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Full Length Research Paper

Inhibitory effect of caffeic acid and its derivatives on human liver cytochrome P450 3A4 activity

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Effects of caffeic acid (CAF) and its derivatives on human liver CYP3A4 activity were evaluated by using diazepam as a substrate. It was found that CAF inhibited CYP3A4 activity by uncompetitive inhibition with IC50 0.72 µM, whereas ester and amide analogues inhibited CYP3A4 by competitive inhibition with IC50 value of 0.31, 0.37, 0.46, 0.49, 0.53, 0.58, 0.75 and 0.82 µM for ethyl 1- (3',4'-dihydroxyphenyl) propen amide (EDPA), phenmethyl 1- (3',4'-dihydroxyphenyl) propen amide (PMDPA), phenethyl 1- (3',4'-dihydroxyphenyl) propen amide (PEDPA), phenylethyl 1- (3',4'-dihydroxyphenyl) propenate (PC), ethyl 1- (3',4'-dihydroxyphenyl) propenate (EC), octyl 1- (3',4'-dihydroxyphenyl) propen amide (ODPA), octyl 1- (3',4'-dihydroxyphenyl) propenate (OC) and phenylmethyl 1- (3',4'-dihydroxyphenyl) propenate (BC), respectively. The K_i values of CAF, PMDPA, EC, PC, ODPA, PEDPA, OC, EDPA and BC were 0.24, 0.29, 0.49, 0.56, 0.57, 0.59, 0.62, 0.62 and 1.03 µM, respectively. However, CAF and its derivatives had high potential to inhibit CYP3A4. Therefore, consumption of herbal medicine containing CAF and its derivatives that are concomitant with other medications should be cautiously monitored.

Key words: Cytochrome P450 3A4, caffeic acid, caffeic acid amide, caffeic acid ester.

INTRODUCTION

In developing countries, herbal medicines are important in the primary health care, while in international traditional trades, herbal medicine products have increased (Subehan et al., 2006). Polyphenols are phytochemicals prevalent in herbal medicines, apart from vegetable and fruits which are the major source of antioxidants (Scalbert and Williamson, 2000). Epidemiological studies of polyphenols suggest that consumption of polyphenol-rich foods may prevent cancers, cardiovascular disease, osteoporosis and diabetes (Arts and Hollman, 2005). Caffeic acid (3, 4-dihydrocinnamic acid, CAF) is one of the most common phenolic acid found in fruits, grains, dietary supplements and Chinese medicinal herbs (Jiang et al., 2005). CAF derivatives including amides, esters, sugar ester and glycosides are widely distributed in honey, propolis, coffee beans and olive. The reported biological properties of CAF and its derivatives include antibacterial, antiviral, anti-inflammatory, antioxidant and antiproliferative properties (Fiuza et al., 2004; Son and Lewis, 2002). Cytochrome P450 (CYP) is the superfamily of hemoproteins which metabolize a variety of xenobiotics including drugs, procarcinogens and toxins. In the body, the purpose of drug metabolism is to increase water solubility of xenobiotics which is then easily excreted in the urine or bile. CYPs enzymes are mainly located in the liver and about 30% of the total CYP enzymes represent CYP3A subfamily (Shimada et al., 1994). Cytochrome P450 3A4 (CYP3A4) is the most important CYP enzyme which contribute to the metabolism of more than one-third of the available

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Abbreviations: EDPA, Ethyl 1- (3', 4'-dihydroxyphenyl) propenamide; **ODPA,** octyl 1- (3', 4'-dihydroxyphenyl) propen amide; **PMDPA,** phenmethyl 1- (3', 4'-dihydroxyphenyl) propen amide; **PEDPA,** phenethly 1- (3', 4'-dihydroxyphenyl) propen amide; **EC,** ethyl 1-(3',4'-dihydroxyphenyl) propenate; **OC,** octyl 1- (3',4'-dihydroxyphenyl) propenate; **BC,** phenylmethyl 1-(3',4'-dihydroxyphenyl) propenate; **PC,** phenylethyl 1- (3',4'-dihydroxyphenyl) propenate; **CAF,** caffeic acid; **CYP,** cytochrome P450.

medications (Li et al., 1995). In humans, consumption of polyphenol has been estimated as roughly 1 g in Western countries (Scalbert and Williamson, 2000). The risk of adverse effects may be carried out through pharmacological overdoses during prevention or treatment of diseases.

