide anion and hydrogen peroxide. Inhibition of CYP1A2 activity may reduce carcinogens and free radical generation. Caffeic acid ester analogues possess free radical scavenging property and additionally inhibited procarcinogen activating enzyme that could be successful strategy for cancer chemoprevention. Further study *in vivo* and animal toxicity test need to be explored.

CONCLUSION

EC, OC, BC and PC, caffeic acid esters analogues, were synthesized and evaluated in different free radical scavenging testing system for antioxidant power and inhibition of human liver CYP1A2 catalytic activity. The results show that caffeic acid esters analogues have potential to scavenge superoxide anion, nitric oxide, DPPH radical. Caffeic acid ester analogues possess potential antioxidant property and are potent inhibitors of CYP1A2 activity with mixed-inhibition type. These compounds may play an important role as free radical scavenger and inhibitor of procarcinogen activating enzyme for cancer chemoprevention.

ACKNOWLEDGEMENTS

This research was supported by the Thailand Research Fund (DBG5080008), the Graduate School and Faculty of Pharmacy, Chiang Mai University, Thailand. We are also

grateful for Toxicology section, Chiang Mai Medical Science Centre, Chiang Mai, Thailand for chemical and HPLC instruments.

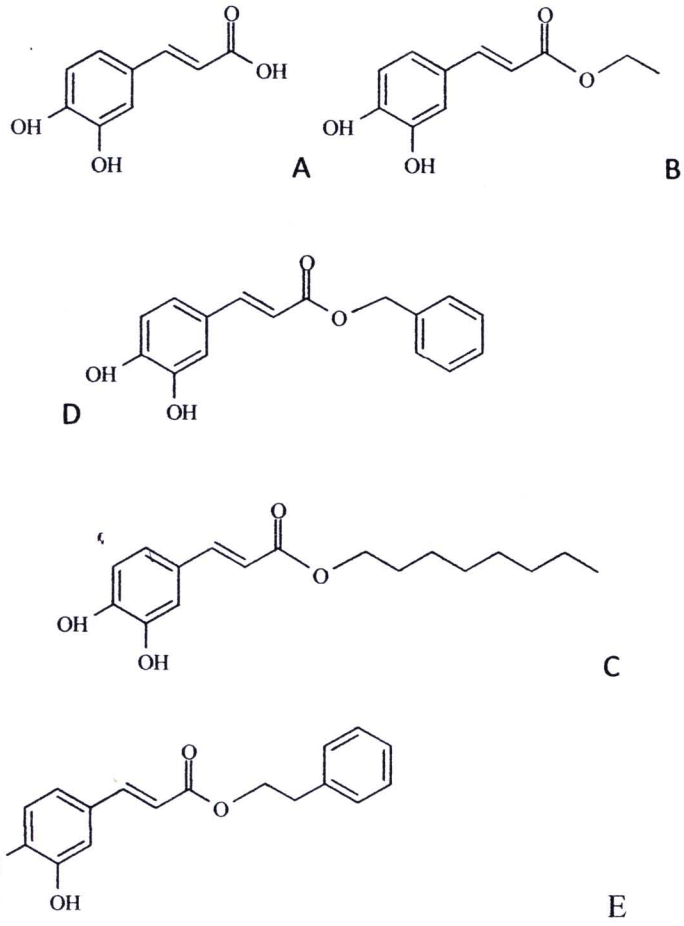

Fig. (1). The structure of caffeic acid ester analogues. A= Caffeic acid , B= Ethyl 1-(3',4'-dihydroxyphenyl)propenate (EC), C= Octyl 1-(3',4'-dihydroxyphenyl)propenate (OC), D=Phenylmethyl 1-(3',4'- dihydroxyphenyl) propenate (BC), E= Phenylethyl 1-(3',4'-dihydroxyphenyl)propenate (PC)

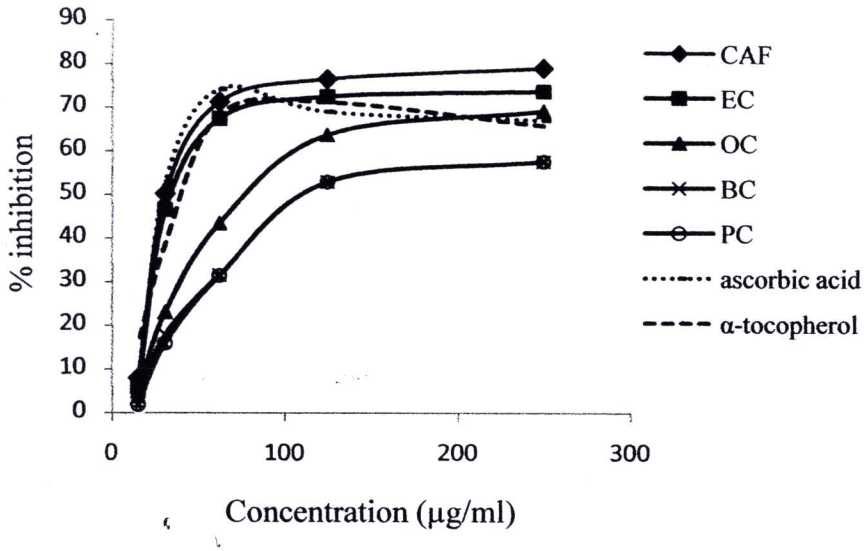

Fig. (2). Percent inhibition of caffeic acid esters on scavenging superoxide anion. The data shows the average values. Values are means of triplicate determination.

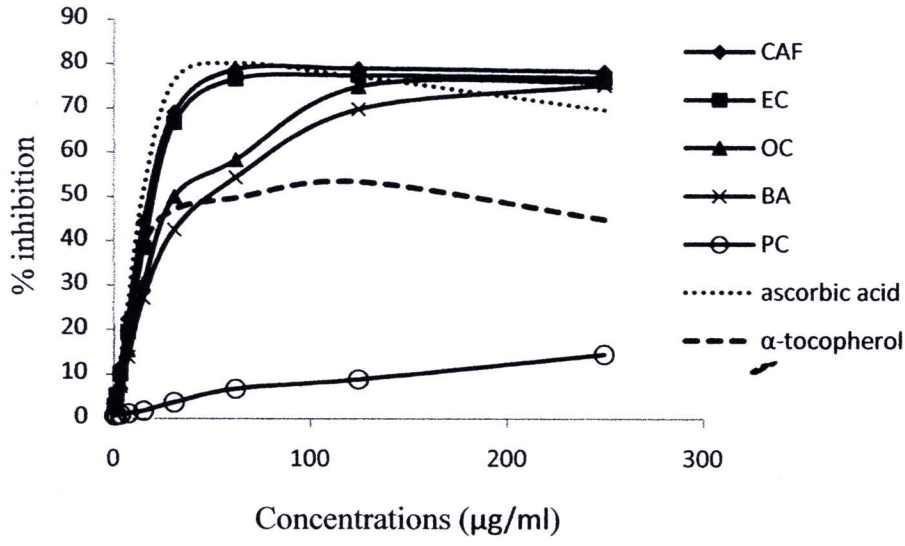

Fig. (3). Percent inhibition of caffeic acid esters on nitric oxide scavenging. The data shows the average values. Values are means of triplicate determination.

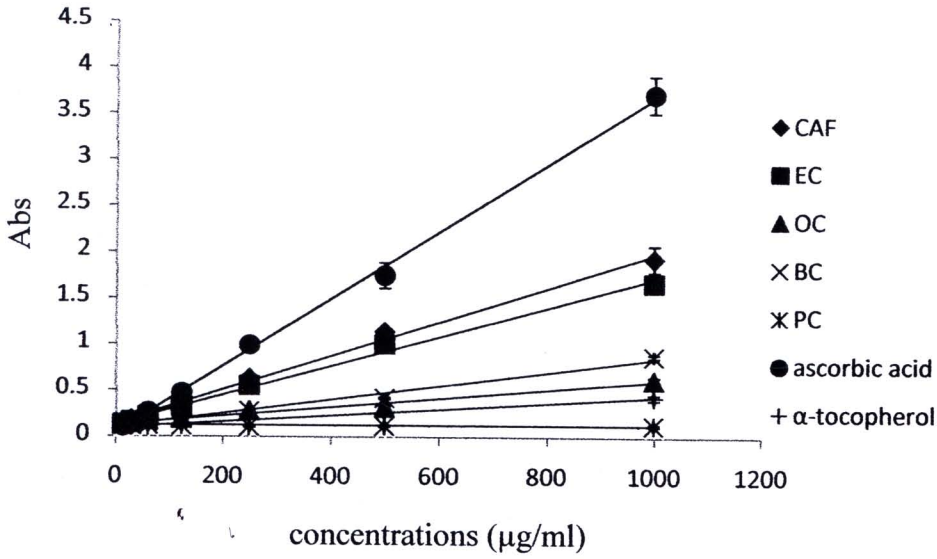

Fig. (4). Reducing power of caffeic acid ester. The data shows the average values. Values are means of triplicate determination

