ผลยับยั้งของเอเชียติโคไซด์และมาดิคาสโซไซด์ต่อเอนไซม์ไซโตโครม พี450 ของมนุษย์

นางสาวธิดารัตน์ วินิจธนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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INHIBITORY EFFECT OF ASIATICOSIDE AND MADECASSOSIDE ON HUMAN CYTOCHROME P450

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เอเชียติโคไซด์และมาดิคาสโซไซด์เป็นสารกลุ่มไตรเทอร์พินอยด์ที่พบในบัวบก เป็นส่วนประกอบหลักที่อยู่ในสารมาตรฐานบัวบกอีซีเอ233 สารทั้งสองมีฤทธิ์ทางเภสัชวิทยาหลายอย่างที่น่าสนใจ เพื่อให้ได้ข้อมูลเบื้องต้นของอันตรกิริยาระหว่างยา การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของเอเชียติโคไซด์และมาดิคาสโซไซด์ในการยับยั้งและชนิดของการยับยั้งเอนไซม์ไซโตโครม พี450 (CYP) ของมนุษย์โดยการศึกษาแบบนอกกาย การศึกษาประกอบด้วย 3 ส่วนคือ การวัดค่า IC_{50} ของการยับยั้งของเอเชียติโคไซด์และมาดิคาสโซไซด์ต่อเอนไซม์ CYP การศึกษาการยับยั้งแบบ mechanism-based inhibition และการศึกษาชนิดของการยับยั้งแบบผันกลับ ในการวัดค่า IC_{50} พบว่าเอเชียติโคไซด์มีผลยับยั้ง CYP2C19 (IC_{50} = 412.68 μM) และ CYP3A4 (IC_{50} = 343.35 μM) และพบว่ามาดิคาสโซไซด์มีผลยับยั้ง CYP2C19 (IC_{50} = 539.04 μM) และ CYP3A4 (IC_{50} = 453.32 μM) สารสำคัญทั้งสองไม่มีผลในการยับยั้ง CYP1A2, CYP2C9 และ CYP2D6 (IC_{50}>1,000 μM) นอกจากนี้เอเชียติโคไซด์ไม่มีผลยับยั้ง CYP2E1 ที่ความเข้มข้นสูงถึง 200 μM และมาดิคาสโซไซด์ไม่มีผลในการยับยั้งการทำงานของ CYP2E1 ที่ความเข้มข้นสูงถึง 1,000 μM จากการศึกษาการยับยั้งแบบ mechanism-based inhibition พบข้อมูลที่แสดงว่า มาดิคาสโซไซด์น่าจะมีผลยับยั้งแบบ mechanism-based inhibition ต่อ CYP2C19 และ CYP3A4 ในส่วนของเอเชียติโคไซด์ไม่สามารถศึกษาได้ เนื่องจากข้อจำกัดในการละลายของสาร ในส่วนของการศึกษาชนิดของการยับยั้งแบบผันกลับ พบข้อมูลที่แสดงว่าเอเชียติโคไซด์น่าจะมีกลไกในการยับยั้งแบบ noncompetitive ต่อ CYP2C19 (Ki = 588 μM) และ CYP3A4 (Ki = 769 μM) และมาดิคาสโซไซด์น่าจะมีกลไกในการยับยั้งแบบ noncompetitive ต่อ CYP2C19 (Ki = 140 μM) และ CYP3A4 (Ki = 692.86 μM) ผลการศึกษานี้ทำให้ได้ข้อมูลความเป็นไปได้ของเอเชียติโคไซด์และมาดิคาสโซไซด์ในการเกิดอันตรกิริยากับยาที่มีการเมแทบอลิซึมด้วยCYP2C19 และ CYP3A4 ควรมีการศึกษาต่อไปว่าผลยับยั้งของสารทั้งสองต่อเอนไซม์ CYP2C19 และ CYP3A4 จะมีผลในทางคลินิกหรือไม่

ภาควิชา เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต
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THIDARAT WINITTHANA: INHIBITORY EFFECT OF ASIATICOSIDE AND MADECASSOSIDE ON HUMAN CYTOCHROME P450. THESIS ADVISOR: ASSOCIATE PROFESSOR POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., THESIS CO-ADVISOR: ASSOCIATE PROFESSOR NUANSRI NIWATTISAIWONG, 114 pp.

Asiaticoside and madecassoside, the triterpenoid compounds found in *Centella asiatica* (Linn.) Urban, are the major constituents in the standardized extract of *C. asiatica* (ECa233). Both compounds possess several interesting pharmacological effects. To obtain the preliminary drug interaction data, the aim of this study was to investigate inhibitory effects and types of inhibition of asiaticoside and madecassoside on human CYPs by using recombinant human CYPs in an *in vitro* study. This study included 3 parts: IC_{50} determination of asiaticoside and madecassoside on human CYP, assessment of mechanism-based inhibition and determination of the type of inhibition using enzyme kinetic study. For IC_{50} determination, asiaticoside inhibited CYP2C19 (IC_{50} = 412.68 μM) and CYP3A4 (IC_{50} = 343.35 μM). Madecassoside inhibited CYP2C19 (IC_{50} = 539.04 μM) and CYP3A4 (IC_{50} = 453.32 μM). Both asiaticoside and madecassoside did not affect CYP1A2, CYP2C9 and CYP2D6 (IC_{50}>1,000 μM). Inhibition of asiaticoside on CYP2E1 was not observed at the concentration up to 200 μM while madecassoside did not affect CYP2E1 (IC_{50}>1,000 μM). As asiaticoside and madecassoside demonstrated inhibitory effects on CYP2C19 and CYP3A4, types of inhibition of both CYPs were performed. In the mechanism-based inhibition study, madecassoside was shown to probably be a mechanism-based inhibitor of CYP2C19 and CYP3A4. Asiaticoside which was limited by its solubility, assessment of its mechanism-based inhibition potential could not be achieved. To determine the type of reversible inhibition, asiaticoside exhibited a pattern of noncompetitive inhibition of CYP2C19 (Ki = 588 μM) and CYP3A4 (Ki = 769 μM). Madecassoside also exhibited a pattern of noncompetitive inhibition of CYP2C19 (Ki = 140 μM) and CYP3A4 (Ki = 692.86 μM). Results from this study provided the potential information that both asiaticoside and madecassoside are probable to cause drug–drug interactions with medicines that are metabolized by CYP2C19 and CYP3A4. Further investigation is needed to evaluate whether these inhibitory effects of both compounds on both CYP isoforms are clinically significant.

Department : Pharmacology and Physiology Student's Signature .....
Field of Study : Pharmacology ..... Advisor's Signature .....
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LIST OF ABBREVIATIONS

α	alpha
β	beta
ºC	degree celcius
[S]	substrate concentration
µg	microgram
µl	microlitre
µM	micromolar
BOMCC	7-benzyloxymethyloxy-3-cyanocoumarin
CIA	collagen II-induced arthritis
CII	collagen II
CK	creatine kinase
con't	continuous
CRP	c-reactive protein
CYP	cytochrome P450
DBOMF	dibenzyloxymethylfluorescein
DMSO	dimethylsulfoxide
e.g.	Exampli gratia
EOMCC	ethoxymethyloxy-3-cyanocoumarin
et al.	et alii
etc.	et cetera
g	gram
G6P	glucose-6-phosphate
G6PD	glucose -6-phosphate dehydrogenase
GI	gastrointestinal
GSH	glutathione

FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
HCl	hydrochloric acid
IC_{50}	median inhibitory concentration
i.c.v.	intracerebroventricular
i.e.	id est
Ig	immunoglobulin
i.g.	intra-gastric
IL	interleukin
iNOS	inducible nitric oxide synthase
i.v.	intravenous
K	dissociation constant
Ki	inhibition constant
Km	the Michaelis constant
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
M	molar
ml	millilitre
m.	meter
MAO	monoamine oxidase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MFO	mixed-function oxidase
mg/kg	milligram per kilogram body weight
mg	milligram
MI	metabolite–intermediate complex
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

MW	molecular weight
ng	nanogram
nM	nanomolar
nm	nanometer
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	sodium hydroxide
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO	nitric oxide
PAHs	polycyclic aromatic hydrocarbons
pg	picogram
pH	potential of hydrogen ion concentration
R^2	coefficient of determination
RFU	relative fluorescence unit
SD	standard deviation
SOD	superoxide dismutase
Tris	tris(hydroxymethyl) aminomethane
v	velocity
v/v	volume by volume
VEGF	vascular endothelial growth factor
Vi	initial velocity
Vmax	the maximum velocity of the reaction
Vs	terminal velocity
w/w	weight by weight
yrs	years

CHAPTER I

INTRODUCTION

Herbal medicines have increasingly been used either as an alternative medicine or a supplement with other medicines. In addition, herbal plants are often used as food supplement. *Centella asiatica* (Linn.) Urban, called in Thai as Bua Bok, is a plant in the Umbelliferae family. *C. asiatica* contains many chemical constituents such as flavonoid glycosides, free amino acids, terpenoids and triterpenes. Major triterpene compounds are asiatic acid, madecassic acid, asiaticoside and madecassoside (Barnes et al., 2006). Whole plants have been used as food ingredient, beverage and traditional medicine. *C. asiatica* was shown to possess several pharmacological effects such as wound healing (Suguna et al., 1996, 1999; Shukla et al., 1999), anticancer (Babu et al., 1995), memory enhancer (Kumar and Gupta, 2002; Gupta et al., 2003), protective effect of gastric ulcer formation (Cheng et al., 2000, 2004) and anti-oxidant (Hamid et al., 2002; Jayashree et al., 2003; Zainol et al., 2003). These beneficial effects demonstrate that *C. asiatica* is an interesting herbal plant to be further developed so as to be use as a herbal medicine in the future. The pharmacological effects of asiaticoside and madecassoside were also studied. Asiaticoside was reported to induce apoptosis of tumor cells and enhance the anti-tumor activity of vincristine (Huang et al., 2004). It also possessed wound healing effect of gastric ulcer and anti-inflammatory (Guo et al., 2004) as well as anxiolytic-like effect (Chen et al., 2006). Madecassoside exhibited anti-inflammatory activity (Li et al., 2007), antidepressant effect (Liu et al., 2004) and anti-rheumatoid arthritic effect (Liu et al., 2008). As mentioned above, both triterpenoid glycosides possess several pharmacological effects similar to those of *C. asiatica*. Thus, both asiaticoside and madecassoside have also been studied by several investigators so as to develop them to be used as drugs for many disease conditions.

