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ประวัติผู้วิจัย

หัวหน้าโครงการ

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ประวัติการศึกษา Ph.D. (Applied Biochemistry)

สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ

เคมีวิเคราะห์, เคมีประยุกต์, Free radical research, Probiotics

ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ โดยระบุสถานภาพในการทำการวิจัยว่าเป็นผู้อำนวยการแผนงานวิจัย หัวหน้าโครงการวิจัย หรือผู้ร่วมวิจัยในแต่ละข้อเสนอการวิจัย

2.1 **หัวหน้าโครงการวิจัย** :

1. การวิจัยและพัฒนาผลิตภัณฑ์น้ำหมักชีวภาพที่ได้จากพืช : Research Director (สวทช.)
2. สถานภาพและความปลอดภัยของผลิตภัณฑ์น้ำหมักชีวภาพที่ได้จากพืชสำหรับการบริโภค (อย.)
3. ส่วนประกอบ คุณสมบัติทางเคมี และชีวภาพของผลิตภัณฑ์น้ำหมักชีวภาพที่ได้จากพืชที่มีผลต่อความปลอดภัยในการบริโภค (สวทช.)
4. ปัจจัยที่มีผลต่อปริมาณเมธานอลที่มีในน้ำหมักชีวภาพจากพืช (อย.)

งานวิจัยที่ทำเสร็จแล้ว :

1. การวิจัยและพัฒนาผลิตภัณฑ์น้ำหมักชีวภาพที่ได้จากพืช: Research Director (สวทช.)
2. ส่วนประกอบ คุณสมบัติทางเคมี และชีวภาพของผลิตภัณฑ์น้ำหมักชีวภาพที่ได้จากพืชที่มีผลต่อความปลอดภัยในการบริโภค (สวทช.)
3. การพัฒนาผลิตภัณฑ์หมักกรดแลคติคโดยใช้หัวเชื้อเริ่มต้นที่มีคุณสมบัติเป็นโปรไบโอติก

4. การประเมินความปลอดภัยและผลเกือบเรื้อรังในการใช้ผลิตภัณฑ์น้ำหมักชีวภาพจากพืช (สวทช.)
5. คุณสมบัติต้านออกซิเดชันของพืชและน้ำหมักชีวภาพจากพืช (สวทช.)
6. คุณสมบัติต้านออกซิเดชันของตำรับยาแผนโบราณและพืชพื้นบ้านไทย (วช)
7. ผลของน้ำหมักชีวภาพและน้ำฟังก์ชั่นนอลต่อการเพิ่มความต้านทานสภาวะเครียดจากออกซิเดชันของข้าว (สวทช)

ผู้ร่วมโครงการวิจัย

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ระดับการศึกษา

ระดับการศึกษา	สาขา/สถาบัน	ปี
ปริญญาเอก	เภสัชศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่	2548-ปัจจุบัน
ปริญญาโท	พิษวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่	2544-2547
ปริญญาตรี	เคมี คณะวิทยาศาสตร์ มหาวิทยาลัยเชียงใหม่	2540-2543
อื่นๆ	ประกาศนียบัตร อังกฤษเพื่อธุรกิจ มหาวิทยาลัยสุโขทัยธรรมธิราช	2548

โครงการที่ได้ทำ

1. โครงการ ประเมินความเสี่ยงต่อสุขภาพของเกษตรกรใน 4ภาคของประเทศไทยจากการได้รับสัมผัสสารเคมีกำจัดศัตรูพืชปี 2550. กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข ผู้ร่วมวิจัย
2. โครงการประเมินความปลอดภัยของน้ำหมักชีวภาพในหนูขาว แบบกึ่งเรื้อรัง. สวทช. ปี 2549 ผู้ร่วมวิจัย
3. โครงการ ผลของสมุนไพรไทยบางชนิดต่อเอนไซม์ที่ใช้เมตาบอลิซึมยา: ไซโตโครมพี 450 2 อี 1 แหล่งทุนคณะเภสัชศาสตร์ มหาวิทยาลัยเชียงใหม่ ปี 2549.