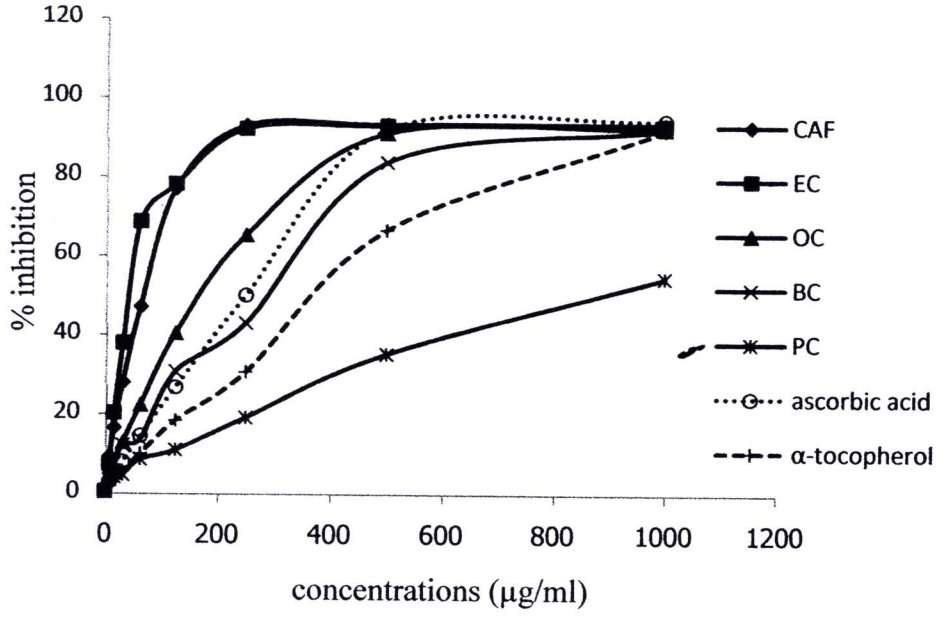

Fig. (5). DPPH radical scavenging of caffeic acid esters. The data shows the average values. Values are means of triplicate determination.

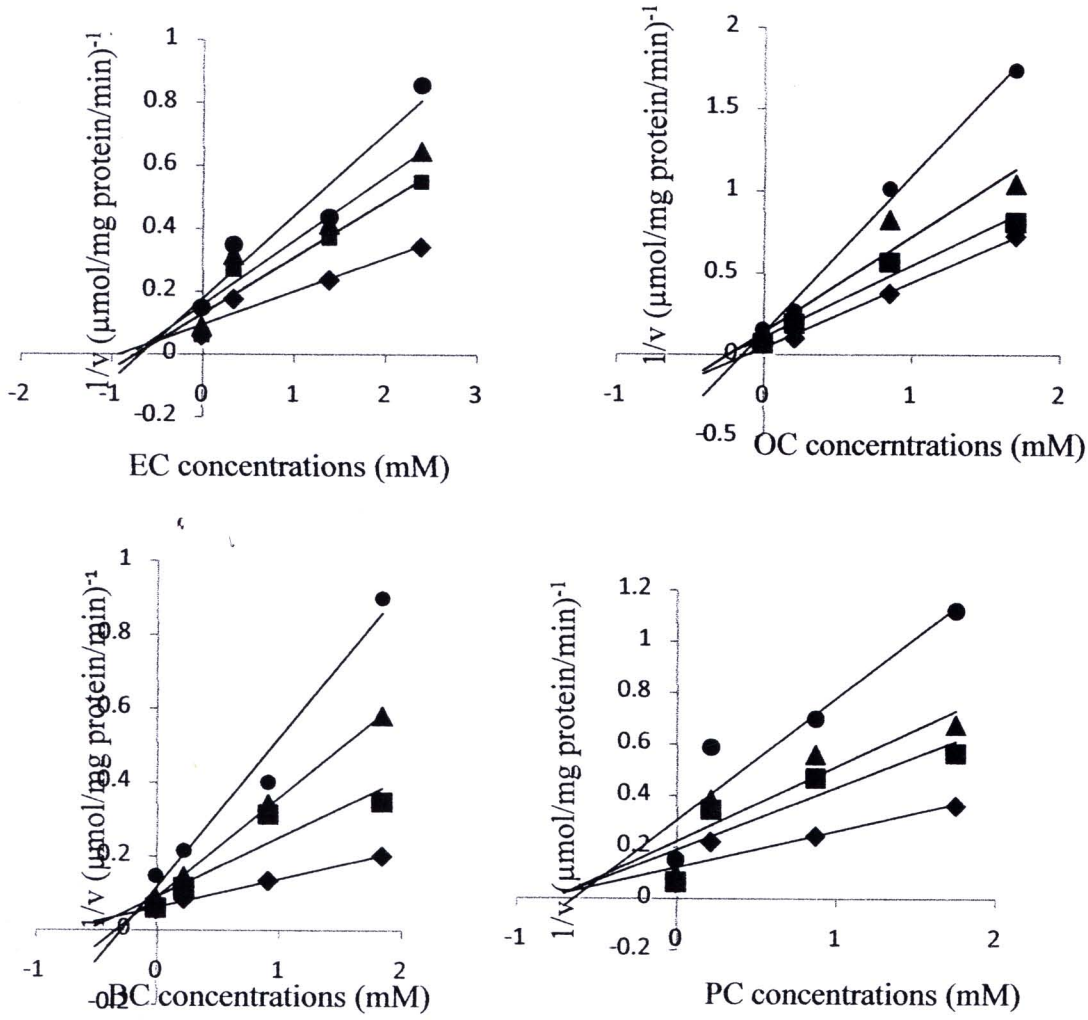

Fig. (6). Dixon plot between 1/v and caffeic acid ester analogues concentrations (mM). Values are average of triplicate determinations. ◆= 1.4 mM, ■= 0.84 mM, ▲=0.70 mM and ●= 0.50 mM of phenacetin concentrations.