A research group at the Faculty of Pharmaceutical Sciences, Chulalongkorn University has been studied the pharmacological and toxicological effects of the standardized extract of *C. asiatica* ECa233 (ECa233). ECa233 containing triterpenoid glycoside of more than 80% with the ratio of madecassoside and asiaticoside content should be within 1.5±0.5, demonstrated several interesting pharmacological effects. It was found to attenuate learning and memory deficited mouse inducing by an intracerebroventricular (i.c.v.) injection of β-amyloid peptide in Morris Water Maze test (Kam-eg et al., 2009). ECa233 was also shown to possess an interesting wound healing effect both in animal (Wannarat et al., 2009). For the toxicity study, ECa233 was shown to be safe in both acute and subchronic toxicity testings (Wannarat et al., 2009).

Beside the toxicity testing, during the drug development process, drug interaction studies are also important so as to assure the safety of a new compound. Generally, drug interaction can occur from either the pharmacokinetic or the pharmacodynamic basis. Metabolism is the most etiology of pharmacokinetic drug interaction. Phase I metabolism catalyzed by cytochrome P450 (CYPs) enzymes is mostly involved in drug interaction. CYPs are found primarily in the liver and small content in other organs such as kidneys, skin, gut, lung, etc. Human CYPs are grouped into 18 families and 39 subfamilies. The major CYPs are in family 1, 2 and 3 which are responsible for xenobiotic metabolism. Such isoforms include CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 (Coleman, 2005). Like other enzymes, CYPs can be inhibited and some isoforms can be induced by exogenous compounds or even other internal factors. Inhibition and/or induction of CYPs that catalyze many currently used medicines are important causes of drug interactions. For the inhibition inhibitors can either inhibit the enzymes either reversibly or irreversibly depending upon the type of inhibitors. Ketoconazole, imipramine, miconazole, etc. are examples of known reversible inhibitors of CYP

activities (Coleman, 2005) whereas ticlopidine, erythromycin, verapamil etc. are known irreversible inhibitors of CYP activities (Obach et al., 2007).

In an attempt to evaluate the drug interaction potential of ECa233, effects of ECa233 on CYP enzymes were investigated in the animals *in vivo* in rats and *in vitro* using rats liver micosomes (Kornphimol Kulthong et al., 2009) as well as *in vitro* using recombinant human CYP (Pitchayapa Seeka, 2008). In the *in vivo* study in rats, ECa233 did not affect CYP1A1, CYP 1A2, CYP 2B1/2B2, CYP 2E1 and CYP 3A activities. However, ECa233 inhibited CYP2B1/2B2 activities in the *in vitro* study using rat liver microsomes (Kornphimol Kulthong et al., 2009). In the *in vitro* study using recombinant human CYPs, ECa233 did not inhibit CYP1A2, CYP2C9, CYP2D6 and CYP2E1 activities but inhibited CYP2B6 (IC_{50} = 871.14 µg/ml), CYP2C19 (IC_{50} = 365.18 µg/ml) and CYP3A4 (IC_{50} = 210.98 µg/ml) (Pitchayapa Seeka, 2008). These preliminary data indicated the possibility that ECa233 had a potential to cause drug-interaction with drugs that are metabolized by CYP2B6, CYP2C19 and CYP3A4. Moreover, ECa233 possessed a potential possibility to decrease risks of toxicity/mutagenesis/carcinogenesis from the compounds that are bioactivates by these CYP isoforms.

As mentioned earlier, ECa233 contains more than 80% of asiaticoside and madecassoside. To elaborate the effect of ECa233 on CYPs whether the inhibitory effects were attributed from these 2 triterpene glycoside, the aim of this study was to investigate the inhibitory effects of both asiaticoside and madecassoside on human CYPs using recombinant human CYPs in an *in vitro* study. In case that any CYP isoforms were inhibited, the type of inhibition was further investigated whether the inhibition was mechanism-based or reversible. If the inhibition is reversible, the type of reversible inhibition was determined whether the inhibition was competitive, non-competitive or uncompetitive.

Hypothesis

Asiaticoside and madecassoside exhibited an inhibitory effect on human CYPs activities.

Study design and process

Experimental design: *In vitro* studies were performed as following

1. Inhibitory effects of asiaticoside and madecassoside on human CYP activities using recombinant human CYPs (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) were investigated.
2. Mechanism-based inhibition studies of asiaticoside and madecassoside were performed.
3. Kinetic assay study of asiaticoside and madecassoside was performed to characterize the type of reversible inhibition.
4. Data analysis

Expected benefits from the study

1. A preliminary data showing the inhibitory effects of asiaticoside and madecassoside on major human CYPs was obtained. The information will indicate the possibility of drug-drug interaction between asiaticoside or madecassoside and the currently used medicines.
2. The type of inhibition of asiaticoside and madecassoside would be a beneficial precaution information for the administration these compound.

CHAPTER II

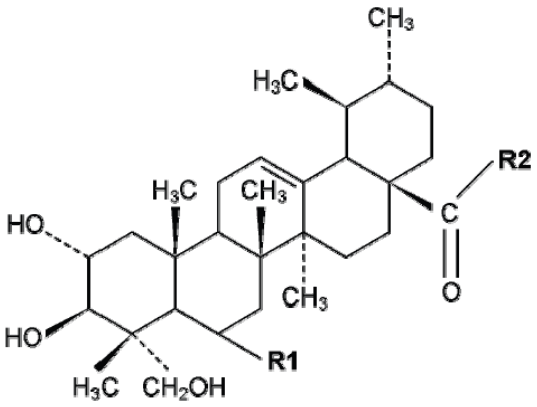
LITERATURE REVIEWS

Centella asiatica (L.) Urban is a member of Umbelliferae family. It flourishes abundantly in moist areas and distributes widely in tropical and subtropical countries up to an altitude of 1,800 m. The plant is clonally propagated by producing stolons that are characterized by long internodes and nodes, on which are borne reniform-cordate leaves and sessile flowers in simple umbels (Figure 1). It is known as Gotu kola in Chinese, Indian Pennywort in English and Bua bok in Thai. It is an important folk medicinal herb by natives of Asia, southern and middle Africa, southeastern United States and Australia, with a long history of therapeutic uses since ancient times.

C. asiatica is claimed to possess a wide range of pharmacological effects such as wound healing, anti-inflammatory, antirheumatic, dermatological, peripheral vasodilator and vulnerary properties. It has also been used in the treatment of inflammations, diarrhea, asthma, tuberculosis, various skin lesions and leprosy, lupus, psoriasis and keloid. In addition, numerous clinical reports demonstrated the ulcer preventive, anti-depressive and sedative effects of *C. asiatica* preparations, as well as their ability to improve venous in sufficiency and microangiopathy (Zheng and Qin, 2007).

C. asiatica contains various compounds such as amino acids, flavonoids, terpenoids, volatile oils, etc. Triterpenoids are major and the most important components of this plant. The triterpenes obtained from this plant are mainly pentacyclic triterpenic acid and their respective glycosides, principally asiatic acid, asiaticoside, madecassic acid and madecassoside. The structures of these compounds were shown in Figure 2. These compounds are the active ingredients of *C.asiatica*. Asiaticoside and madecassoside have also been studied for the pharmacological activities.

Figure 1 *Centella asiatica* (L.) Urban



Asiatic acid	R_1 = H	R_2 = H
Madecassic acid	R_1 = OH	R_2 = H
Asiaticoside	R_1 = H	R_2 = Glucose-Glucose-Rhamnose
Madecassoside	R_1 = OH	R_2 = Glucose-Glucose-Rhamnose

Figure 2 Chemical structures of pentacyclic triterpenes from *C. asiatica*

(Jia and Lu et al., 2008)

Pharmacological effects of asiaticoside and madecassoside

1. **Wound healing effect**

Asiaticoside was reported to possess wound-healing ability. It had a stimulating effect on the epidermis and promoted keratinisation. It was thought to act

by an inducing action on the synthesis of collagen and mucopolysaccharides in connective tissue (Barnes et al., 2006).