Then, food and drug interaction had recently come into focus (Chavez et al., 2006). Interaction has occurred as a result of inhibiting or inducing the CYP enzymes. The inhibition of CYP is becoming important, because the inhibitor could interrupt drug metabolism and lead to serious toxic effects. In a previous study, it was reported that caffeic acid and its amide analogues potently inhibited human liver microsome CYP1A2 (Jaikang et al., 2009). However, inhibition of CYP3A4 activity by caffeic acid and its analogues have not been studied. In this study, evaluation of the effects of caffeic acid and its analogues on CYP3A4 activity *in vitro* using diazepam as substrate and pooled human liver microsomes as a source of CYP3A4 was carried out. In addition, the mechanism of inhibition was investigated by Cornish-Bowden and Dixon plot analysis.

MATERIALS AND METHODS

Chemicals

Caffeic acid, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) and β-nicotinamide adenine dinucleotide phosphate oxidized form (NADP) were purchased from Sigma Aldrich (St Louis, MO, USA), while diazepam, flunitrazepam and oxazepam were obtained from the Toxicology section, Chiang Mai Medicinal Science Center, Chiang Mai, Thailand. Ethyl 1-(3', 4'-dihydroxyphenyl) propen amide (EDPA), octyl 1-(3', 4'-dihydroxyphenyl) propen amide (ODPA), phenmethyl 1-(3', 4'-dihydroxyphenyl) propen amide (PMDPA), phenethly 1- (3', 4'-dihydroxyphenyl) propen amide (PEDPA), ethyl 1-(3',4'-dihydroxyphenyl) propenate (EC), octyl 1-(3',4'-dihydroxyphenyl) propenate (OC), phenylmethyl 1-(3',4'-dihydroxyphenyl) propenate (BC) and phenylethyl 1-(3',4'-dihydroxyphenyl) propenate (PC) were obtained from the Health Product Research Unit, Faculty of Pharmacy, Chiang Mai University. However, all chemicals had the highest purity that is commercially available.

Preparation of human liver microsome

Livers were obtained from most subjects who died in traffic accident. The protocols were approved by the Ethical Committee for Human Research, Faculty of Medicine, Chiang Mai University (protocol No. 06OCT131501). Human microsomes were prepared by a differential centrifugation via the methods of Kusirisin et al. (2009). Briefly, livers were washed with cool 0.9% normal saline 5 times to eliminate the hepatic blood and were homogenized in 3 volumes (w/v) of Tris buffer.

The homogenate was centrifuged at 9,000 g for 30 min at 4°C, while the resulting pellet was centrifuged at 105,000 g for 60 min at 4°C. The pellets, which contain 0.1 M potassium phosphate buffer, pH 7.4, 20% (v/v) glycerol, 1 mM EDTA and 0.25 M sucrose, were resuspended in the storage buffer, and the microsome was stored at -70°C until use. The protein concentration was determined by bicinchonic acid (BCA) assay kit with bovine serum albumin as the standard.

Determination of CYP3A4 activity

Diazepam was used as a substrate and flunitrazepam was used as an internal standard to determine CYP3A4 activity. The incubation mixture (1 ml) contained 0.1 mg microsomal protein, 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 0.4 mM magnesium chloride and NADPH generating system, which consisted of 0.5 mM NADP, 20 mM NADPH, 2 mM G-6-P and 1 IU/ml G-6-PD, diazepam (0.35 to 3.47 mM) and CAF or its derivatives. After 60 min, the reaction was stopped by the addition of 200 µl cool methanol. Then, 50 µl of 100 µg/ ml flunitazepam was added to the mixture, before it was centrifuged at 13,000 RPM for 10 min 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 and an 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). A wavelength used for identification of the diode array detector was 230 nm. Diazepam, funitrazepam and oxazepam were separated by Agilent C8 column (250 × 4 mm, 5 µm, Agilent®) and the column oven was set up at 25°C. The mobile phase consisted of 0.125% (v/v) isopropylamine in water: methanol (30: 70 v/v) with a flow rate of 0.7 mL/ min and the injection volume was 10 µl.