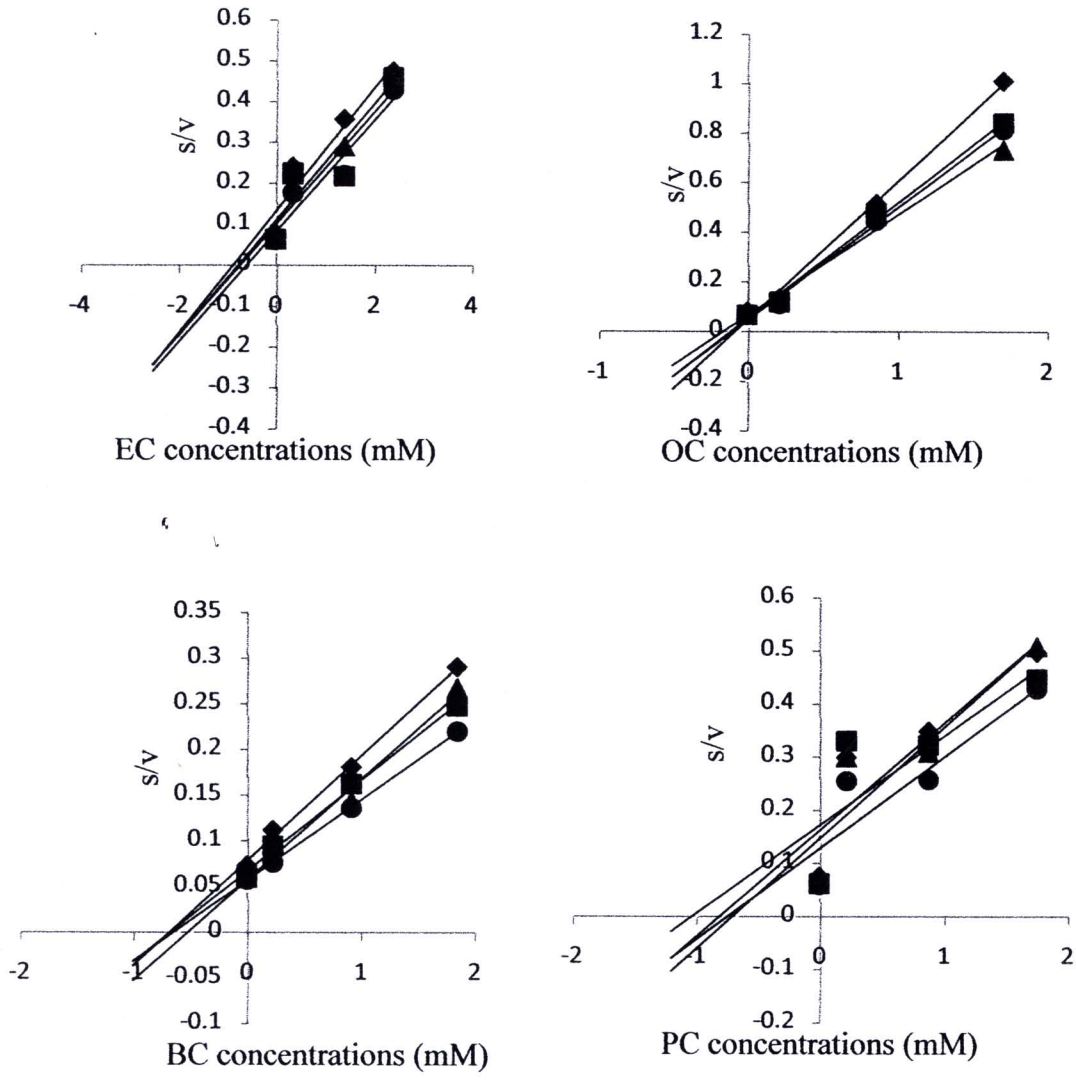

Fig. (7). Cornish-Bowden plot between s/v and caffeic acid ester analogues concentrations (mM). Values are average of triplicate determinations. ◆= 1.4 mM, ■= 0.84 mM, ▲=0.70 mM and ●= 0.50 mM of phenacetin concentrations.

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Submitted manuscript ส่งไปเพื่อพิจารณาลงตีพิมพ์ ในวารสาร Food Chemical Toxicology (impact factor 2.114; 2009)

Inhibition of Procarcinogen activating enzyme CYP1A2 Activity and Free Radical Scavenging of Caffeic acid and its amide analogues

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Key words: caffeic acid, caffeic acid amide analogues, free radical scavenging, CYP1A2, procarcinogen activating enzyme

Abstract

Caffeic acid (CAF) and its amide analogues, ethyl 1-(3',4'-dihydroxyphenyl)propen amide (EDPA), octyl 1-(3',4'-dihydroxyphenyl) propen amide (ODPA) , phenmethyl1-(3',4'-dihydroxyphenyl) propen amide (PMDPA) and phenethyl 1-(3',4'-dihydroxyphenyl) propen amide (PEDPA) were investigated for the inhibition of procarcinogen activating enzyme (CYP1A2), nitric oxide, superoxide anion, DPPH radical and hydroxyl radical scavenging activities. It was found that they inhibited CYP1A2 enzyme by uncompetitive inhibition. Apparent Ki values of CAF, EDPA, ODPA, PMDPA, PEDPA were 1.34, 0.68, 0.55, 1.77 and 0.53 nM, respectively. Moreover, they potentially scavenged nitric oxide radical with IC 50 values of 19.15, 45.77, 148.97, 59.12 and 79.11 μg/ ml, respectively. The IC50 values of superoxide anion scavenging were 36.47, 44.56, 728.27, 586.14, and 124.34 μg/ ml, respectively. Ability of them to DPPH radical scavenging shown IC50 values were 74.49, 61.32, 245.11, 241.62 and 878.12 μg/ml and hydroxyl radical scavenging *in vitro* model shown IC 50 values were 4.18, 4.36, 4.60, 4.63 and 4.84 ng/ml, respectively. Overall, caffeic acid and its amide analogues are an in vitro inhibitor of human CYP1A2 catalytic activity and free radical generation. They may useful to develop to potential cancer chemopreventive agent that block CYP1A2-mediated chemical carcinogenesis.

1. Introduction

Cytochrome P450 (CYP) is important phase I enzymes in the biotransformation of xenobiotics and their subsequent clearance from the body. Among of the different CYP isoform, cytochrome P450 1A2 (CYP1A2) is constitutively expressed in human liver where it is capable of metabolizing and activating numerous promutagens. Many carcinogens and mutagens are activated by CYPs (Eaton et al., 1995; Guengerich and Shimada, 1991). CYP1A2 is an important enzyme in metabolism of xenobiotics and procarcinogens such as polycyclic aromatic hydrocarbon, nitrosamine, and aromatic and heterocyclic amines (Konstandi et al., 2006; Pavanello et al., 2007; Skupinska et al., 2009). Increasing of CYP1A2 activity can activate procarcinogen metabolism to reactive metabolite and therefore, elevate the risk of carcinogens development (Skupinska et al., 2009). High CYP1A2 activity has been suggested to be a susceptibility factor for lung, bladder and colon cancer where exposure to such compounds has been implicated in the a etiology of the disease (Pavanello et al., 2007). Moreover, The CYPs are also potentially a significant source of reactive oxygen species. Both superoxide anions and hydrogen peroxide can be formed during the metabolism of compounds by CYPs (Karuzina and Archakov, 1994; Puntarulo and Cederbaum, 1998; Stupans et al., 2002). Various natural and synthetic compounds are capable of reducing CYP1-mediated bioactivation of PAHs and thus may protect against carcinogenic potential of these chemicals (Sparfel et al., 2004). CYP1A2 was proposed as a target for cancer chemoprevention (Piton et al., 2005). Therefore, it is important to identify inhibitors of CYP1A2 that could be used to block the initiation stage of chemical carcinogenesis(Androutsopoulos et al., 2010).

Caffeic acid (3, 4-dihydroxycinnamic acid, CAF) and its analogues are found in many agricultural products such as fruits, vegetables, wine, olive oil and coffee. They are usually found as various simple derivatives including amides, esters, sugar esters, and glycosides, and flavonoid- linked derivatives. The CAF and its analogues have been reported to possess wide spectrum biological effects such as antibacterial, antiviral, anti-inflammatory, antiatherosclerotic, antioxidative, antiproliferative, immunostimulatory, neuroprotective properties(Son and Lewis, 2002) and induce phase 2 enzyme such as heme oxygenase-1 activity(Jaikang and Chaiyasut, 2010). There is widespread agreement that compounds especially from natural sources capable of protecting against ROS mediated damage may have potential applications as antioxidants in prevention and/or curing of diseases. However, synthetic antioxidants are widely used because they are effective and cheaper than natural types (Pinho et al., 2000). There are many researches to study of synthesized of CAF derivatives about antioxidant properties. However, the information of CAF on CYP1A2 catalytic activity is less to observe. Then, we evaluated the effects of caffeic acid and its analogues on CYP1A2 activity *in vitro* using phenacetin as a substrate and pooled human liver microsome as a source of CYP1A2 and studied free radical scavenging property for preventing cancer.

2. Materials and methods

2.1 .Chemicals and apparatus

Caffeic acid amide analogues were obtained from Health product research Unit Faculty of Pharmacy Chiang Mai University, 1,1-diphenyl-2-picrylhydrazyl,(DPPH), nicotinamide adenine dinucleotide (NADH), Nitroblue tetrazolium(NBT), phenazine

methosulfate (PMS) ,dicyclohexyl carbodiimide (DCC) tetrahydrofuran, DMAP, caffeic acid, thiobarbutric acid (TBA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, nitro blue tetrazolium (NBT) and phenazonium methosulphate (PMS) were purchased from Sigma and Aldrich (St. Louis, MO). Other chemicals and solvents were purchased from Merck chemicals. Potassium ferricyanide, ferrous chloride, ferric chlorid, methanol and trichloroacetic acid (TCA) were obtained from E. Merck (Darmstadt. Germany). butylated hydroxytoluene (BHT), and butylated hydroxyanisol (BHA) were obtained from Sigma Chemical Co.(Sigma–Aldrich GmbH. Sternheim. Germany). All other chemicals and solvents are of analytical grade.

2.2. Preparation of Human microsome

Human liver samples (n=9) were obtained from subjects who died after traffic accident. In accordance with Thai law, Ethical Committee for Human Research was approved from Faculty of Medicine, Chiang Mai University through the Department of Forensic Medicine (protocol No. 06OCT131501). Liver microsomes were prepared by different centrifugation using a previously described method (Kusirisin et al., 2009). The microsome pellets were kept in Tris buffer pH 7.4 and stored at -70 °C until use. Protein contents were determined by bicinchonic acid (BCA) protein assay kit using bovine serum albumin as a substrate.

2.3. Determination of Human CYP1A2 activity

Phenacetin *O*-deethylation was used to assess CYP1A2 activity. Incubation mixture of 1 ml contained 0.1 mg microsomal protein, 100 mM potassium phosphate pH 7.4, 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 (0.2-2.8 mM) and caffeic acid amide analogues (0.025-0.25 µg/ml). Reaction was stopped after 60 mins by the addition of 200 µl cooled methanol and 50 µl of caffeine 100 µg/ ml was added as an internal standard. The mixtures were centrifuged at 13,000 RPM for 5 mins and the supernatant was filtrated and injected into the analytical high performance liquid chromatography (HPLC). HPLC systems consisted of an Agilent 1100 Series high-performance liquid chromatography, Agilent 1100 series diode- array detector, HPLC pumps, autosampler, column oven, and diode- array which the system were monitored and controlled by the HP chem. station computer program (Agilent). Wavelengths used for the identification of with the diode array detector at 253 nm. Acetaminophen was separated by water C18 column (250×4 mm, 5 µm), column oven was set up at 25°C. Mobile phase consisting of water: methanol: acetronitrile: tetrahydrofuran (55:20:20:5), with flow rate 0.7 ml/ min and the injection volume was 5 µl.