Publication

1. Jaikang C. and Chaiyasut C. Caffeic acid and its derivatives on heme oxygenase 1 inducer in Hep G2 cell line. J Medicinal Plants Research. (inpress)
2. Kusirisin W, **Jaikang C**, Chaiyasut C, Narongchai P. Effect of polyphenolic compounds from Solanum torvum on plasma lipid peroxidation, superoxide anion and cytochrome P450 2E1 in human liver microsomes. Med Chem. 2009 Nov;5(6):583-8
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4. Kusirisin W., **Jaikang C.**, Chaiyasut C., Narongchai P., Srichairattanakool S. Effect of ploy phenolic compounds from Solanum torvum on drug metabolizing enzymes(cytochrome P450 2E1) and lipid peroxidation in diabetic patients. 19th International Congress of Nutrition ,2009, Bangkok Thailand.

5. **Jaikang C.**, Niwatananun K., Narongchai S., Narongchai P., Chaiyasut C. Inhibitory effects of caffeic acid and its amide derivatives on human liver microsome P4501A2. The 3 rd Asian Pacific Rgional ISSX Meeting. 2009. Bangkok, Thailand.

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Full Length Research Paper

Caffeic acid and its derivatives as heme oxygenase 1 inducer in Hep G2 cell line

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Effect of 10, 50 and 100 µg/ml caffeic acid and its esters and amides derivatives on heme oxygenase 1 (HO-1) activity induction in Hep G2 cell line was studied. It was found that caffeic acid ester derivatives could induce HO-1 activity more than amide derivatives. The longer side chain and aromatic side chain affected HO-1 activity. At 10 µg/ml concentration, phenylmethyl 1-(3', 4'-dihydroxyphenyl) propenate (PMDP) had 2.1-fold more activity when compared with control. At 50 and 100 µg/ml concentrations, phenylethyl 1-(3', 4'-dihydroxyphenyl) propenate (PEDP) was the highest HO-1 inducer, whose activity was 2.1- and 2.3-fold greater, respectively, when compared with the control group. Caffeic acid and all derivatives induced HO-1 activity on a higher level than curcumin, positive control. This study implied that caffeic acid and its derivatives could be used to induce HO-1 to protect tissues against free radicals.

Key words: Caffeic acid, caffeic acid derivatives, heme oxygenase 1.

INTRODUCTION

Oxidative stress is the inappropriate exposure to reactive oxygen species (ROS), such as superoxide anion radicals and hydroxyl radicals. High levels of ROS damage cells and are involved in several human pathologies (Loguercio and Federico, 2003). Because ROS formation is a naturally occurring process, mammalian cells have developed several protective mechanisms to prevent ROS formation or to detoxify ROS. These mechanisms are called antioxidants as well as protective enzymes (Hwang et al., 2009). Cytoprotective enzymes not only provide a major mechanism by which cells combat the toxicities of ROS, but their induction is also highly effective and sufficient for protecting cells against oxidative stress.

Heme oxygenase (HO) is an enzyme found in the endoplasmic reticulum. It catalyzes the first and rate-limiting step in the oxidative degradation of free heme to produce carbon monoxide, ferrous iron, and biliverdin. This sole physiological pathway of heme degradation plays a critical role in the regulation of endothelial heme levels. Heme oxygenase-1 (HO-1) is a critical protein in response to oxidative injury. Under basal conditions, HO-1

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Abbreviations: **HO,** heme oxygenase; **HO-1,** heme oxygenase-1; **HO-2,** heme oxygenase-2; **HO-3,** heme oxygenase-3; **CAPE,** caffeic acid phenethylester; **ROS,** reactive oxygen species; **EDP,** ethyl 1-(3',4'-dihydroxyphenyl) propenate; **ODP,** octyl 1-(3',4'-dihydroxyphenyl) propenate; **PMDP,** phenylmethyl 1-(3',4'-dihydroxyphenyl)propenate; **PEDP,** phenylethyl 1-(3',4'-dihydroxyphenyl) propenate; **EDPA,** ethyl 1-(3',4'-dihydroxyphenyl) propen amide; **ODPA,** octyl 1-(3',4'-dihydroxyphenyl) propen amide; **PMDPA,** phenmethyl1-(3',4'-dihydroxyphenyl) propen amide; **PEDPA,** phenethyl 1-(3',4'-dihydroxyphenyl) propen amide; **Nrf2,** nuclear factor-erythroid-2-related factor; **ARE,** antioxidant responsive elements; **ROS,** reactive oxygen species.