The activity of asiaticoside was studied in normal as well as delayed-type wound healing. In guinea pig punch wounds, topical applications of 0.2% solution of asiaticoside produced 56% increased in hydroxyproline, 57% increased in tensile strength, increased collagen content and better epithelisation. In streptozotocin diabetic rats of which where healing was delayed, topical application of 0.4% solution of asiaticoside over punch wounds increased hydroxyproline content, tensile strength, collagen content and epithelisation thereby facilitating the healing. Asiaticoside was also actived by the oral route at 1 mg/kg in the guinea pig punch wound model. Asiaticoside 40 mg/disk concentration enhanced angiogenesis in the chick chorioallantoic membrane model (Shukla et al., 1999).

The application of asiaticoside at low doses of 10^{-8} to 10^{-12}% (w/w) facilitated burn wound repair. The topical application of low doses (10 pg, 1 ng, or 100 ng/wound area) of asiaticoside increased monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and interleukin (IL)-1ß levels in burn wound exudates. Asiaticoside (10 pg to 100 ng/ml) enhanced MCP-1 production in HaCaT cells, but it had no direct effect on VEGF production. Furthermore, asiaticoside (10 pg to 100 ng/ml) increased the IL-1ß production in THP-1 macrophages with MCP-1, but it had no effect on IL-1ß production without MCP-1 or with lipopolysaccharide (LPS). These results showed that the improvement of burn wound healing by asiaticoside might be due to the promotion of angiogenesis during skin wound repair as a result of the stimulation of VEGF production caused by the increase in MCP-1 expression in keratinocytes and the increase in IL-1ß expression in macrophages induced cooperatively by asiaticoside plus MCP-1 (Kimura et al., 2008).

Liu et al. (2008) studied the effect of madecassoside on burn wound healing in mice and its possible mechanism of action. Oral administration of madecassoside (6, 12, 24 mg/kg) facilitated wound closure in a time-dependent manner and reached its

peak effect, nearly completely wound closure, on day 20 in the group receiving the highest dose of 24 mg/kg of madecassoside. Madecassoside at the doses of 12 and 24 mg/kg decreased nitric oxide levels and malondialdehyde content in the burn skin tissue but reduced glutathione whereas hydroxyproline levels were increased in the same skin tissue. In addition, madecassoside enhanced skin angiogenesis. These results concluded that madecassoside had significant wound-healing activity. Mechanisms of madecassoside on wound healing included antioxidative activity, collagen synthesis and angiogenesis.

Wound healing properties of madecassoside were studied by combining with copper, zinc, and manganese salts that were frequently used for their antibacterial and epidermal repair properties. This study was performed for 22 days with daily topical application, in 12 healthy volunteers of 25.2 yrs average age, The effects of this emulsion on wound healing were determined after roof removal of section blister on volar forearm areas. The results showed that madecassoside associated with copper/zinc/manganese salts significantly decreased the inflammatory reaction. A significantly better and faster re-epidermization was obtained. After 22 days of treatment, the wounds were significantly less visible on the areas treated with madecassoside combined with copper/zinc/manganese salts (Rougier and Humbert, 2008).

2. Protective effect on gastric ulcer formation

Cheng et al. (2004) studied the healing effects of *C. asiatica* water extract and asiaticoside on acetic acid induced gastric ulcers in rats. Different concentrations of *C. asiatica* extract and asiaticoside were orally administered to rats with gastric ulcers. They were found to reduce the size of the ulcers at day 3 and 7 in a dose-dependent manner, with a concomitant attenuation of myeloperoxidase activity at the ulcer tissues. In addition, they were found to promote epithelial cell proliferation and angiogenesis. The expression of basic fibroblast growth factor, an important

angiogenic factor, was also upregulated in the ulcer tissues in rats treated with *C. asiatica* extract or asiaticoside.

3. Anti-inflammatory activity

Madecassoside was examined for the protection against myocardial reperfusion injury in rabbit heart *in vivo*. The ischemia reperfusion model was established. Treatment with madecassoside (3.2, 1.6 and 0.8 mg/kg) i.v. during ischemia reperfusion injury attenuated myocardial damage which was characterized by the decreasing of infarct size, decreasing of lactic dehydrogenase (LDH) and creatinine kinase (CK) release. Activities of superoxide dismutase (SOD) were diminished and malondialdehyde (MDA) level was increased obviously in control group whereas pretreatment with madecassoside significantly blunted the decrease of SOD activity, markedly reduced the levels of MDA, C-reactive protein (CRP) and cardiomyocyte apoptosis as well as upregulated the expression of Bcl-2. It was concluded that madecassoside exhibited protective effect against myocardial ischemia reperfusion injury, anti-lipid peroxidation, enhancement of SOD activity, anti-inflammatiory and anti-apoptosis (Li et al., 2007).

Guo et al. (2004) studied effects of *C. asiatica* water extract and asiaticoside on the expression and activity of inducible nitric oxide synthase (iNOS) during gastric ulcer healing in rats. Different concentrations of *C. asiatica* extract (0.10 g/kg and 0.25 g/kg) and asiaticoside (5 mg/kg and 10 mg/kg) were orally administered to rats with acetic acid-induced gastric ulcers. They were found to reduce the size of the ulcers at days 1, 3 and 7 after ulcer induction in a dose-dependent manner, with a concomitant attenuation of iNOS activity and protein expression at the ulcer tissues. The levels of nitrite and nitrate ($NO_{(X)}$-), the stable end-products of nitric oxide (NO), in the gastric ulcer tissues were also decreased. N-[3(aminomethyl) benzyl]acetamidine, a highly selective inhibitor of iNOS, was found to produce similar but more potent inhibition on iNOS activity at a dose of 0.1 mg/kg. These findings

suggested that *C. asiatica* water extract and asiaticoside had an anti-inflammatory property that was brought about by inhibition of NO synthesis and thus facilitates ulcer healing.

4. Anti-tumor activity

The MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay was used to evaluate inhibitory effect of asiaticoside combined with vincristine on proliferation of several cancer cell lines. The median inhibition concentration (IC_{50}) values of asiaticoside for KB, KBv200, MCF-7, and MCF-7/ADM cells detected by MTT assay were 1.11±0.13 mg/ml, 1.82±0.08 mg/ml, 1.58±0.15 mg/ml and 3.25±0.46 mg/ml, respectively. Multidrug resistant KBv200 and MCF-7/ADM cancer cells displayed similar sensitivity to asiaticoside as their parental counterparts (KB, and MCF-7 cells). Moreover, asiaticoside induced apoptosis of KB cells. At sub-cytotoxicity concentration, asiaticoside showed a synergistic effect with vincristine in these 4 cell lines. The apoptosis rates were much higher in asiaticoside plus vincristine groups than in the vincristine or the asiaticoside groups. Bcl-2 phosphorylation levels were higher in the combination group than in the vincristine or the asiaticoside alone groups. The activated caspase-3 protein was only presented in the combination group. Asiaticoside plus vincristine enhanced S-G (2)/M arrest, up-regulated Cyclin B1 protein expression, and down-regulated P34 (cdc2) protein expression in KB cells. These results showed that asiaticoside induced apoptosis and enhance anti-tumor activity of vincristine in cancer cells which might be useful in cancer chemotherapy (Huang et al., 2004).

5. Anxiolytic effect

The anxiolytic activity of asiaticoside was examined in male mice by using a number of experimental paradigms of anxiety, with diazepam as a positive anxiolytic control. In the elevated plus-maze test, diazepam (1 and 2 mg/kg) or asiaticoside (5 or

10 mg/kg) increased the percentage of entries into open arms and the percentage of time spent on open arms. In the light/dark test, as with 1 mg/kg diazepam, asiaticoside (10 and 20 mg/kg) increased the time spent in the light area and the movement in the light area without altering the total locomotor activity of the animals. In the hole-board test, asiaticoside at 10 mg/kg significantly increased head-dipping counts and duration similar to diazepam (0.3 mg/kg). These findings concluded that asiaticoside exhibited an anxiolytic-like effect (Chen et al., 2006).

Liu et al. (2004) suggested that madecassoside demonstrated antidepressant effects through monoamine oxidase (MAO) inhibition in rat brain. Effects of madecassoside on the depression behavior of mice were observed by forced swimming test and reserpine antagonist test. Madecassoside at the dosage of 10, 20 and 40 mg/kg intragastrically significantly reduced the immobility time of mice in forced swimming test. With acute administration (3 days), all three dosages of madecassoside significantly inhibited the activity of MAO-A in hippocampus. The high dosage (40 mg/kg) of madecassoside significantly inhibited the activity of MAO-A in hypothalamus, while all the 3 dosages had no significant effects on the activity of MAO-A in cortex. With chronic administration (21 days), three dosages of madecassoside had no significant effects on the activities of MAO-A in cortex and hypothalamus but the high dosage significantly enhanced the activity of MAO-A in hippocampus. With acute administration, madecassoside at dosages of 10 and 20 mg/kg significantly inhibited the activity of MAO-B in cortex. Madecassoside in dosage of 10 mg/kg significantly inhibited the activity of MAO-B in hypothalamus while madecassoside at the dosage of 20 mg/kg significantly enhanced the activity of MAO-B in hippocampus. With chronic administration, madecassoside of 3 dosages produced no significant effects on the activities of MAO-B in 3 different rat brain regions.