2.4. Kinetic analysis of phenacetin O-deethylation in human liver microsome

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

2.5. Nitric oxide radical scavenging activity

Nitric oxide scavenging activity was measured following the method described by (Govindarajan et al., 2004). Briefly, the mixture contained 10 mM sodium nitroprusside in phosphate buffer and CAF and its analogues of different concentration were incubated at 25

°C for 150 min. Then 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequence coupling with napthylethylene diamine was measured at 546 nm.

2.6. Superoxide radical scavenging activity

Measurment of superoxide anion scavenging activity of CA and its analogues was measured following (Gulcin, 2006). Superoxide radicals are generated in PMS-NADH system by oxidation of NADH and assayed by reduction of NBT. The superoxide radicals were generated in 3 ml of Tris-HCl buffer (16mM, pH 8.0) containing 1 ml of NBT (50μM) solution, 1 ml NADH (78 μM) solution and various concentration of CAF and its derivatives 100 μl. The reaction was started by adding 1 ml of PMS solution (10μ M) to the mixture. The reaction mixture was incubated at 25 °C for 15 min and absorbance was measured at 560 nm. The inhibition percentage of superoxide anion generation was calculated.

2.7. Scavenging of DPPH radical

The stable DPPH was used for determination of free radical scavenging activity of CAF and its derivatives following the method of *(Banerjee et al., 2005).* Aqueous dilution of CA and its analogues was added to 3 ml of 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 mins, and the percent of inhibition was calculated.

2.8. Hydroxyl radical scavenging activity

The assay was based on the competition between deoxyribose and test compounds for hydroxyl radical generated by Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) according to the method of (Halliwell et al., 1987). One millilitre of the final reaction solution consisted of aliquots of various concentrations of CAF and its analogues, 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 future heated in a boiling water bath for 15 mins after addition of 1 ml of 2.8% (w/v) trichloroacetic acid and 1 ml of 1% (w/v) 2-thiobarbituric acid. The color development was measured at 532 nm.

2.9. Statistical analysis

Data was expressed as mean ± S.D. of five values and analysed by one way ANOVA followed by Duncan. *P* values of less than 0.05 were considered statically significant.

3. Results

Caffeic acid amide analogues in this study were ethyl 1-(3', 4'-dihydroxyphenyl) propenamide (EDPA), octyl 1-(3', 4'-dihydroxyphenyl) propenamide (ODPA), phenmethyl 1-(3', 4'-dihydroxyphenyl) propenamide (PMDPA) and phenethly 1-(3', 4'-dihydroxyphenyl) propenamide (PEDPA) and all structures shown in Fig. 1.

3.1. The effects of caffeic acid and it amide derivatives on phenacetin O-deethylation in human liver microsome

The mean values of percent inhibition of 150 μg/ ml of CAF, EDPA, ODPA, PMDPA and PEDPA when using 1.4 μM of phenacetin were 45.38, 96.65, 81.92, 93.74 and 90.60 respectively. Among the CAF and its derivatives studied, EDPA and PMDPA were

more strongly inhibited phenacetin *O*-deethylation in a concentration-dependent manner. Mean IC50 values of caffeic acid and its amide analogues were shown in Table 1.

According to characterization of the inhibition kinetic of CYP1A2, which was obtained from Cornish-Bowden (Fig.2) and Dixon plots (Fig.3), all plots indicated that CAF and its amide analogues inhibited phenacetin *O*-deethylation by uncompetitive inhibition. The Ki values of caffeic acid and its analogues were obtained from liner regression. EDPA had the lowest Ki value (0.124 μM), whereas Ki value of PEDPA, CAF, ODPA, and PMDPA were 0.143, 1.148, 0.25 and 0.439 μM respectively.

3.2. Nitric oxide scavenging activity

Nitric oxide (NO) is an important chemical generated by maccropharge, endothelial cells, and neurons and is involved in the regulation of various physiological processes. Excess concentration of NO is associated with several diseases. Moreover, NO can react with superoxide to from the peroxynitrite anion, which is a potential strong oxidant that can decompose to hydroxyl radical and nitrus oxide (Awah et al., 2010). Effect of caffeic acid and its amide derivatives were used to inhibit NO released from sodium nitroprusside as shown in Fig. 4. IC50 scavenging of nitric oxide of CAF, EDPA, ODPA, PMDPA, PEDPA were 19.15, 45.77, 148.97, 59.12 and 79.11 μg/ml, respectively.

3.3. Superoxide radical scavenging activity

The superoxide radical is a highly toxic radical which generated by biological and photochemical reactions. In the PMS-NADH-NBT system, superoxide anions were derived from dissolved oxygen by the PMS-NADH coupling reaction and could reduce to NBT (Hseu et al., 2008). The percentage inhibition of superoxide radical generation by caffeic

acid and its amide derivatives compared with ascorbic acid and α-tocopherol was shown in Fig. 5. Our results indicated that caffeic acid and its amide derivatives scavenge superoxide radical, which was decreased in the order, CAF>EDPA>PEDPA>PMDPA>ODPA with IC 50 value 36.47, 44.56, 124.34, 586.14 and 728.27μg/ml respectively.

3.4. Scavenging of DPPH radical

DPPH is a stable nitrogen free radical compound that has been used to determine the free radical-scavenging ability of samples (Su et al., 2008). DPPH colour decreased significantly when exposing hydrogen atom or electron. The DPPH free radical-scavenging activities of caffeic acid, amide derivatives and standards were presented in Fig. 6. The CAF and its amide derivatives showed a variation of IC50 ranging from 61.32 to 878.12 μg/ml. The EDPA was found the most powerful DPPH radical scavenging as evidenced by low IC50 values. Their scavenging activity of DPPH radicals decreased in the following order: EDPA> CAF > PMDPA> ODPA > PEDPA with IC 50 value of 61.32, 74.49, 241.62, 245.11, and 878.12 μg/ml, respectively.

3.5. Hydroxyl radical scavenging activity

Hydroxyl radicals are the most reactive and can induce severe damage to the biomolecules. Competition between deoxyribose and the sample against hydroxyl radical generated from the Fe^{3+}/ ascorbate/ EDTA/ H_2O_2 system was measured for determination of hydroxyl radical scavenging activity. Fig.7. shown the ability of caffeic acid and its amide derivatives at different concentrations to scavenge hydroxyl radical compared with ascorbic acid and α-tocopherol as standards. The IC 50 shown that CAF was the highest hydroxyl

radical inhibition (4.18 ng/ ml). Whereas, IC50 of EDPA, ODPA, PMDPA and PEDPA were 4.36, 4.60, 4.63 and 4.84 ng/ ml, respectively.

4. Discussion

Caffeic acid and its analogues are widely used as antioxidants. They have been reported to exert a variety of beneficial and harmful effects. This study, we added to the beneficial data by evaluating CAF and its amide analogues for their ability to inhibit activity of procarcinogen activating enzyme (CYP1A2) activity in pooled human liver microsome and free radical scavenging properties. The modulation of CYPs would be important from a clinical point of view, as these enzymes can activate or inactivate xenobiotics, including therapeutic agents (Yoo et al., 2007). Many carcinogens and mutagens are activated by CYPs (Eaton et al., 1995; Guengerich and Shimada, 1991). The CYP1A2 is a primary metabolism of various foods, drugs such as acetaminophen, theophylline, quinolone, mexiletine (Abd El-Aty et al., 2008). CYP1A2 is carcinogen activating enzymes and also can produce free radicals(Kim et al., 2008). Ryu and Chung, (2003) showed that the long term consume of dietary supplement, gingo biloba can induce CYP1A2 activity levels which may increase the incidence of colorectal cancers caused by procarcinogens activated by CYP1A2. Previous studied have shown that hydrocinnamic acid such as chlorogenic acid, caffeic acid and ferrulic acid inhibited CYP1A2 when using methoxyresorufin *O*-demethylation and hamsters liver microsome (Teel and Huynh, 1998). In this study, we demonstrated that CAF and its amide analogues significantly inhibited CYP1A2 enzyme in human liver microsome with low micromolar K_i values. The type of mechanism inhibition of CYP1A2 was incompetitive inhibition. The results suggested that CAF and its analogues are bind with enzyme substrate complexes which do not change to products. Moreover, CAF and its amide analogues inactivation of CYP1A2 was time- and concentration-

dependent and required NADPH. CYP1A2 enzyme is composed of hydrophobic and aromatic amino acids with polar amino acids for hydrogen bonding being present near the heme centre (Korhonen et al., 2005) and CYP activities are related to substance lipophilicity (Lewis, 2004). It is possible that hydrogen bonding and hydrophobic interaction contribute to the inhibitory activities. The substances which have an *ortho*-or *para*- hydroxyl group can potent inhibit CYP1A2 (Appiah-Opong et al., 2008). The CAF and its amide analogues consisted two hydroxyl groups in *meta* and *para* position and benzene ring especially PMDPA and PEDPA which have two benzene rings. Then, they have high power to inhibit the CYP1A2 activities than CAF that is parent compound. The ODPA has long carbon side chain which cause more lipophilicity than CAF and EDPA. Therefore, the ODPA is more potent CYP 1A2 inhibitor than CAF and EDPA.