Determination of CYP3A4 enzyme kinetic

Different concentrations of diazepam were used in kinetic experiments for CYP3A4 activity. Michaelis-Menten parameters were estimated by two enzyme kinetic approaches. Cornish-Bowden and Dixon plots were used for determining the type of inhibition and value of the inhibitory constant (Ki).

Statistical analysis

Data were expressed as mean ± S.D. of three values and analysed by one way ANOVA followed by Duncan multiple range test. Consequently, *p* values of less than 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

CYP3A4 is the most important drug-metabolizing enzyme in the human liver and small intestine and it is involved in approximately 50% of all drug metabolisms in clinical use, including acetaminophen, diazepam, erythromycin, codeine, cyclosporine A, midazolam, rifampicin and quinidine (Fluck et al., 2010). Kilicarslan et al. (2001) found that diazepam is metabolized by oxazepam using CYP3A4 enzyme. In this study, we used diazepam as a probe and quantified the amount of oxazepam to evaluate CYP3A4 activity.

CAF and its analogues are found in fruits, vegetable wine, olive oil and coffee. Simple derivatives of CAF,

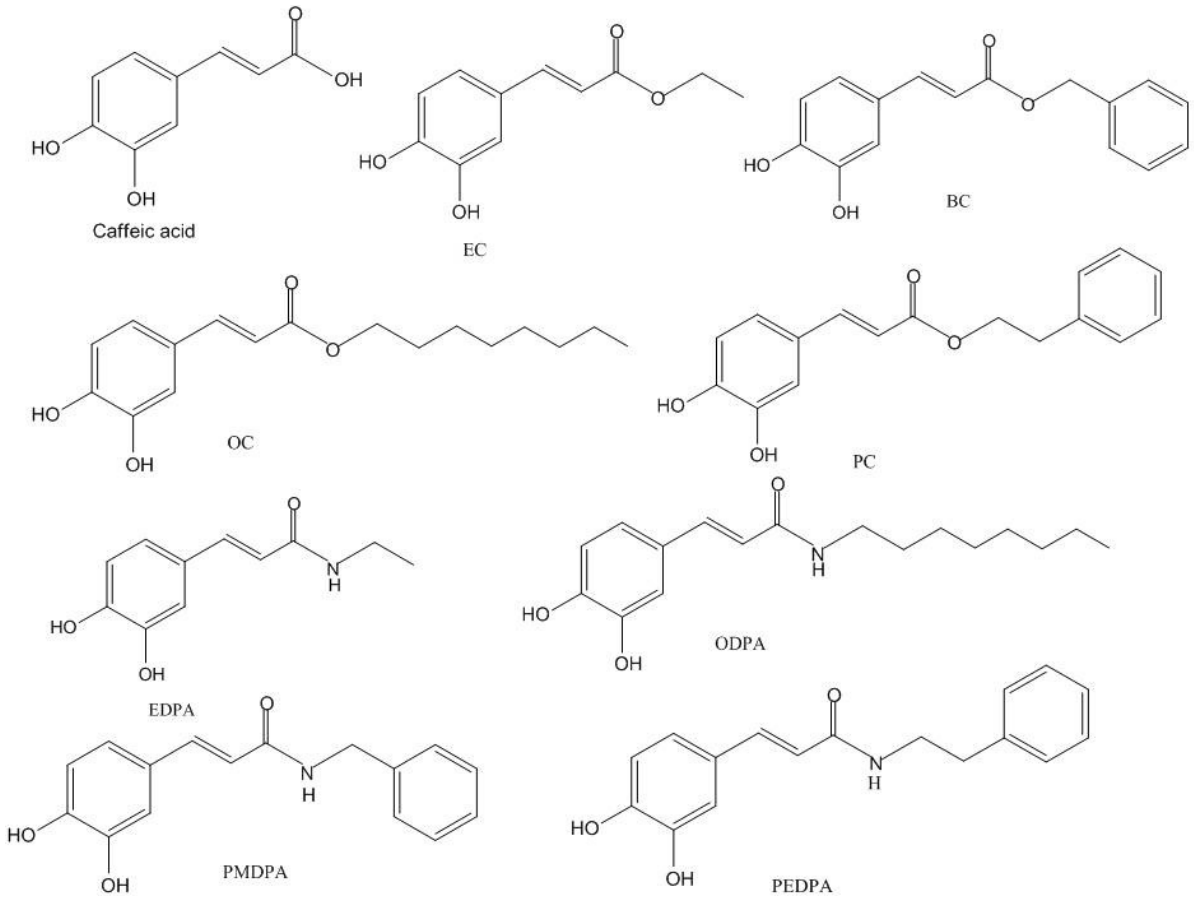


Figure 1. The structure of caffeic acid and its derivatives.

including esters and amides, have been reported to possess a wide spectrum of biological effects, such as: antioxidation activity, induction of phase 2 enzymes and anticancer activity (Jaikang and Chaiyasut, 2010). The structures of CAF and its analogues in this study are shown in Figure 1. The percent inhibition of CYP3A4 activity in human liver microsome using diazepam at a concentration of 2.1 µM and the concentration of CAF and its derivatives at 0.0625 µg/ mL is shown in Figure 2. The EDPA exhibited strong inhibition of 53.31 ± 2.46% on CYP3A4 activity when compared with the control. The inhibitory effect