is expressed at low levels in endothelial, kidney, liver, and spleen cells, and is induced by the oxidative stress produced by hyperthermia, oxidized lipoproteins, inflammatory cytokines, hypoxia, nitric oxide, and heavy metals (Martin et al., 2004). Induction of HO-1 is an important cellular protective mechanism against oxidative injury both in vitro and in vivo and has been implicated in a cytoprotective mechanism to prevent tissues from oxidative damage (Lee et al., 2007). A number of natural antioxidant compounds contained in plants have been demonstrated to be effective to induce HO-1 in Hep-G2 cells, such as curcumin, flavonoids, organosulfur compounds and isothiocyanate (Li Volti et al., 2008).

Caffeic acid (3, 4-dihydroxy cinnamic acid) and its derivatives are distributed in fruits, vegetables, wine and propolis. They are usually found as various simple derivatives including amides, ester, sugar ester and glycosides. The physiological function of caffeic acid and its derivatives are known to have antibacterial, antiviral, anti-inflammatory, antioxidative, antiproliferative, immunostimulatory and neuroprotective properties (Son and Lewis, 2002; Wu et al., 2007). Previous studies showed that caffeic acid phenethylester (CAPE), a derivative of caffeic acid and major component of bee honey, propolis, has potential to induce HO-1 in cell injury (Wang et al., 2008). However, the other derivatives of caffeic acid have not been evaluated. To study caffeic acid and its derivatives on HO-1 activity induction, we used eight caffeic acids, including amide and ester derivatives and compared their properties to induce HO-1 activity in Hep G2 cell line.

MATERIALS AND METHODS

Chemicals

Caffeic acid, caffeic acid ester and amide derivatives were obtained from the Health Product Research Unit, Faculty of Pharmacy, Chiang Mai University. Hemin, curcumin, NADPH, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, trypan blue and BSA kit were purchased from Sigma Chemical Co. (St., Louis, USA). Dimethyl sulfoxide (DMSO) and chloroform were purchased from Merck Co. (Germany). Dulbeccos Minimal Essential Medium (DMEM), trypsin and the antibiotics were from Gibco (Paisley, Scotland).

Cell line

The Hep G2 cells were obtained from Dr. Suchart Kothan, Faculty of Associated Medical Science, Chiang Mai University. The cells were cultured in DMEM supplemented with 10% FBS, and penicillin (100 units/ml)–streptomycin (100 mg/ml) at 37°C in a humidified 5% CO_2 atmosphere. Medium was changed every 2 days and subculture of cells was performed using Trypsin/EDTA. Cell counts were performed using trypan blue and a Fuchs-Rosenthal haemocytometer.

Cell culture and treatments

The Hep G2 cells were plated at a density of 1×10^6 cells/well into 6 well culture plates. After overnight growth, the cells were treated with caffeic acid and its derivatives (10, 50 and 100 µg/ml final concentrations) dissolved in DMSO (2% final volume) for 48 h. The treatments were trypsinized and washed with phosphate buffer saline pH 7.4. The cells were lysed by lyses buffer and centrifuged at 3000 × *g* for 20 min. The obtained supernatant was stored at -70°C before use. Protein concentrations were measured by BSA kit assay (Sigma Chemical Co. St., Louis, USA).

Biliverdin reductase preparation

Preparation of biliverdin reductase was by the modified method (Farombi et al., 2008). Briefly, rat livers were homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose and centrifuged at 10,000*g* for 30 min at 4°C, followed by centrifugation at 100,000 g of the supernatant for 60 min at 4°C, to obtain the microsomal fraction as a pellet. The pellet was re-suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 10% glycerol and stored in -70°C before used.

Heme oxygenase 1 activity assay

HO-1 activity assay was done by the applied method of Motterlini et al. (2000). 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 redcuctase, 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.