6. Anti-rheumatoid arthritic effect

Liu et al. (2008) studied the therapeutic potential and the underlying mechanisms of madecassoside on collagen II (CII)-induced arthritis (CIA) in mice. Madecassoside (10, 20 and 40 mg/kg) was orally administered to mice from the day of the antigen challenge for twenty consecutive days. The results showed that madecassoside relieved the severity of the disease dose-dependently as charadterized by the reduced clinical scores and the elevation of body weights of the mice. Histopathological examination indicated that madecassoside relieved the infiltration of inflammatory cells and synovial hyperplasia as well as protected joint destruction. Moreover, madecassoside reduced the serum level of anti-CII IgG, suppressed the delayed type hypersensitivity against CII in ears, and moderately suppressed CII-stimulated proliferation of lymphocytes from popliteal lymph nodes in CIA mice. In an *in vitro* study, madecassoside was ineffective in the activation of macrophages caused by lipopolysaccharide. It was concluded that madecassoside substantially prevented mouse CIA and might be the major active constituent of *C. asiatica* that was responsible for the clinical uses for rheumatoid arthritis. The underlying mechanisms of action may be mainly through regulating the abnormal humoral and cellular immunity as well as protecting joint destruction.

Xenobiotic Biotransformation

Xenobiotics are the foreign compounds that humans are exposed daily. They are absorbed across the lungs, skin, or more commonly, ingested either unintentionally or intentionally as compounds presented in food, drink or drugs for therapeutic. Xenobiotic biotransformation or metabolism is a metabolic process that alters the chemical structure of xenobiotics, which are foreign compounds leading to termination or alteration of biological activity. In general, lipophilic xenobiotics are transformed to more polar and hence more readily excretable products. The principal organs of metabolism include liver, kidneys and the GI tract, but drugs may be metabolized at other sites, including lungs, plasma, etc.

Biotransformation reactions are classified as phase I or functionalisation reactions, and phase II or conjugation reactions. Phase I reactions introduce a new functional group on the parent compound, generally resulting in loss of pharmacological activity; however, active and chemically reactive intermediates may also be generated. Phase II conjugation reactions lead to the formation of a covalent linkage between a functional group on the parent compound (or on a phase I metabolite) with endogenously derived glucuronic acid, sulphate, glutathione, amino acids or acetate. These highly polar conjugates are generally inactive and are excreted rapidly in the urine and faeces.

Phase I metabolism

Phase 1 metabolism includes oxidation, reduction, hydrolysis and hydration reactions, as well as other rarer miscellaneous reactions. Oxidation is the most common type of biotransformation. It includes side-chain hydroxylation, aromatic hydroxylation, deamination, N-, O-, and S-dealkylation, sulphoxide formation, dehydrogenations, and deamination of mono- and diamines. Reduction is relatively uncommon. It includes reduction of nitro, nitroso, and azo groups. Hydrolysis is a common biotransformation route for esters and amides. Hydration can be regarded as a

specialised form of hydrolysis where water is added to the compound without causing the compound to dissociate. In most cases, the final product contains a chemically reactive functional group, such as -OH, $-NH_2$, -SH, -COOH, etc., which are further acted upon by the phase II or conjugative enzymes.

Oxidations involving cytochrome P450 (the microsomal mixed-function oxidase)

Phase I metabolism is dominated by the mixed-function oxidase (MFO) system and this is known to be involved both in the metabolism of endogenous compounds (steroid hormones, thyroid hormones, fatty acids, prostaglandins and derivatives) as well as in the biotransformation of drugs (or other xenobiotics). The mixed-function oxidase system found in microsomes (endoplasmic reticulum) of many cells (notably those of liver, kidney, lung and intestine) performs many different functionalisation reactions. All of these reactions require the presence of molecular oxygen and NADPH as well as the complete mixed-function oxidase system [cytochrome P450 (CYP), NADPH-cytochrome P450 reductase and lipid]. All reactions involve the initial insertion of a single oxygen atom into the drug molecule. A subsequent rearrangement and/or decomposition of this product may occur, leading to the final products seen. The general cytochrome P450-catalysed reaction is:

$$NADPH + H^+ + O_2 + R\mathbf{H} \xrightarrow{CYP} NADP^+ + H_2O + R\mathbf{OH}$$

Where RH represents an oxidisable drug substance and ROH, the hydroxylated metabolite. As can be seen from the above reaction, reducing equivalents (derived from NADPH + H^+) are consumed and only one atom of the molecular oxygen is incorporated into the substrate generating the hydroxylated metabolite, whereas the other oxygen atom is reduced to water (Ionescu and Caira, 2005).

Components of the MFO system

Components of the MFO system include the CYP, the NADPH- cytochrome P450 reductase and lipids.

CYP

CYP is the terminal oxidase component of an electron transfer system presented in the endoplasmic reticulum and responsible for the oxidation of almost all drugs. CYP is classified as a haem-containing enzyme (a haemoprotein) with iron protoporphyrin IX as the prosthetic group as shown in Figure 3. Mammalian CYPs are found mostly in the endoplasmic reticulum but also in mitochondria. In contrast to the porphyrin moiety, which is constant, the protein part of the enzyme varies markedly from one isoenzyme to the other subsequently determining differences in their properties, substrate specificities and sensitivity to the inhibitors as well as inducers (Gibson and Skett, 2001).

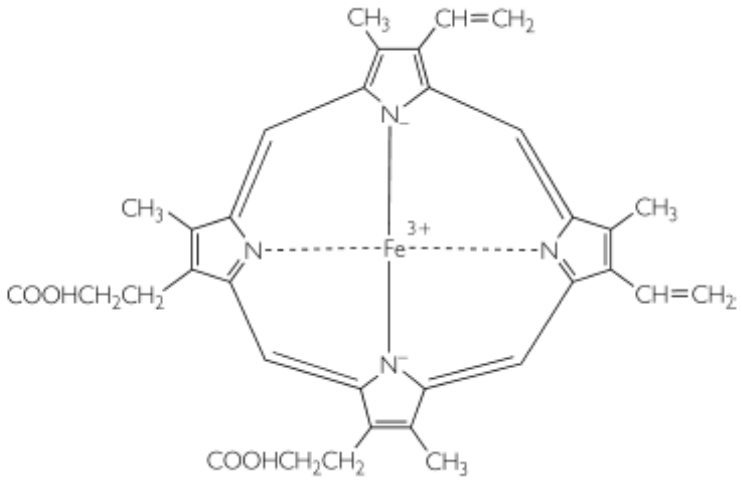


Figure 3 Structure of ferric protoporphyrin IX, the prosthetic group of CYP (Gibson and Skett, 2001)

CYPs exist as multiple forms of monomeric molecular weight of approximately 45,000–55,000 daltons. The haem of CYP is non-covalently bound to the apoprotein and serves as the locus for oxygen binding/activation and the binding site for some, but not all drugs. CYPs are classified into families and subfamilies by

the similarity of their amino acid sequence. Within a given CYPs gene family, all the component genes in that family are greater than 40% identical in their sequence to each other. Individual gene families are further sub-divided into gene sub-families where the nomenclature dictates that two CYPs belong to the same gene sub-family when they are 70% (or greater) similar in their sequence. Over 50 human CYPs have now been isolated, unequivocally identified and characterised to date, including the steroid hydroxylases (e.g. CYP11A1, CYP21A2, etc.) and the major human liver drug-metabolising CYP including CYP1A2, CYP2A6, CYP2C8/9, CYP2C18/19, CYP2E1, CYP2D6 and CYP3A4 (Figure 4).

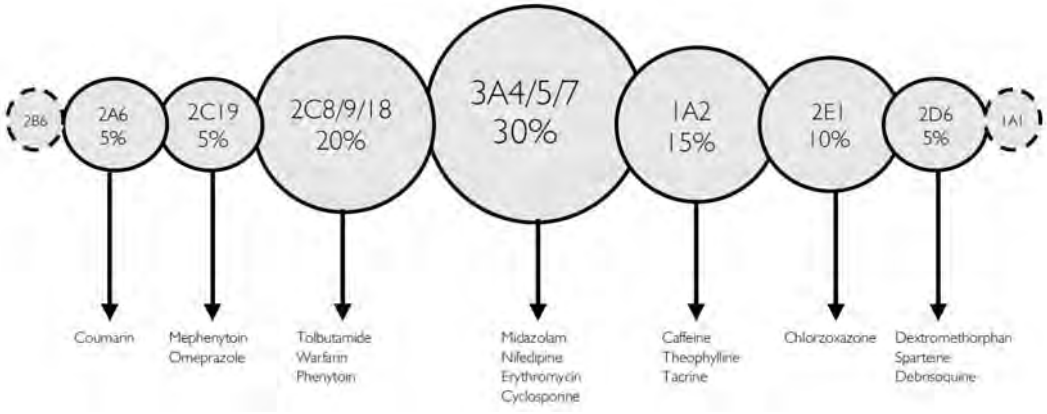


Figure 4 Human liver CYPs and their representative substrates (Gibson and Skett, 2001)

CYP1A2 originates from a gene on chromosome 15 in humans and it is linked with oestrogen metabolism, as it is capable of oxidizing this series of hormones. Increased levels of this enzyme are also associated with colon cancer. CYP1A2 oxidizes planar aromatic molecules that contain aromatic amines. CYP1A2 orientates aromatic amines, some of which are quite large, in such a way as to promote the oxidation of the amine group. Consequently, this enzyme is able to metabolize a variety of drugs that resemble aromatic amines: these include caffeine, theophylline, etc. CYP1A2 tends to be inhibited by molecules that are planar and possess a small

volume to surface area ratio. It is blocked by the methylxanthine derivative furafylline and miconazole. It is inducible by polycyclic aromatic hydrocarbons and amines.