of PMDPA, EC, ODPA, PEDPA, CAF, PC, BC and OC on CYP3A4 activity were 45.51±5.60, 43.63±2.37, 42.01±3.06, 40.61±4.37, 34.70±2.68, 30.54±2.38, 27.70±3.39 and 22.63±1.95%, respectively, whereas the IC50 values of EDPA, PMDPA, PEDPA, PC, EC, ODPA, CAF, OC and BC were 0.31, 0.37, 0.46, 0.49, 0.53, 0.58, 0.72, 0.75 and 0.82 µM, respectively. CAF and its derivatives inhibited CYP3A4 activities in a concentration-dependent manner, with IC50 values ranging from 0.31 to 0.82 µM, indicating that they are strong inhibitors against diazepam metabolism. The CAF analogues showed comparable inhibition on CYP3A4 activity. From the result of this study, amide analogues showed higher potential to inhibit diazepam metabolism than ester analogues with the same side chains for 1.1 to 2.6 folds. It is possible that the high polarity of substrates contained in the hydroxyl groups may contribute to the inhibitory effect on CYP3A4 activity enzyme (Ho et al., 2001). In addition, Bu (2006) reported that lipophilicity property decreased CYP3A4 activity.

The Ki values were calculated with linear regression, and it was seen that the Ki values of CAF, PMDPA, EC, PC, ODPA, PEDPA, EDPA, OC and BC were 0.24, 0.29, 0.49, 0.56, 0.57, 0.59, 0.62, 0.62 and 1.03 µM, respectively. According to characterization of the inhibition kinetic of CYP3A4, these values were obtained from Dixon plots (Figure 3) and Cornish-Bowden plots (Figure 4). CAF inhibited CYP3A4 by uncompetitive inhibition, while ester and amide analogues were affected by competitive inhibition.

Normally, the active site of CYP3A4 appears capable of accommodating a wide range of structure from simple molecules to macromolecules. It shows that CYP3A4 enzyme tends to oxidize the lipophilic substances or basic compounds (Bertz and Granneman, 1997). This is consistent with previous observation amid CYP3A4 pharmacophore models and t he apparent lack of