Several chemopreventive agents are known as mechanism-based inactivators of CYPs (Chun et al., 2001). CAF and its analogues inhibited CYP1A2 activity which may reduce of procarcinogenic substrate metabolism. Therefore, its modulation can dramatically decrease the compound's toxicity and carcinogenesis. Kuenzig et al., (1984) showed CAF and ferrulic acid could react with nitrite in vitro and blocked the elevation of serum *N*-nitrosodiumthymine levels in rats which play a role in the body's defence against carcinogenesis.

Oxygen activation by CYP can also result in the production of reactive oxygen species (ROS). Superoxide anion radical can be produced from decomposition of the oxygenated CYP complex, while hydrogen peroxide can be fromed either from the dismutation of superoxide anion or the decomposition of the peroxy CYP complex. Free radicals are produced by endogenous and exogenous chemicals metabolic processes in

human cells. Oxidative stress, the result of increasing oxidative metabolism, produces ROS such as hydroxyl radical (OH•), superoxide anion radical (O_2•) and hydrogen peroxide (H_2O_2), which can damage various substrates including lipid, proteins and nucleic acid and lead to cancer, cardiovascular disease, and related macular degeneration and to aging. Human body has multiple mechanisms especially enzymatic and non-enzymatic antioxidant systems to protect the cellular molecules against ROS induced damage (Bebianno et al., 2005; Ognjanovic et al., 2010; Sun, 1990). However the innate defense may not be enough for severe or continued oxidative stress. Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS in human body. Antioxidants are vital substances which possess the ability to protect the body from damage caused by free radical induced oxidative stress (Ozsoy et al., 2008). There is an increasing interest in natural antioxidants, e.g. polyphenols, present in medicinal and dietary plants, which might help prevent oxidative damage (Lee et al., 2003; Pacifico et al., 2009; Shaker et al., 2010).

There is widespread agreement that compounds, especially from natural sources capable of protecting against ROS mediated damage, may have potential applications as antioxidants in prevention and/or curing of diseases. However, synthetic antioxidants are widely used because they are effective and cheaper than natural types. This experiment shows that amide analogues of CAF have more potential to scavenge free radicals than CAF due to amide analogues contain three hydrogens that can donate hydrogen to reactive oxygen species but caffeic acid are two hydrogens donating (Son and Lewis, 2002). Then, CAF and amide analogues potentially inhibited procarcinogen activating enzyme and free radical scavenging that could be apply for primary cancer chemoprevention. In the future, toxicity test and side effects should be evaluated.

5. Conclusion

Our results clearly show that the caffeic acid and its amide analogues have high potential to inhibit CYP1A2 using phenacetin as a substrate and human liver microsome as a source of CYP1A2 enzyme. The type of inhibition of CAF and its amide analogues are uncompetitive inhibition. Free radical such as nitric oxide, super oxide radical, hydroxyl radical and DPPH radical were evaluated for the scavenging properties. The CAF and its amide analogues exhibit strong scavenging free radicals properties. From these evidents, the CAF and its amides analogues may use as chemopreventing agents.

6. Conflict of interest

The authors declare that there are no conflicts of interest.

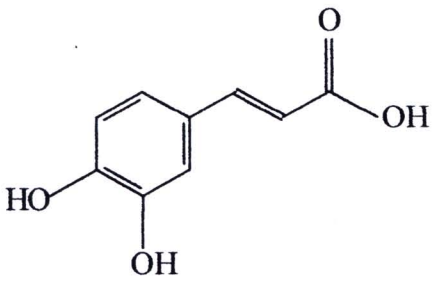
Acknowledge ment

This work was supported by grants from the Thailand Research Fund (DBG5080008), the Graduate School and Faculty of Pharmacy, Chiang Mai University Thailand. We also thank Toxicology section, Chiang Mai regional Medical Science centre for HPLC instruments and chemicals.

Table. 1 Kinetic parameters of phenacetin O-deethylation.

Substances	*K_{max}^{app}	*V_{max}^{app} (μg/ml/mg protein/h)$^{-1}$	Ki (nM)	IC_{50} (μg/ml)	Type of inhibition
CAF	2.37	2.37	1.34	0.282	Noncompetitive inhibition
EDPA	1.59	20.57	0.68	0.318	Noncompetitive inhibition
ODPA	1.29	31.15	0.55	0.297	Noncompetitive inhibition
PMDPA	3.09	19.57	1.77	0.394	Noncompetitive inhibition
PEDPA	0.41	19.23	0.53	0.253	Noncompetitive inhibition

*Calculation of K_{max}^{app} and V_{max}^{app} used 1.4 μM phenacetin



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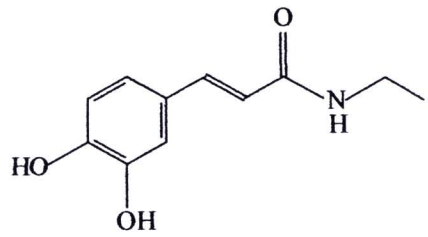

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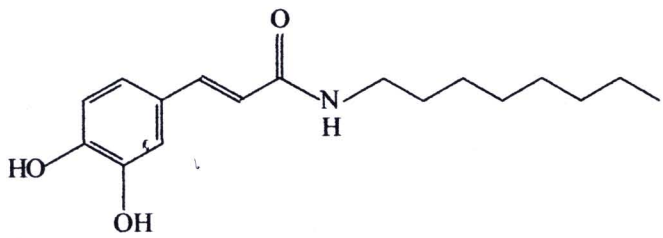

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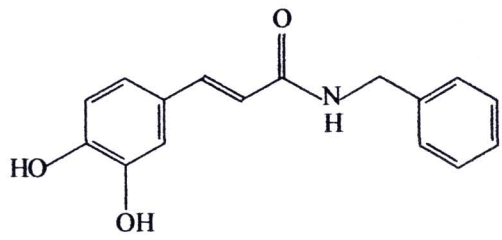

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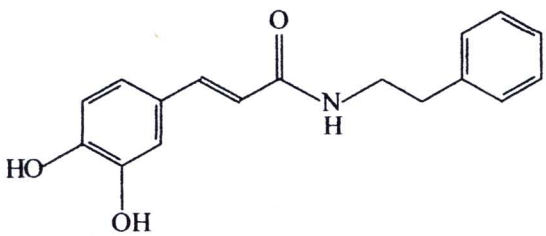

PEDPA

Figure 1. The structure of caffeic acid and its amide analogues.