CYP2C9 has evolved to process relatively small, acidic and lipophilic molecules, although no basic amino acid residues have been found in the active site to attract and bind acidic molecules. There are a large number of substrates for this CYP, which include tolbutamide, dapsone, warfarin, etc. The active site is large and it appears that there is more than one place where drugs can bind. With warfarin, there appears to be a primary recognition site, which is too far from the catalytic area of the enzyme (the iron) for the drug to be metabolized. It has been suggested that the events of the catalysis of the substrate automatically promote binding of the next substrate molecule, possibly through allosteric mechanisms. Sulphafenazole is a potent inhibitor and miconazole also inhibits this CYPs. It is inducible by rifampicin and phenobarbitone.

CYP2C19 is also inducible and differs by only around 10 per cent of its amino acids from CYP 2C9. But CYP2C19 does not oxidize acidic molecules, indicating that the active sites and access channels are subtly different. CYP2C19 metabolizes omeprazole, a common ulcer medication, barbiturates, citalopram, mephenytoin, etc. CYP2C19 is inducible by rifampicin and polymorphisms in this gene exist. Tranylcypromine and miconazole act as potent inhibitors of CYP2C19.

CYP2D6 is responsible for more than 70 different drug oxidations. This enzyme is non-inducible, but is strongly subjected to polymorphisms. Since there may be no other way to clear these drugs from the system, poor metabolizers may be at severe risk from adverse drug reactions. Relatively little is found in the gut, and it comprises about 2–4 per cent of the CYPs in human liver. There are several groups of drugs that are metabolized by CYP2D6; these include antiarrhythmics, tricyclic antidepressants, selective serotonin reuptake inhibitor, antipsychotic, beta-blockers and analgesics. Quinidine and miconazole inhibit this enzyme.

CYP2E1 comprises around 7 percent of human liver CYP. It oxidizes small heterocyclic agents, ranging from pyridine through to ethanol, acetone and other small ketones (methyl ethyl ketone). Ethanol and acetone are strong inducers of this isoform. Many of its substrates are water-soluble and it is often implicated in toxicity, as the metabolites it forms can be highly reactive and toxic to tissues. It is responsible for the oxidation of paracetamol. There is a polymorphism associated with this gene that is more common in Chinese people. The mutation correlates with a two-fold increased risk of nasopharyngeal cancer linked to smoking. Many sulphur-containing agents block this enzyme, such as carbon disulphide, diethyl dithio carbamate. Imipramine also inhibits this CYP.

CYP3A4 are responsible for the metabolism of more than 120 drugs. CYP3A4 comprises around 28 per cent of human total CYPs. It is also found in the intestinal walls in considerable quantity. Its major endogenous function is to metabolize steroids, but its active site is so large and flexible that a vast array of different molecules can undergo at least some metabolism by this enzyme. There are many substrates of this CYP such as cyclosporine, paracetamol, codeine, diazepam, erythromycin, lidocaine, lovastatin, taxol, warfarin, etc. CYP3A4 are inducible by many anticonvulsants such as phenobarbitals, carmabazepine and phenytoin; rifampicin, St John's Wort, etc. Azole antifungal agents such as ketoconazole and fluconazole as well as anti-HIV agents such as ritonavir are reported to inhibit CYP3A4 (Coleman, 2005).

NADPH-cytochrome P450 reductase

NADPH-cytochrome P450 reductase is a flavin-containing enzyme, consisting of one mole of FAD (flavin adenine dinucleotide) and one mole of FMN (flavin mononucleotide) per mole of apoprotein. The enzyme exists in close association with CYP in the endoplasmic reticulum membrane. It represents an essential component of the MFO system in that the flavoprotein transfers reducing equivalents from NADPH + H^+ to CYP as shown in Figure 5.

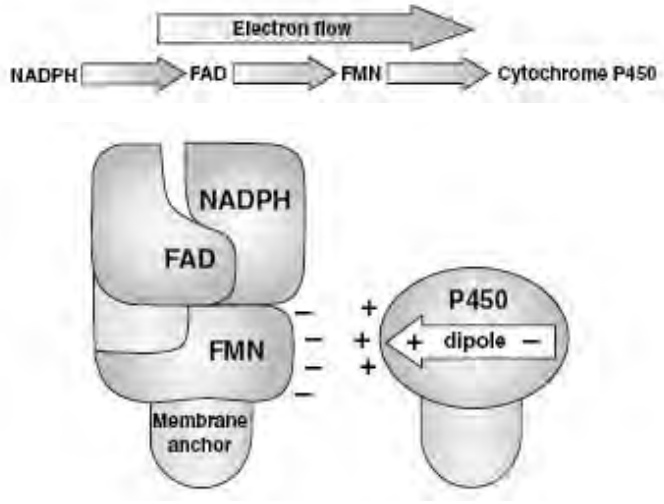


Figure 5 Position of NADPH-cytochrome P450 reductase in relation to CYP enzyme and the direction of flow of electrons necessary for CYP catalysis (Coleman, 2005)

NADPH-cytochrome P450 reductase is thought to act as "a transducer" of reducing equivalents by accepting electrons from NADPH and transferring them sequentially to CYP (Gibson and Skett, 2001).

Lipid

The lipid component was originally identified as phosphatidylcholine. Later studies showed that fatty acid composition of the phospholipids could be critical in determining functional reconstitution of MFO activity. It has been suggested that lipid may be required for substrate building, facilitation of electron transfer or even providing a "template" for the interaction of CYP and NADPH-cytochrome P450 reductase molecules. Nevertheless, it must be stressed that the precise mode of action of lipids is still unknown (Gibson and Skett, 2001).

Enzyme Kinetics

The fundamental of the kinetic characterization of enzymatic reactions has been explained by the Michaelis-Menten equation as following:

$$v = \frac{Vmax[S]}{Km+[S]} \qquad \text{Eq. 1}$$

In this case, v is the velocity of the reaction, [S] is the substrate concentration, Vmax (also known as V or Vm) is the maximum velocity of the reaction. Vmax is only an ‘‘estimation’’ of the maximum velocity of the reaction, since the true maximum velocity is never reached at a finite substrate concentration. Km or the Michaelis constant is operationally defined as the concentration of substrate at which half-maximal velocity of the reaction is achieved. From this equation, quantitative descriptions of enzyme-catalyzed reactions, in terms of rate and concentration, can be made. Data that is described by the Michaelis-Menten equation takes the shape of a hyperbola when plotted in two-dimensional fashion with velocity as the y-axis and substrate concentration as the x-axis (Figure 6).

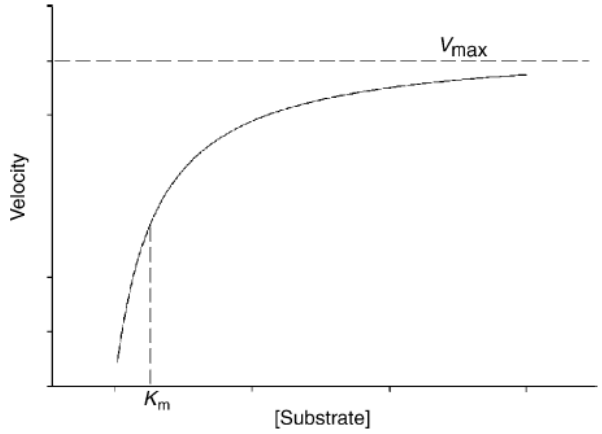


Figure 6 Hyperbolic plot of a reaction following Michaelis-Menten kinetics (Zhang et al., 2008)

Generally, the most commonly used graphical plot in analyzing enzyme kinetic data is the double-reciprocal plot, as called Lineweaver-Burk plot (Figure 7). In this analysis the Michaelis-Menten equation is rewritten so that the results can be plotted as a straight line where 1/v is plotted along the y-axis and 1/[S] along the x-axis. In this plot, the slope of the line best fitting the data points is equal to Km/Vmax, the y-intercept is equal to 1/Vmax and the x-intercept is equal to -1/Km. However, this type of plotting suffers from potentially large errors in 1/v. At very small values of v, even small errors lead to very large errors in 1/v.