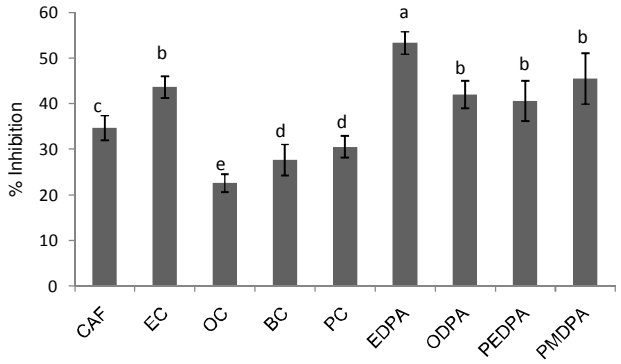
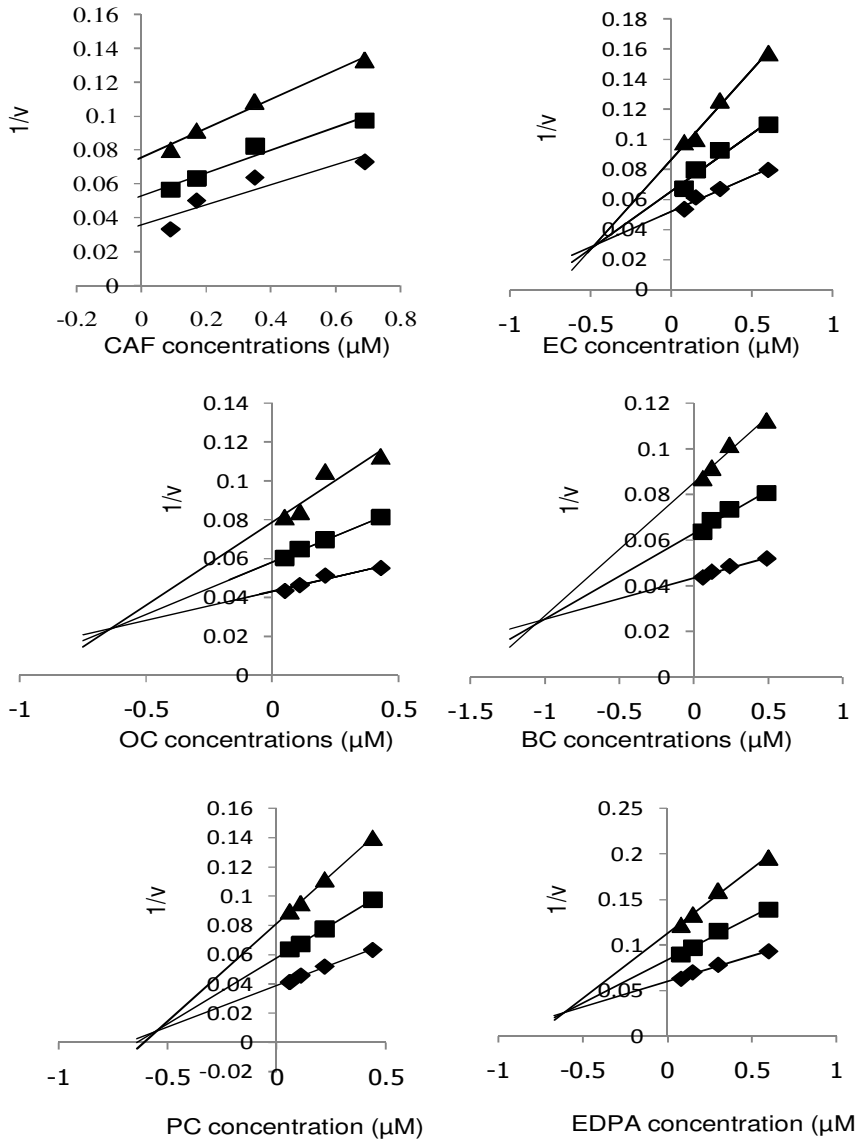


Figure 2. Percent inhibition of CYP3A4 activity when using diazepam at a concentration of 2.1 µM and CAF and its derivatives at 0.0625 µg/ml. Bars represent the mean ± S.D. of 3 independent experiments. Samples represented with different small letters are significantly different from other groups ($p<0.05$).