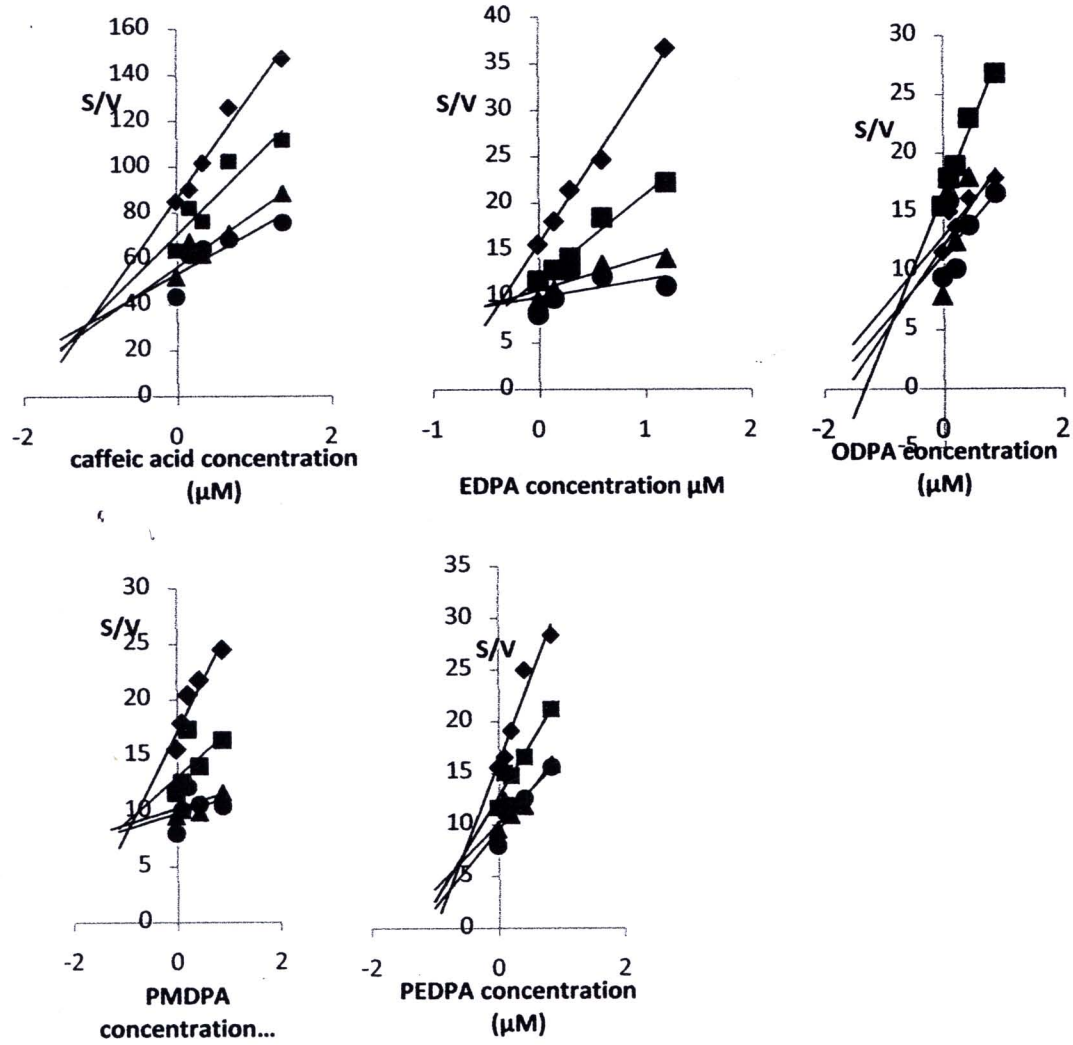

Figure 2 Cornish- Bowden plot of CAF and its derivatives on phenacetin *O*-deethylation when phenacetin concentration were◆ 2.8 mM, ■ 1.4 mM, ▲0.72 mM, ●0.56 mM.

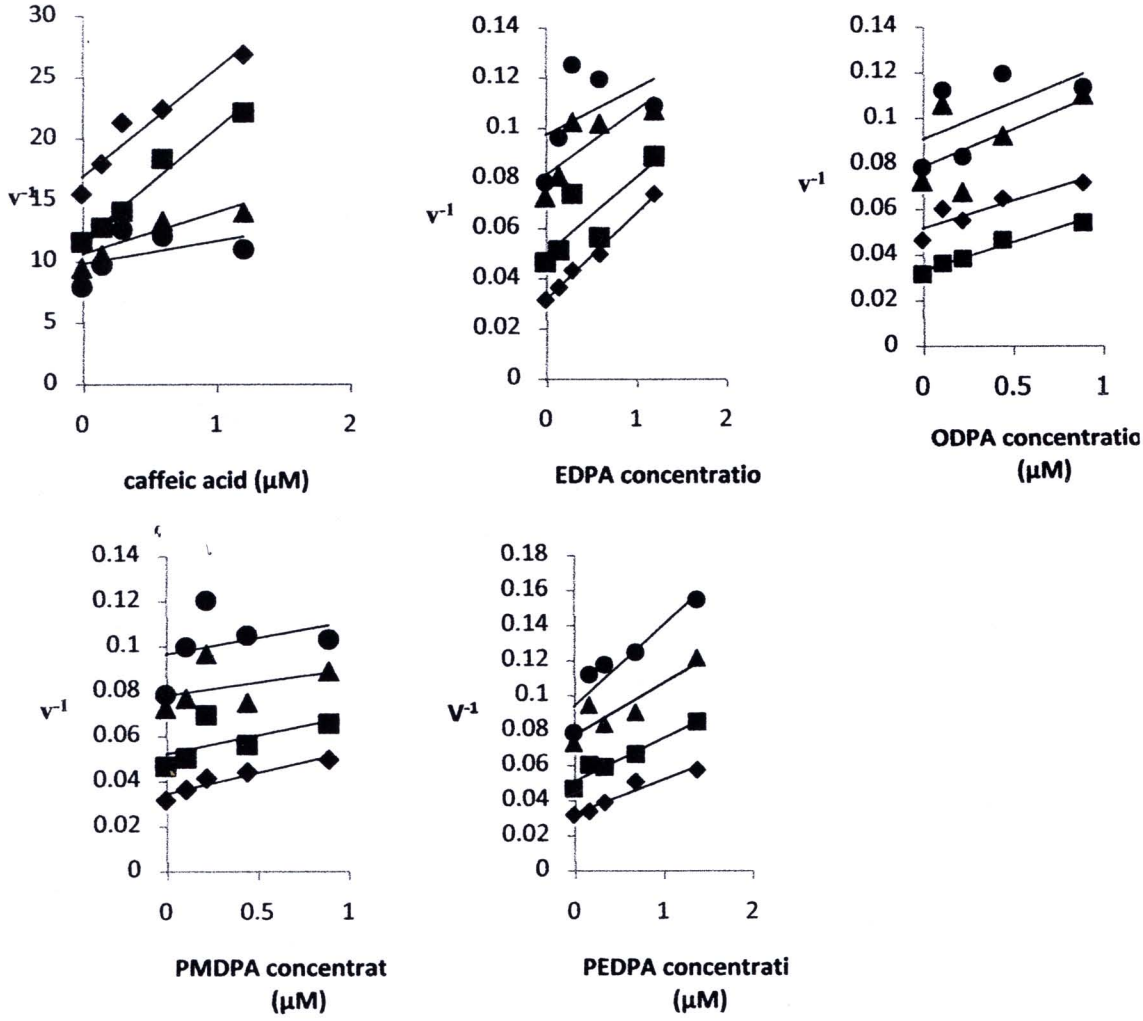

Figure. 3 Dixon plot of caffeic acid and its derivatives on phenacetin *O*-deethylation when phenacetin concentration were◆ 2.8 mM, ■ 1.4 mM, ▲0.72 mM, ●0.56 mM.

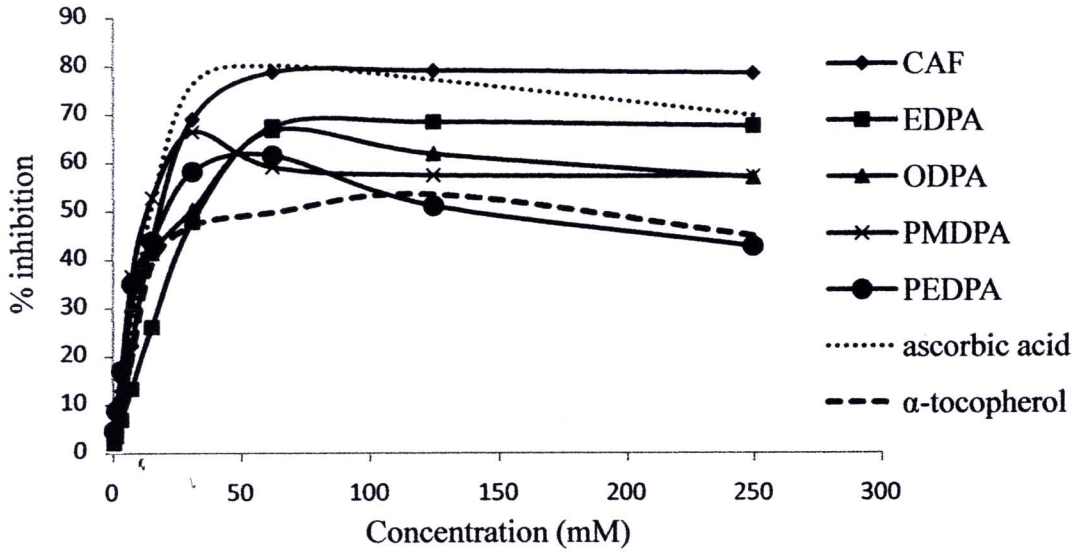

Figure. 4 Percent inhibition of nitric oxide of CAF and its amide analogues.