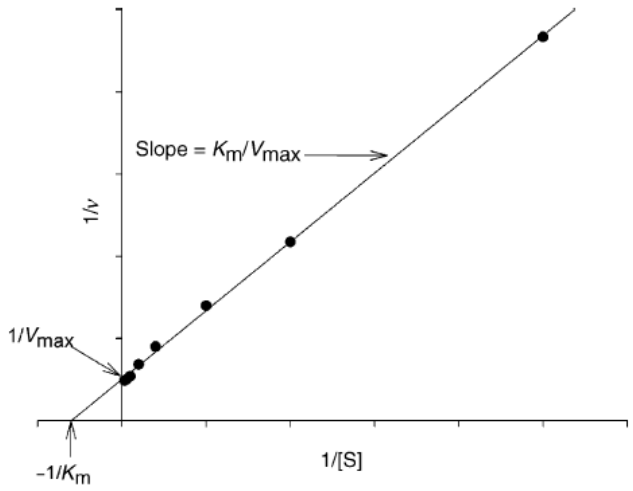


Figure 7 Lineweaver-Burk (double-reciprocal) plot of a reaction that follows Michaelis-Menten kinetics (Zhang et al., 2008)

Another type of graphical plot used to determine kinetic values is the Hanes-Woolf plot (Figure 8). The Hanes-Woolf plot avoids taking reciprocal concentrations. [S]/v is plotted along the y-axis and [S] is plotted along the x-axis. In this plot, the slope of the line best fitting the data points is equal to 1/Vmax, the y-intercept is equal to Km/Vmax and the x-intercept is equal to -Km. This plot suffers less from the error issues of Lineweaver-Burk plot. Therefore, this type of plotting may be the most preferred graphical plot for estimating kinetic values.

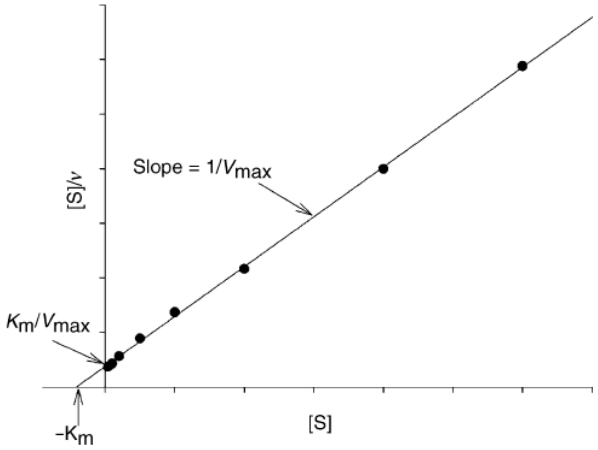


Figure 8 Hanes-Woolf plot of a reaction that follows Michaelis-Menten kinetics (Zhang et al., 2008)

The graphical plot for determining inhibition constant (Ki) is the constructed plots with intercepts (1/Vmax) or slopes (Km/Vmax) of double-reciprocal graphs and inhibitor concentrations. The so-called second plots, intercepts (1/Vmax) or slopes (Km/Vmax) are plotted along the y-axis and [I] plotted along the x-axis (Figure 9). A perpendicular from the point of intersection to x-axis gives an estimate of the negative of Ki (Zhang et al., 2008).

a)

b)

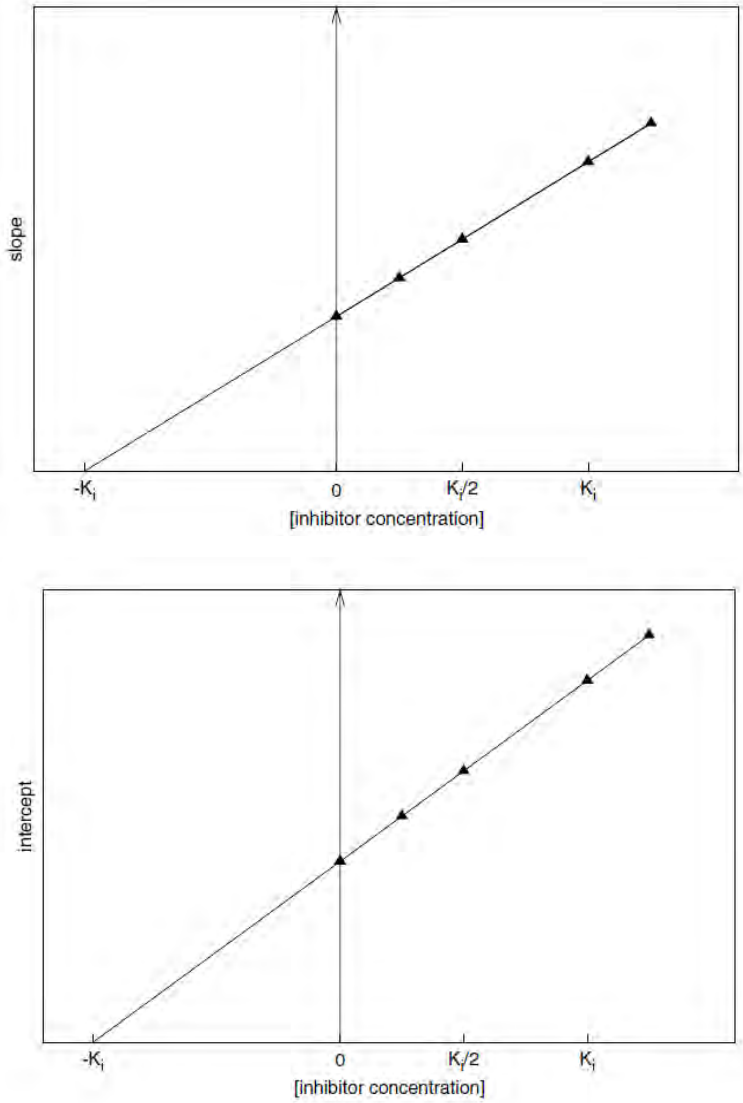


Figure 9 The second plot of the slopes (a) or the intercept (b) of Lineweaver-Burk plots at various inhibitor concentrations. For competitive and noncompetitive inhibition, the second plot of the slopes is used. For uncompetitive inhibition, the second plot of the intercepts is used (Smith and Simons, 2005).

Inhibition of drug metabolism

Inhibition of drug metabolism by pre- or co-administration of other drugs or xenobiotics is reflected by exacerbated pharmacodynamic activity or adverse effects of relative overdosing at the usual therapeutic doses. In the context of the common practice of polypharmacy, another topic of great interest arises, namely drug-drug interaction. This inhibition of drug metabolism by drugs or xenobiotics can take place in several ways, including the destruction of preexisting enzymes, inhibition of enzyme synthesis or by complexing and thus inactivating the drug metabolising enzyme. Thus, the inhibition of CYPs is clinical importance for both therapeutic and toxicological reasons.

Mechanism of CYP inhibition

CYP Inhibition can occur through two main processes: reversible inhibition, including competitive, noncompetitive, uncompetitive and mixed (Figure 10), and irreversible inhibition or mechanism-based inhibition (Figure 11). Which type of inhibition occurs with various drugs can depend on many factors, such as drug concentration and the characteristics of a particular CYP isoform. Many drugs can act as competitive inhibitors with one CYP and non-competitive with others.

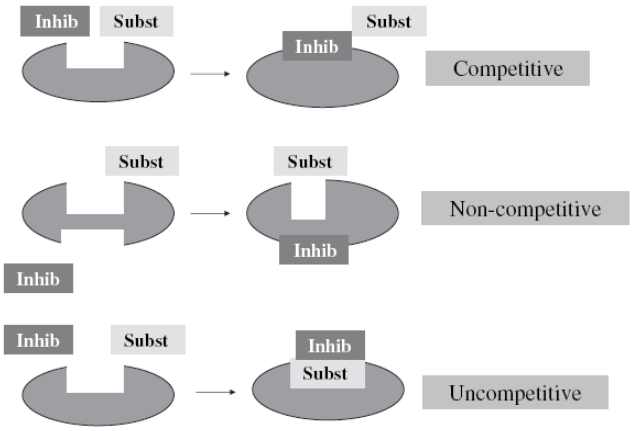


Figure 10 Main types of reversible enzyme inhibition (Coleman, 2005)

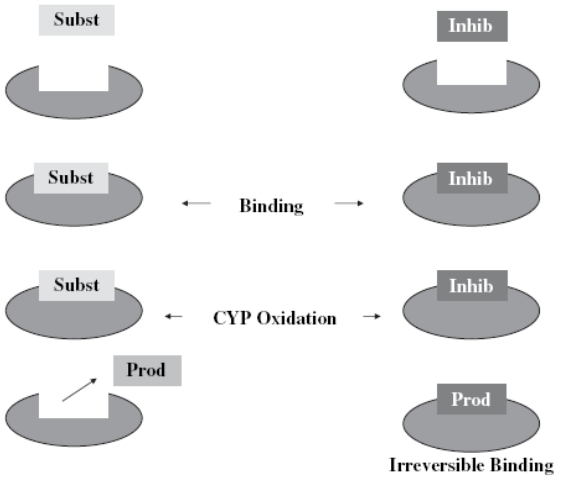


Figure 11 Scheme of normal substrate CYP binding (left) and mechanism-based inhibition (right) (Coleman, 2005)

Competitive inhibition

The most commonly observed type of enzyme inhibition is that of competitive inhibition. In this case, both substrate and inhibitor are competing for the enzyme active site and their binding is mutually exclusive. When the inhibitor is bound to the enzyme, the complex is incapable of reacting (turning over substrate). As shown by the Lineweaver-Burk plot, competitive inhibition has no effect on Vmax but Km is increased (Figure 12).