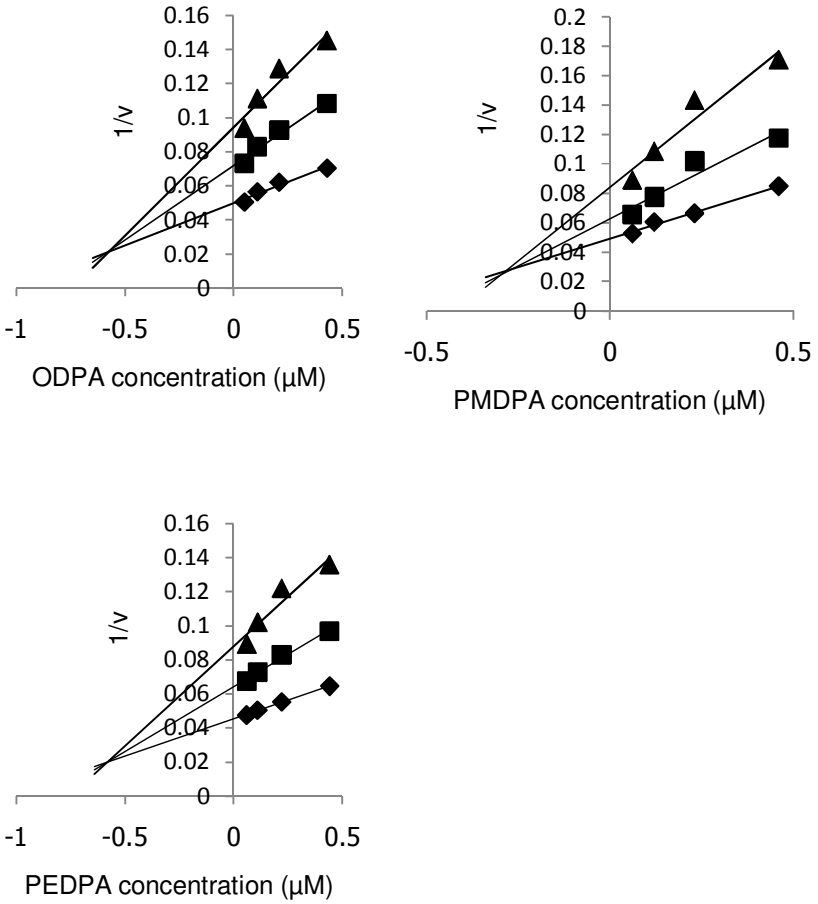
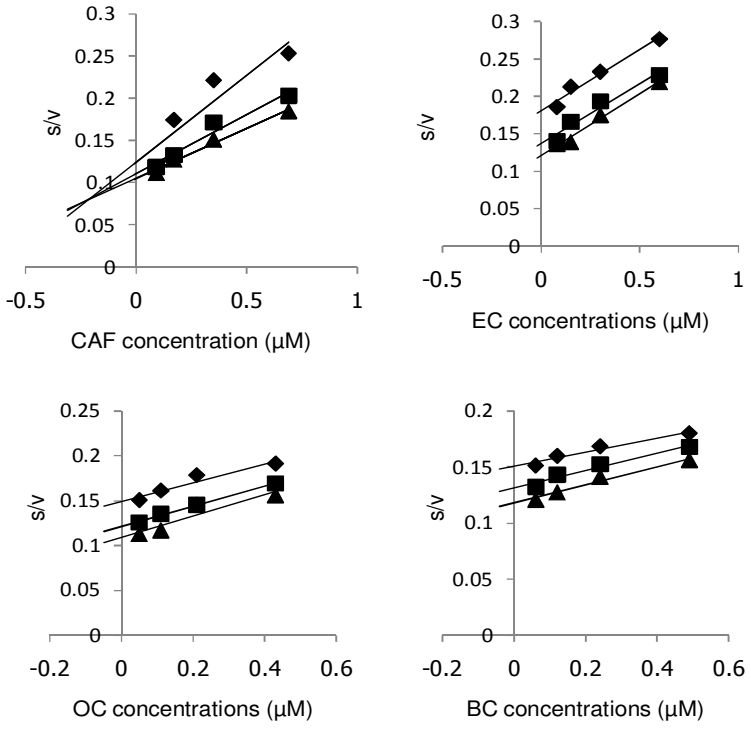


Figure 3. Dixon plot of CAF and its derivatives on CYP3A4 activity when diazepam was ▲ 1.39, ■ 2.08 and ◆ = 3.47 μM.