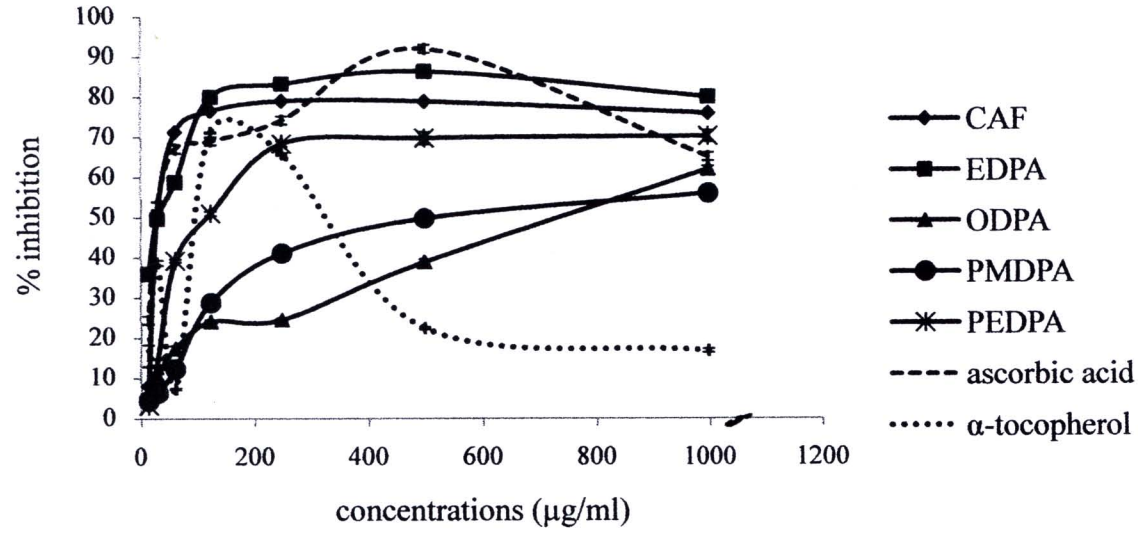

Figure.5 Superoxide radical scavenging activities of CAF and its amide analogues.

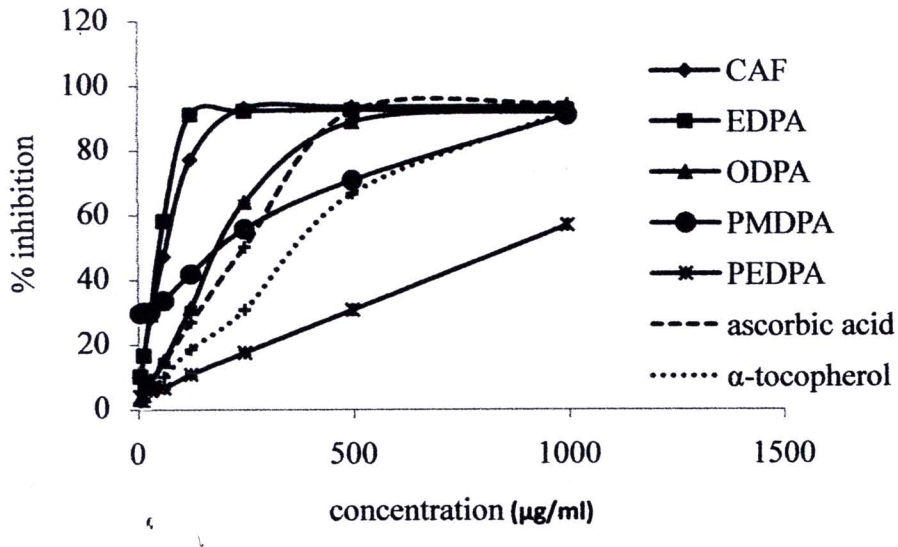

Figure. 6 Percent inhibition of DPPH radical by CAF and its amide analogues.

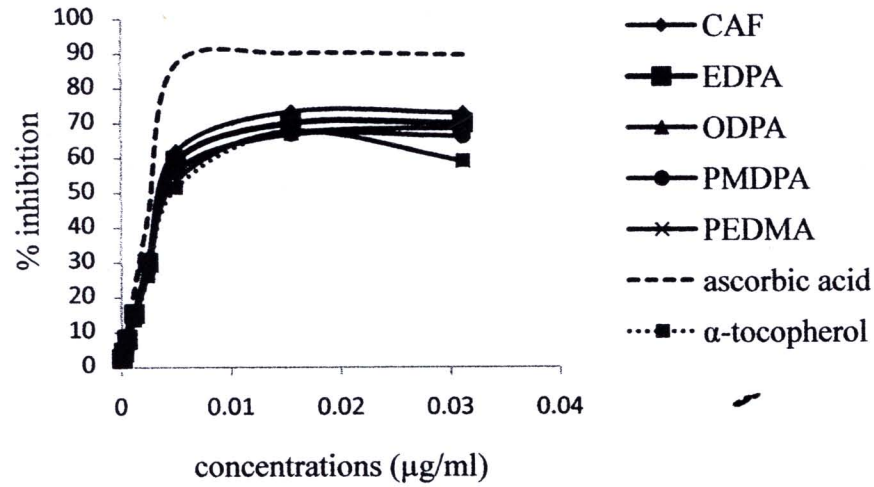

Figure.7 Hydroxyl radical scavenging activities of CAF and its amide analogues.

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รายละเอียดงานวิจัยที่ส่งเข้าร่วมเสนอผลงานในงาน International Society for the Study of Xenobiotixs. 3nd Asian Pacific Regional Meeting Understanding Xenobiotics for better Drug Development and therapy. 10-12 May 2009 Bangkok Thailand.

Inhibitory Effects of Caffeic Acid and Its Amides Derivatives on Human Cytochrome P450 1A2

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Abstract

Caffeic acid (CAF) and its analogues are found in fruits, vegetables and coffee bean. Caffeic amide analogues possess antioxidant activity and free radical scavenging potential. The effects of caffeic acid and its amide derivatives on CYP1A2 activity in human liver microsomes using phenacetin as a probe substrate were examined. Ethyl 1-(3',4'-dihydroxyphenyl)propen amide (EDPA), octyl 1-(3',4'-dihydroxyphenyl) propen amide (ODPA) , phenmethyl1-(3',4'-dihydroxyphenyl) propen amide (PMDPA)and phenethyl 1-(3',4'-dihydroxyphenyl) propen amide (PEDPA) were synthesized by using PyBOP as a coupling agent. The formation of the CYP-specific metabolite following co-incubation with CAF or its amide derivatives were determined to establish Ki values for this enzyme. The inhibition of CAF and its amide derivatives (EDPA, ODPA, PMDPA, PEDPA) on CYP1A2 according to Dixon and Cornish-Bowden plots were uncompetitive. The inhibitory potency CAF and its amide derivatives on phenacetin *O*-deethylation was comparable with Ki values of 1.34, 0.68, 0.55, 1.77 and 0.53 nM, respectively. The results indicate that CAF and its amide derivatives exhibit uncompetitive mode of

inhibition on CYP1A2 activity in human liver microsome. This may lead to important implication in prevention of CYP1A2-mediated chemical carcinogenesis.

Key words: CYP1A2, Caffeic acid, Caffeic acid amide derivatives, human liver microsome

Introduction

More than 90% of all cancers are related to environmental factor. The majority of carcinogenic chemicals do not produce their biological effects but require metabolic activation before interact with cellular macromolecule. Cytochrome P450 (CYP) is a superfamily of haemoprotein enzymes are carcinogen activating enzymes and produce free radicals. CYP 1A2 is constitutively expressed in human liver where it is capable of metabolizing and activating numerous promutagens and procarcinogens such as 2- acetylaminofluorine, acetanilide, aflatoxin B1, 4-aminobiphenyl and other arylamines. Epidermiolocal studies suggest that consumption of fruits and vegetable can prevent cancer because many phytochemicals present in diets may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation and scavenge free radicals.

Caffeic acid (3, 4-dihydroxycinnamic acid, CAF) and its analogues are found in many agricultural products such as fruits, vegetables, wine, olive oil and coffee. They are usually found as various simple derivatives including amides, esters, sugar esters, and glycosides, and flavonoid- linked derivatives. The CAF and its analogues have been reported to possess wide spectrum biological effects such as antibacterial, antiviral, anti-inflammatory, antiatherosclerotic, antioxidative, antiproliferative, immunostimulatory and neuroprotective properties. In this study, we evaluated the effects of caffeic acid and its analogues on CYP1A2 activity *in vitro* using phenacetin as a substrate and pooled human liver microsome as a source of CYP1A2.

Methods

Synthesis of caffeic acid analogues

Ethyl 1-(3',4'-dihydroxyphenyl)propenamide (EDPA), octyl 1-(3',4'-dihydroxyphenyl) propenamide (ODPA) , phenmethyl1-(3',4'-dihydroxyphenyl) propenamide (PMDPA)and phenethyl 1-(3',4'-dihydroxyphenyl) propenamide (PEDPA) were synthesized by using PyBOP as a coupling agent. The residue was purified using column chromatography and the structure was confirmed by FT-IR, H^1-NMR, C^{13}-NMR, GC-MS and spectrophotometer.