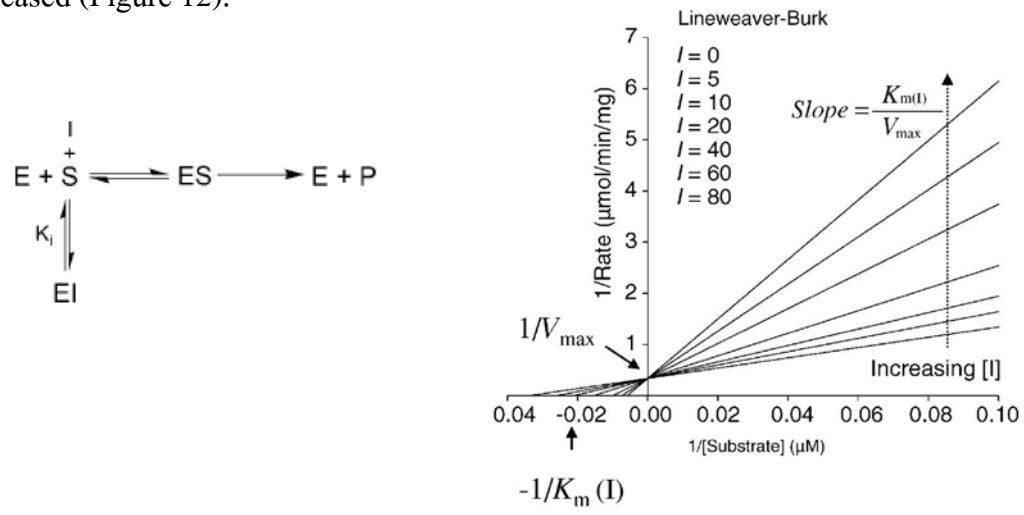


Figure 12 Scheme of and the Lineweaver-Burk plots of competitive inhibition (Zhang et al., 2008)

Noncompetitive Inhibition

Non-competitive inhibition does not involve the inhibitor and substrate competing for the same active site. There is another site, known as the allosteric site, which is distant from the active site. Once a ligand binds this allosteric site, the conformation of the active site is automatically changed and it becomes less likely to bind the substrate and product formation tails off. This process of allosteric binding is another example of the endogenous control of product formation, perhaps by another product/substrate from a related or similar pathway. The net result is to slow or even halt product formation at the main site, depending on how much allosteric binding occurs. The Lineweaver-Burk plot will show a falloff in Vmax (enzyme cannot run at maximal rate) but Km does not change, that is, the affinity of the substrate for the site is unchanged (Figure 13).

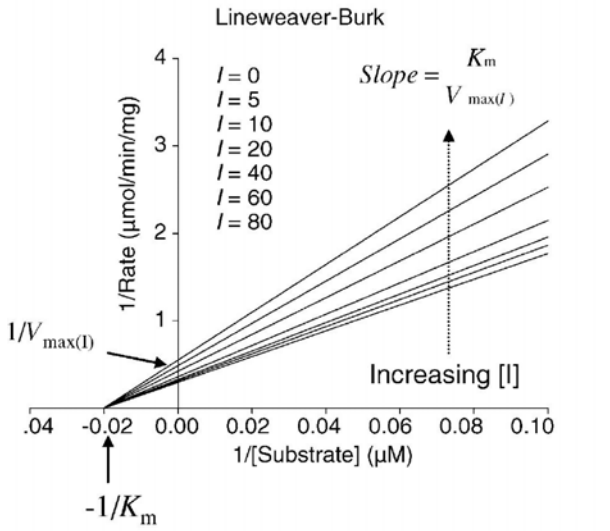


Figure 13 The Lineweaver-Burk plots of noncompetitive inhibition

(Zhang et al., 2008)

Uncompetitive Inhibition

This is an unusual form of inhibition, where the inhibitor binds only to the enzyme/substrate complex. This has the effect of stimulating enzyme/substrate complex formation so increasing affinity (fall in Km), although the enzyme/substrate/inhibitor complex is non-functional, so the Vmax falls. This appears

to be a relatively rare form of inhibition of human CYPs by therapeutic drugs. However some dietary agents such as the flavonoid tangeretin, found in citrous fruits, are an uncompetitive inhibitor of CYP3A4 in human liver microsomes. The Lineweaver-Burk plot will show a falloff in Vmax but Km is decreased (Figure 14).

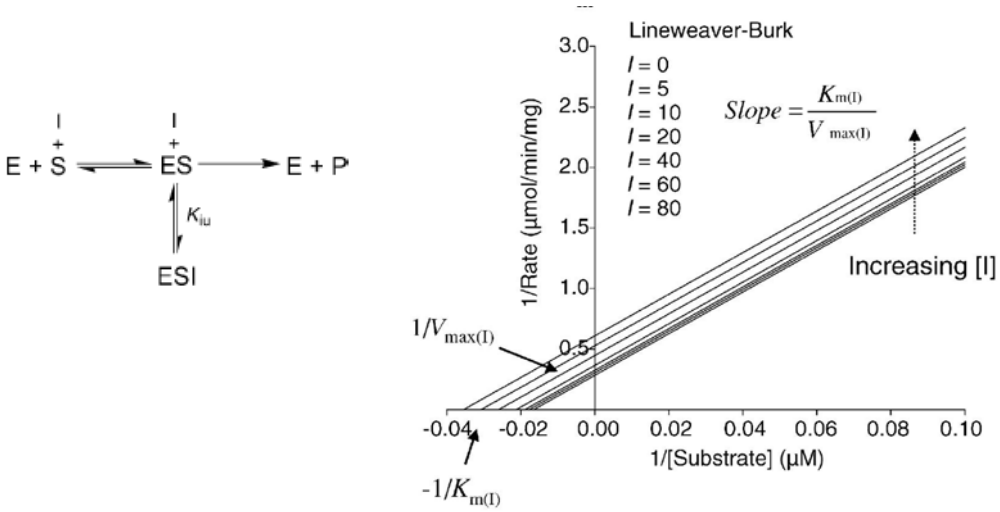


Figure 14 Scheme and the Lineweaver-Burk plots of uncompetitive inhibition (Zhang et al., 2008)

Mixed Inhibition

In the case of mixed inhibition, both the maximal velocity and the concentration at which half-maximal velocity is achieved are changed as compared to the absence of inhibitor. The mechanism that produces mixed inhibition is much more complex than that of competitive inhibition since the formation of an enzyme–inhibitor complex is not necessarily a dead-end reaction. In this case, substrate can bind to the enzyme-inhibitor complex that can subsequently dissociate to form an enzyme-substrate complex that can undergo catalysis to produce product (Coleman, 2005). The Lineweaver-Burk plots are shown in Figure15.

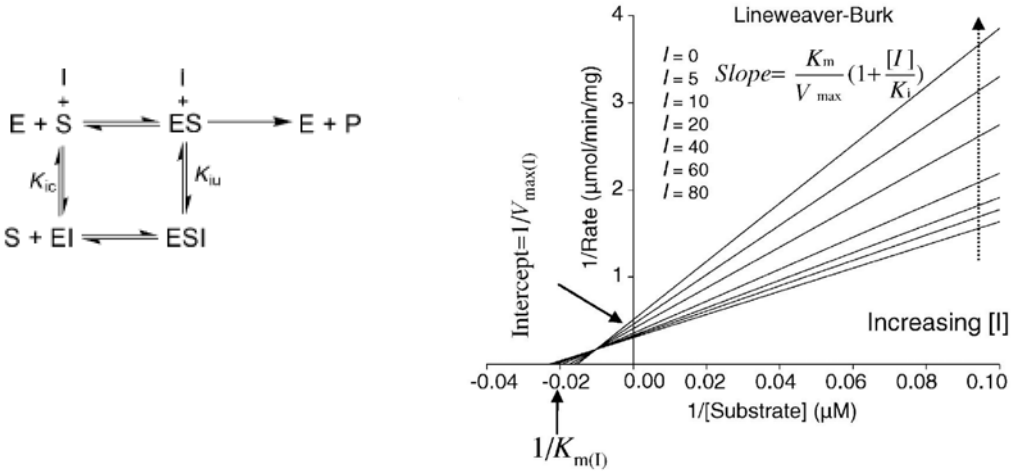


Figure 15 Scheme and the Lineweaver-Burk plots of mixed inhibition (Kic = Ki competitive and Kiu = Ki uncompetitive) (Zhang et al., 2008)

Mechanism-based inhibition

Mechanism-based enzyme inactivation kinetic refers to the irreversible inhibition of an enzyme via a catalytically formed reactive intermediate that binds covalently (typically) to the enzyme active site prior to release and causes permanent inactivation of the enzyme. The inhibition becomes stronger over time (Figure 16) and does not progress without co-factors (NADPH). Unlike competitive inhibition, withdrawal of the drug causing the inactivation does not immediately result in restoration of enzyme function. In fact, it can take several days for complete turnover of an enzyme pool and restoration of full metabolic function.