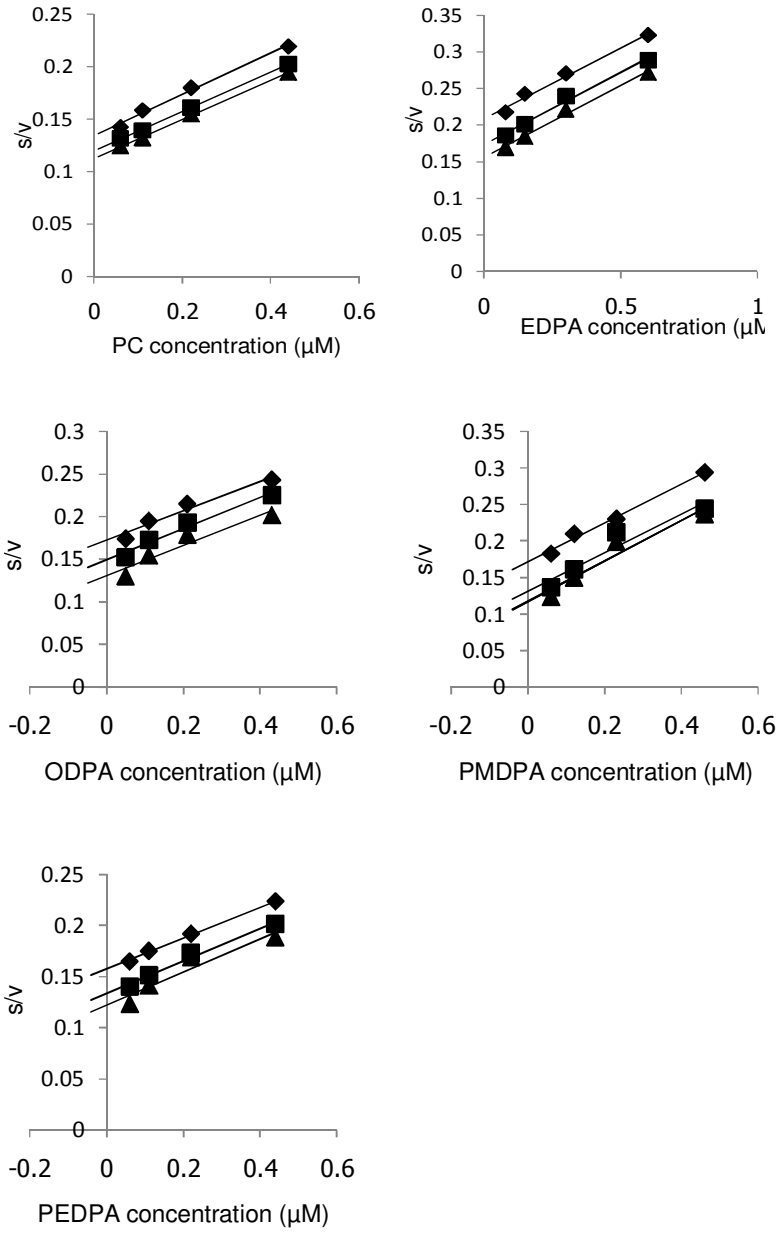


Figure 4. Cornish-Bowden plot of CAF and its derivatives on CYP3A4 activity when diazepam was ▲ 1.39, ■ 2.08 and ◆ 3.47 μM.

functionality in the amino acid residues comprising CYP3A4 active site, which is composed of leucine 210, leucine 211 and aspartic acid 214 (Fowler et al., 2002). N-containing heterocyclic in the structure appeared to have increased the potential of CYP3A4 inhibition, although CAF amide derivatives were more potent to inhibit CYP3A4 than ester derivatives. It seems like this reflects the increased binding energy afforded by the lone pair electron on the nitrogen atom ligating the CYP heme iron at about 6 kcal/ mol as suggested for the prototypic CYP inhibitors, including ketoconazole, sulfaphenazole, carbamazepine, quinidine, verapamil (Riley et al., 2001) and grapefruit juice (Row et al., 2006). Moreover, the grapefruit juice is composed of CAF, coumaric acid, sinapic acid and ferrulic acid (Peleg et al., 1991). Nonetheless, identification of the herbs bioactive compounds will help to understand the mechanism involved and the consumer will be able to avoid the side effects of the herbs-drug interaction.

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