Statistical analysis

Differences in the data among the groups were analyzed by using one-way ANOVA combined with the Bonferroni test. Values were expressed as a mean ± SD and differences between groups were considered to be significant at $p < 0.05$.

RESULTS

We used 4 amide and 4 ester derivatives of caffeic acid and grouped according to their functional groups and side chain group. Ethyl 1-(3', 4'-dihydroxyphenyl) propenate (EDP) and octyl 1-(3', 4'-dihydroxyphenyl) propenate (ODP) were caffeic acid esters that had an aliphatic side

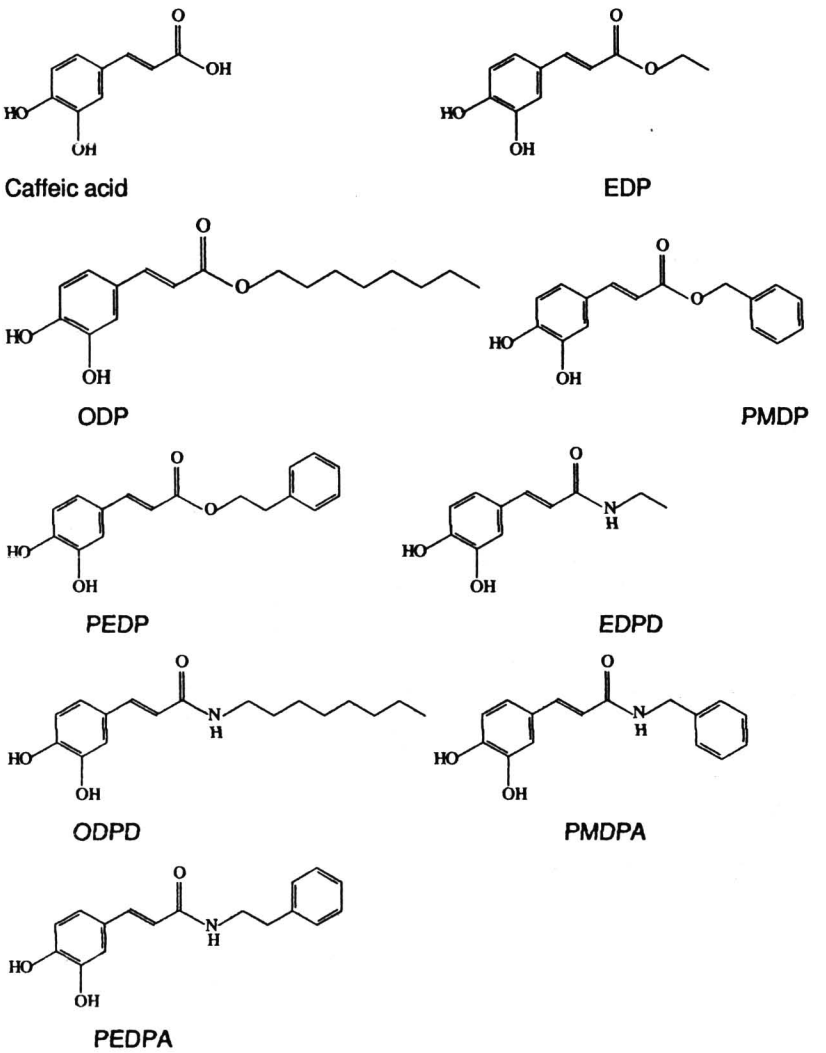

Figure 1. The structure of caffeic acid and its derivatives.

chain. Phenylmethyl 1-(3', 4'-dihydroxyphenyl)propenate (PMDP) and phenylethyl 1-(3',4'-dihydroxyphenyl) propenate (PEDP) were caffeic acid esters that had an aromatic side chain. Ethyl 1-(3', 4'-dihydroxyphenyl) propen amide (EDPA) and octyl 1-(3', 4'-dihydroxyphenyl) propen amide (ODPA) were the representative of caffeic acid amide that had an aliphatic side chain. Phenmethyl1-(3', 4'-dihydroxyphenyl) propen amide (PMDPA) and phenethyl 1-(3', 4'-dihydroxyphenyl) propen amide (PEDPA) were caffeic acid amides that had an aromatic ring side chain. The structures of caffeic acid and its derivatives are shown in Figure 1.