Preparation of human liver microsome

Human liver samples were obtained from Department of Forensic Medicine, Faculty of Medicine Chiang Mai University. Liver microsomes were prepared by differential centrifugation using previously described method (Potter *et.al*, 1973). The microsomal pellets were kept in Tris buffer pH 7.4 and stored at -70°C until use. Protein contents were determined by bicinchonic acid (BCA) protein assay kit using bovine serum albumin as a standard.

Determination of Human 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 analogues (0.025-0.25 μg/ml). Reaction was stopped after 60 min by the addition of 200μl cooled methanol and 50 μl of caffeine100μg/ml was added as an internal standard. The mixtures were 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 chem. station computer program (Agilent). Wavelengths used for the identification of with the diode array detector at 253 nm. Acetaminophen was

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 water: methanol: acetronitrile:tetrahydrofuran(55:20:20:5), with flow rate 0.7 ml/ min and the injection volume was 5 µl.

Data analysis

All incubation for enzyme activity assays were in triplicate. The results of the inhibition study were analyzed by Dixon and Cornish- Bowden plots to determine the type of inhibition and the value of the inhibitory constant (Ki)

Results

This study was designed to evaluate selected synthesized caffeic acid analogues for their ability to modulate cytochrome P450 1A2 which their structures are shown in figure 1. Characterize of inhibition kinetic patterns of CYP1A2, Cornish-Bowden and Dixon plots were obtained by using different concentrations of substrate. All plots indicated that caffeic acid and its derivatives inhibited phenacetin O- deethylation by uncompetitive inhibition. The Ki values of caffeic acid and its analogues were shown in table 1.

Discussion

This study was designed to evaluated caffeic acid and its analogues for their ability to modulate CYP1A2 activity. In the present study, we demonstrated that caffeic acid and analogues inhibited phenacetin *O*-deethylation, probed CYP1A2 activity. All of the kinetic inhibition of CYP1A2 was uncompetitive. The majority of carcinogenic chemicals do not produce their biological effects but require metabolic activation before interact with cellular macromolecule. Cytochrome P450s (CYP) is carcinogen activating enzymes and produce free radicals. Caffeic acid and its derivatives inhibited CYP1A2 activity which may reduce of procarcinogenic substrate metabolism including aromatic amines, nitrosamines, planar polyaromatic amine and amides. This CYP1A2 isoform is involved in the metabolism of

several xenobiotics, including chemical carcinogen. Therefore, its modulation can dramatically decrease the compound's toxicity and carcinogenesis.

In conclusion, caffeic acid and its amide derivatives are uncompetitive inhibitor of CYP1A2 activity in pooled human microsome. These findings suggest that caffeic acid and its amide analogues have potential to prevent chemical carcinogenesis.

References

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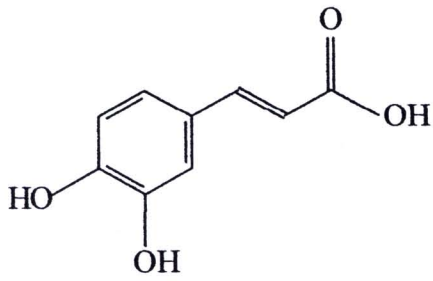
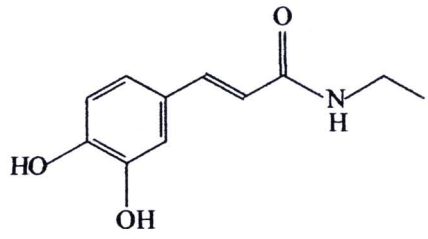
Table 1

Type of inhibition and Ki value of CAF and its derivatives

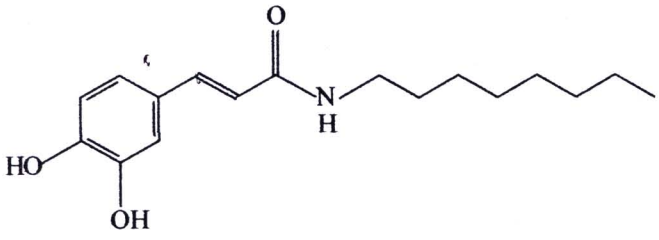
Substances	type of inhibition	Ki (nM)
CAF	uncompetitive	1.34
EDPA	uncompetitive	0.68
ODPA	uncompetitive	0.55
PMDPA	uncompetitive	1.77
PEDPA	uncompetitive	0.53



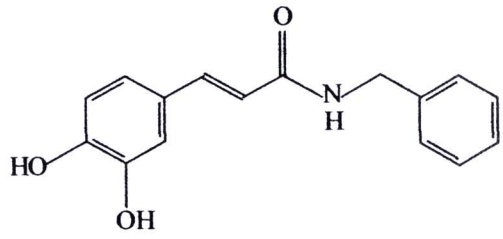
Caffeic acid



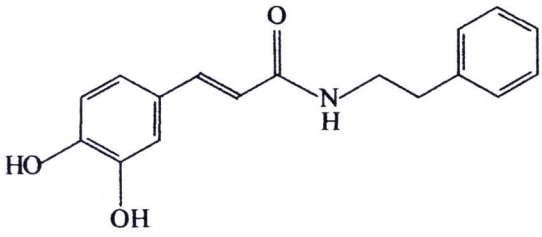
EDPA



ODPD

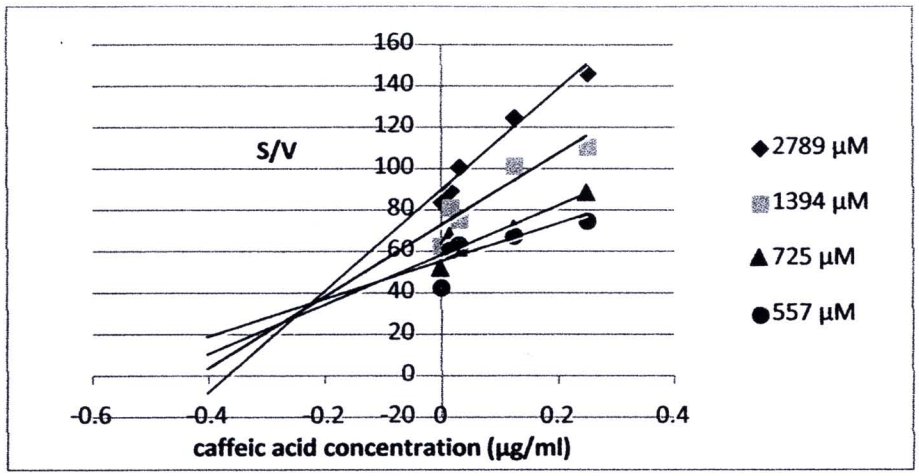


PMDPA

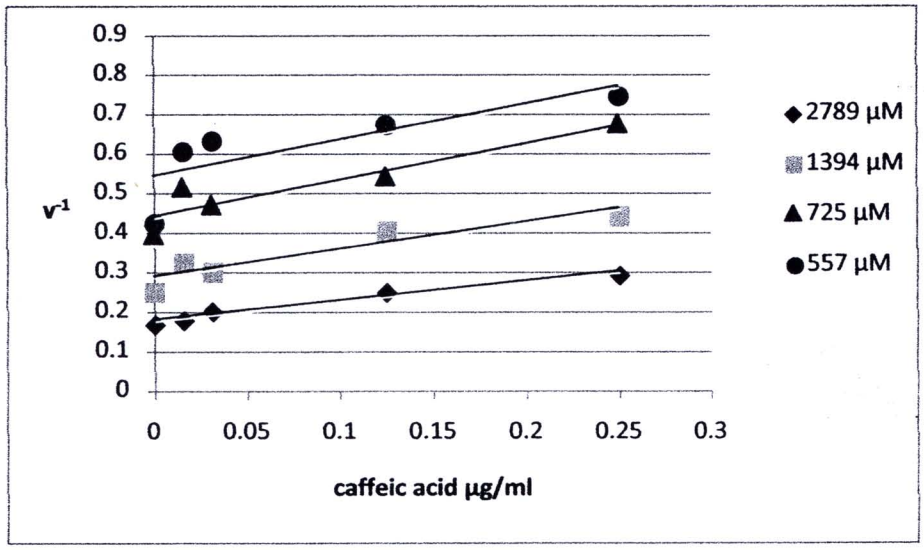


PEDPA

Figure 1. Chemical structures of caffeic and its amide derivatives.



A



B

Fig 2. The effect of caffeic acid on phenacetin *O*-deethylation in human liver microsome (A) Cornish-Bowden plot and (B) Dixon plot