The method to determine HO-1 activity in this experiment has been shown to give the same results as the more refined mRNA assay that is compatible with mRNA HO-1 expression (Motterlini et al., 2000). In this experiment, we selected caffeic acid and its derivatives at concentration 10, 50 and 100 µg/ ml that had no toxicity to the Hep G2 cell line (data not shown). After treating the cells for 48 h, HO-1 activity was significantly increased when compared with the control (untreated group). The order of HO-1 induction at 10 µg/ml of caffeic acid with ester and amide substituted group was as following: EDP< CAF< ODP< EDPA< ODPA< PEDP< PMDPA< PEDPA< DMPD (Figure 2). EDP was classified as having the lowest activity level, whereas PMPD was classified as having the highest activity level. The others were classified as the medium activity level group. PMPD was the highest stimulating HO-1 activity agent with HO-1 activity of 4.07 µM bilirubin/mg protein/h (2.1-fold when compared with control group) and higher than curcumin, which was the positive control.

Figure 3 shows the HO-1 activity treated with 50 µg/ml caffeic acid and its derivatives. The order of HO-1

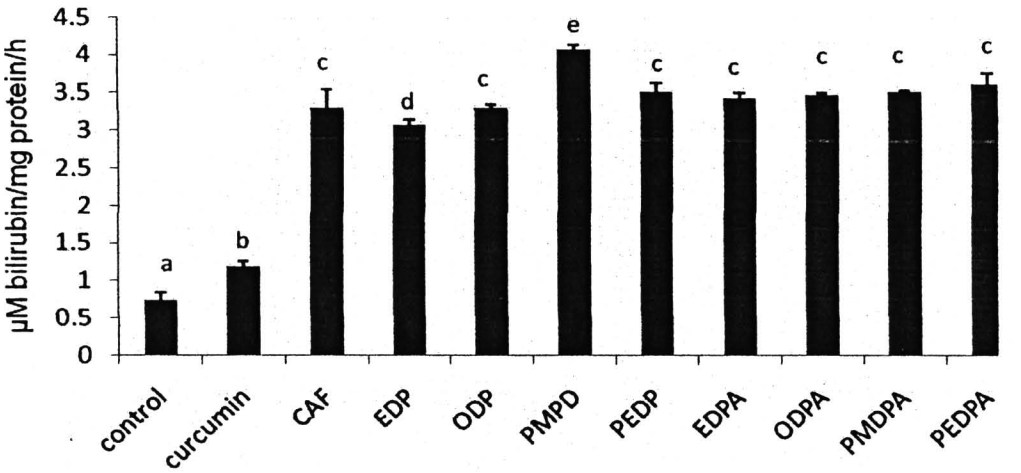

Figure 2. HO-1 activity in Hep G2 cells incubated for 48 h in complete medium with 10 μg/ml of caffeic acid and its derivatives. The activity is expressed as μM bilirubin formed/mg protein/h. Bars represent the mean ±SD of 5 independent experiments. Samples represented with different small letters are significantly different from other groups ($p < 0.05$).

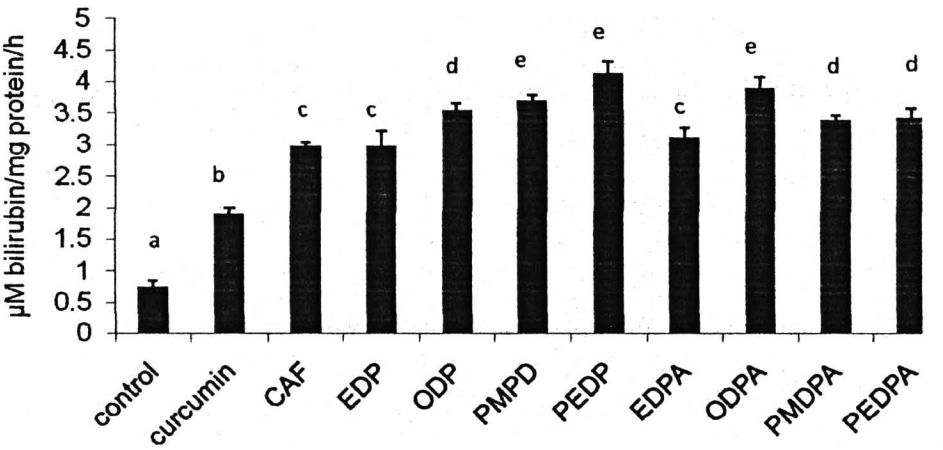

Figure 3. HO-1 activity in Hep G2 cells incubated for 48 h in complete medium with 50μg/ml of caffeic acid and its derivatives. The activity is expressed as μM bilirubin formed/mg protein/h. Bars represent the mean ± SD of 5 independent experiments. Samples represented with different small letters are significantly different from other groups ($p < 0.05$).

induction was as following: EDP< CAF< EDPA< PMDPA< PEDPA< OPD< PMPD< ODPA<PEDP. PEDP was the highest stimulating HO-1 activity agent, with HO-1 activity level of 4.14 μM bilirubin/ mg protein/ h. This value was 2.1-fold of the control group and 1.2-fold of curcumin. Figure 4 showed the HO-1 activity of the cells that were treated with 100 μg/ml caffeic acid and derivatives. HO-1 activity of such treatments ranged from lower to higher as following: CAF< EDP< PMDPA< EDPA< PMPD< ODPA< ODP< PEDPA< PEDP. PEDP was the highest stimulating HO-1 activity agent with 4.32 μM bilirubin/mg protein/h, 2.3-fold when compared with the control group. When compared between ester and amide derivatives to induce HO-1, it was found that ester derivatives had higher ability to induce HO-1 activity.

In this experiment, curcumin was used as the positive control substance. Curcumin had the potential to induce HO-1 activity in a dose-response relationship. Curcumin, at 10 and 50 μg/ml, induced HO-1 significantly less than caffeic acid and its derivatives by about 2-3 times. However, curcumin, at a higher concentration, 100 μg/ml, had the same potential to induce HO-1 activity as caffeic acid and its derivatives at the same concentration, except for PEDP.

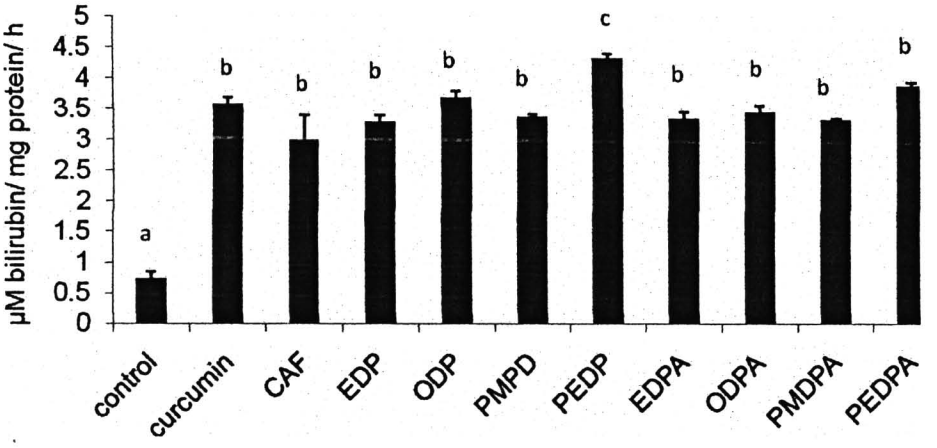

Figure 4. HO-1 activity in Hep G2 cells incubated for 48 h in complete medium with 100 µg/ml of caffeic acid and its derivatives. The activity is expressed as µM bilirubin formed/mg protein/h. Bars represent the mean ±SD of 5 independent experiments. Samples represented with different small letters are significantly different from other groups ($p < 0.05$).

DISCUSSION

Human cells have developed several protective mechanisms to prevent oxidative stress. Phase 2 enzymes are key responders providing protective actions, which can be highly induced by synthetic and natural chemical agents. There are many phytochemicals found in fruits and vegetables normally present in the human diet. Consumption of fruits and vegetables is associated with a decrease in cancer risk and diseases associated with free radicals. Plants constituents such as curcumin (Scapagnini et al., 2002; Balogun et al., 2003; Jeong et al., 2009) caffeic acid phenethyl ester (Scapagnini et al., 2002), and carnosol (Martin et al., 2004) are able to induce HO-1 expression. In this study, we reported the ability of caffeic acid ester and amide derivatives to enhance the activity of HO-1 in Hep G2 cells. Caffeic acid ester derivatives such as PEDP (caffeic acid phenethyl ester), and PMDP (caffeic acid benzyl ester) are naturally occurring substances present in bee honey and propolis (Orsolic et al., 2003). Our results showed that the pattern of inducibility was different in each of the caffeic acid derivatives. Changing the chemical structure can significantly affect the potency of HO-1 induction.

Caffeic acid and its derivatives enhance HO-1 induction more than curcumin, the known standard for induction of HO-1. The presence of hydroxyl groups in the *ortho* position on the aromatic ring is already known to enhance noticeably the HO-1 inducer potency of plant constituents (Foresti et al., 2005). Caffeic acid and its derivatives have a hydroxyl group in the *ortho* position, but curcumin does not. This might be the reason why caffeic acid and its derivative are stronger HO-1 inducers than curcumin.

In this study, it was shown that caffeic acid ester analogues with a long side chain, such as, ODP, PMDP and PEDP, had a high potential to induce HO-1 activity when compared with caffeic acid amide analogues. Caffeic acid esters were hydrolysed to caffeic acid as the major metabolite (Celli et al., 2007; Lee et al., 2007; Wang et al., 2007) and alcohols (Rajan et al., 2001; Celli et al., 2007). HO-1 was induced in response to alcohol, thiol-containing dietary anti-oxidant, triterpenoid and lipopolysacharide (Drechsler et al., 2006; Hsu et al., 2008). Treatment with caffeic acid ester may induce HO-1 activity expression through caffeic acid, alcohol and caffeic acid ester. Caffeic acid amides were more stable than caffeic acid esters (Son and Lewis, 2002). Therefore, only the caffeic acid amide compound might be able to induce HO-1 activity. From our experiments, the chemicals with long side chain groups were more potent HO-1 inducers than those with short side chain groups. Ardhaouia and his colleagues (Ardhaoui ,.a et al 2004) reported that hydrophobicity of chemicals with long side chains was greater than those with short side chains. Therefore, those chemicals with hydrophobic properties might easily penetrate cell membranes and induce signal activation. Nrf2 plays a key role in the transcriptional regulation of the HO-1 gene expression through interaction with ARE. Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor Keap 1. Upon stimulation by inducers, however, Nrf2 dissociates from Keap 1 and translocates into the nucleus where it dimerizes with some cofactors like small Maf protein and binds to ARE. This will lead to activation of a battery of highly specialized proteins, including HO-1(Lau et al., 2008; Kim et al., 2010). HO-1 induction by caffeic acid and its derivatives might occur via signalling through the nuclear factor-erythroid-2-related factor Nrf2/ ARE pathway. This hypothesis was supported by CAPE induced HO-1 expression observed in several cell types. The molecular mechanism in the regulation of CAPE-

mediated HO-1 expression has been studied in some cell types (Wang et al., 2008; Wang et al., 2010). CAPE disrupts the Nrf2-Keap1 complex, leading to increase Nrf2 binding to ARE. Compounds stimulated nuclear translocation of Nrf2 by inactivating the Nrf2-Keap1 complex, which was associated with a significant increase in the activity and HO-1 mRNA (Balogun et al., 2003). The other caffeic acid derivatives, which have a similar structure to CAPE, may have the same mechanism. It was, therefore, possible that preconditioning Hep G2 cells by caffeic acid and its derivatives may enhance activation of the Nrf2/ARE pathway and induction of phase II detoxification/ antioxidant enzymes upon oxidative stress, thereby resulting in increased resistance to oxidative damage. Mechanisms of HO-1 induction in Hep G2 by caffeic acid and its derivatives will be further investigated.

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