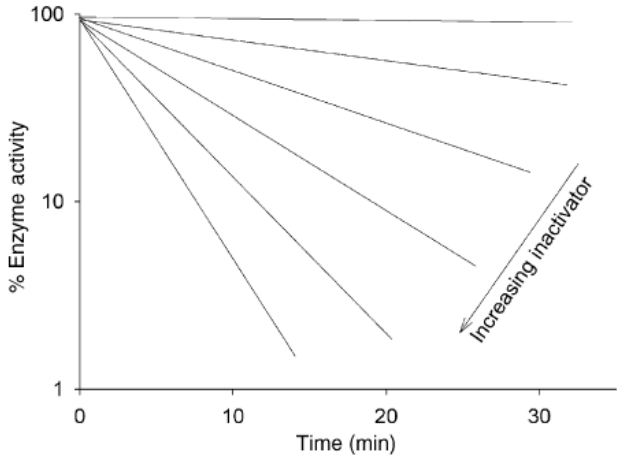


Figure 16 The decreasing enzyme activity with respect to the time and concentration dependence of mechanism-based inactivation kinetics (Zhang et al., 2008)

Mechanism-based inhibition includes CYP suicide inactivation process and metabolite–intermediate complex (MI) formation. CYP suicide inactivation involves metabolism of drugs to products that denature the CYP. In this case, the inactivator for the target enzyme is the substrate that in the process of catalytic turnover is metabolized to a reactive intermediate that inactivates the enzyme irreversibly. The latter is MI complexation in which CYP produces a metabolite with the capacity to bind tightly to the CYP heme. The difference between CYP inactivation and MI complexation is that, in the latter, the hemoprotein is not actually destroyed, even though it is rendered catalytically inert. There are most by three pathways for inactivation of the enzyme by the reactive intermediate: covalent modifications on the apoprotein, on the heme, and crosslink between apoprotein and heme (Zhang et al., 2008).

The impacts of CYP inhibition on drug therapy, drug-drug interaction, adverse drug reaction and drug toxicity are due to the modulation of CYP which is a particular enzyme involving in the metabolism of the drug substrate. Example of substrates, inhibitors and inducers of the human CYP isoform involved in xenobiotic biotransformation are shown in Table 1.

Table 1 Examples of substrates, inhibitors, and inducers of the major human CYP enzymes (Coleman, 2005; Parkinson and Ogilvie, 2008)

CYP isoforms	Substrates	Inhibitors	Inducers
CYP1A2	Caffeine 7-Ethoxyresorufin Phenacetin Tacrine Tizanidine Theophylline Amitryptyline Imipramine Fluvoxamine Clozapine Propranolol R-warfarin Zolmitriptan	Amiodarone Cimetidine Furafylline Fluvoximine Ticlopidine Acyclovir Ciprofloxacin Famotidine α-Naphthoflavone	Polycyclic aromatic hydrocarbons (PAHs) Amines in barbecued Flame-broiled meat Insulin Tobacco Omeprazole
CYP2C9	Amitryptyline Dapsone Fluoxetine Fluvestatin NSAIDS Phenytoin Tamoxifen S-warfarin Tolbutamide	Isoniazid Fluvastatin Fluvoxamine Lovastatin Sulphafenazole Fluconazole Amiodarone Miconazole	Rifampicin Phenobarbital

Table 1 (con't) Examples of substrates, inhibitors, and inducers of the major human CYP enzymes (Coleman, 2005; Parkinson and Ogilvie, 2008)

CYP isoforms	Substrates	Inhibitors	Inducers
CYP2C19	Barbiturates Mephenytoin PPIs Phenytoin R-warfarin Fluoxetine	Cimetidine Fluoxetine Ketoconazole Miconazole Ticlopidine	Carbamazepine Prednisone Rifampicin
CYP2D6	TCAs Antipsychotics Anti-arrhythmics Beta-blockers MDMA SSRIs Opiates Imipramine Loperamide Tamoxifen	Amiodarone Cimetidine Ranitidine Chlorpheniramine Quinidine SSRIs St John's wort	Not conventionally induced
CYP2E1	Benzene Chlorzoxazone Ethanol Halothane Paracetamol	Sulphides Imipramine Diethyldithiocarbamate	Ethanol Acetone Isoniazid

Table 1 (con't) Examples of substrates, inhibitors, and inducers of the major human CYP enzymes (Coleman, 2005; Parkinson and Ogilvie, 2008)

CYP isoforms	Substrates	Inhibitors	Inducers
CYP3A4	Alprazolam	Amiodarone	Barbiturates
	Amlodipine	Azoles	Carbamazepine
	Atorvastatin	Cimetidine	Glucocorticoids
	Azithromycin	Grapefruit juice	Glitazones
	Buspirone	Macrolides	Nevirapine
	Budesonide	Steroids	Phenytoin
	Carbamazepine	Protease inhibitors	Rifampicin
	Clarithromycin	Mibefradil	St John's wort
	Amiodarone	Verapamil	Simvastatin
	Dextromethorphan	Gestodene	Spironolactone
	Diergotamine		Sulfinpyrazole
	Ergotamine		Efavirenz
	Etoricoxib		Cyproterone acetate
	Indinavir		
	Itraconazole		
	Nifedipine		
	Quinidine		
	Simvastatin		
	Sildenafil		
	Tacrolimus		
	Vincristine		

Biotransformation does not always lead to detoxification. It is also contributed to an increase of human risks to environmental chemical toxicities or tumorigenicity.

Examples of environmental chemicals that are metabolic activated by each individual human CYP is summarized in Table 2.

Table 2 Examples of xenobiotics activated by human CYP **(**Parkinson and Ogilvie, 2008)

CYP isoforms	Xenobiotics
CYP1A2	Acetaminophen 2-Acetylaminofluorene 4-Aminobiphenyl 2-Aminofluorene 2-Naphthylamine Amino acid pyrolysis products Tacrine NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone)
CYP2C9, CYP2C19	Tienilic acid Phenytoin Valproic acid
CYP2D6	NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone)
CYP2E1	Acetaminophen Acrylonitrile Benzene Carbon tetrachloride Chloroform Dichloromethane 1,2-Dichloropropane Ethylene dibromide Ethylene dichloride

Table 2 (con't) Examples of xenobiotics activated by human CYP **(**Parkinson and Ogilvie, 2008)

CYP isoforms	Xenobiotics
CYP2E1	Ethyl carbamate Halothane *N*-Nitrosodimethylamine Styrene Trichloroethylene Vinyl chloride
CYP3A4	Acetaminophen Aflatoxin B_1 and G_1 6-Aminochrysene Benzo[α]pyrene 7,8-dihydrodiol Cyclophosphamide Ifosfamide 1-Nitropyrene Sterigmatocystin Senecionine *Tris*(2,3-dibromopropyl) phosphate

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Equipments

The following instruments were used in the experimentation.

1). Autopipettes 20, 100, 200 and 1000 µl (Gibson, France)

2). Autopipette tips (Gibson, France)

3). Fluorescence Reader VICTOR3V (Perkin Elmer, USA)

4). Multi-channel pipette (Rainin Instrument, USA)

5). Multi-channel pipette tips (Rainin Instrument, USA)

6). pH meter (Beckman Instrument, USA)

7). Reagent reservoir (Rainin Instrument, USA)

8). Sonicator (Elma, Germany)

9). Stop clock (Citizen®)

10). Vortex mixer (Clay Adams, USA)

11). 96-well black plates (Costar, USA)

1.2 Enzymes and Chemicals

The Vivid® CYP450 Screening Kits were purchased from Invitrogen Drug Discovery Solutions, USA. They consisted of five components as following:

1. CYP450 BACULOSOME® Reagent which consisted of recombinant human CYP (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) and rabbit NADPH CYP reductase.
2. Vivid® Substrate included either 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC), ethoxymethyloxy-3-cyanocoumarin (EOMCC) or dibenzyloxymethylfluorescein (DBOMF) depending on CYP isoforms.

3. Vivid® CYP450 Reaction Buffers included either Reaction Buffer I (200 mM potassium phosphate buffer pH 8.0), Reaction Buffer II (100 mM potassium phosphate buffer pH 8.0) or Reaction Buffer III (400 mM potassium phosphate buffer pH 8.0) depending on CYP isoforms.
4. Regeneration System consisted of Glucose-6-phosphate (G6P) and Glucose-6-phosphate dehydrogenase (G6PD) in potassium phosphate buffer pH 8.0
5. $NADP^+$ in potassium phosphate buffer pH 8.0
6. Fluorescent blue standard

Asiaticoside (MW = 959.12) and madecassoside (MW= 975.12) were kindly supplied by Associate Professor Chamnan Patarapanich, Ph.D. Faculty of Pharmaceutical Sciences, Chulalongkorn University.

These following chemicals were used in the study.

Acetonitrile, anhydrous (Labscan Asia, Thailand),

Dimethylsulfoxide (DMSO) (Sigma Chemical Co. Ltd., USA),

Erythromycin ethylsuccinate (Siam Pharmaceutical, Thailand),

Ethanol and hydrochloric acid (HCl) (Merck, Germany),

Imipramine HCl (Siam Chemi-Pharm (1997), Thailand),

Ketoconazole HCl (Siam Pharmaceutical, Thailand),

Miconazole nitrate (Government Pharmaceutical Organization, Thailand),

Ticlopidine HCl (Greater Pharma, Thailand),

Trizma® base (MW = 121.1) (Sigma Chemical Co. Ltd., USA)

Ultrapure water of 18 $MQcm^{-1}$ specific resistance was obtained by ELGASTAT MAXIMA UF® (ELGA Ltd. England).

2. Methods

2.1 Determining the inhibitory effects of asiaticoside and madecassoside on CYP activities using recombinant human CYPs

Inhibitory effects of asiaticoside and madecassoside on CYP activities were determined using the Vivid® CYP450 Screening Kits Protocol (www.invitrogen.com). In the assay, the Vivid® Substrates (BOMCC or EOMCC) were metabolized by a specific CYP enzyme into a product (7-hydroxycoumarin) that was highly fluorescent in the aqueous solutions (Figure 17).

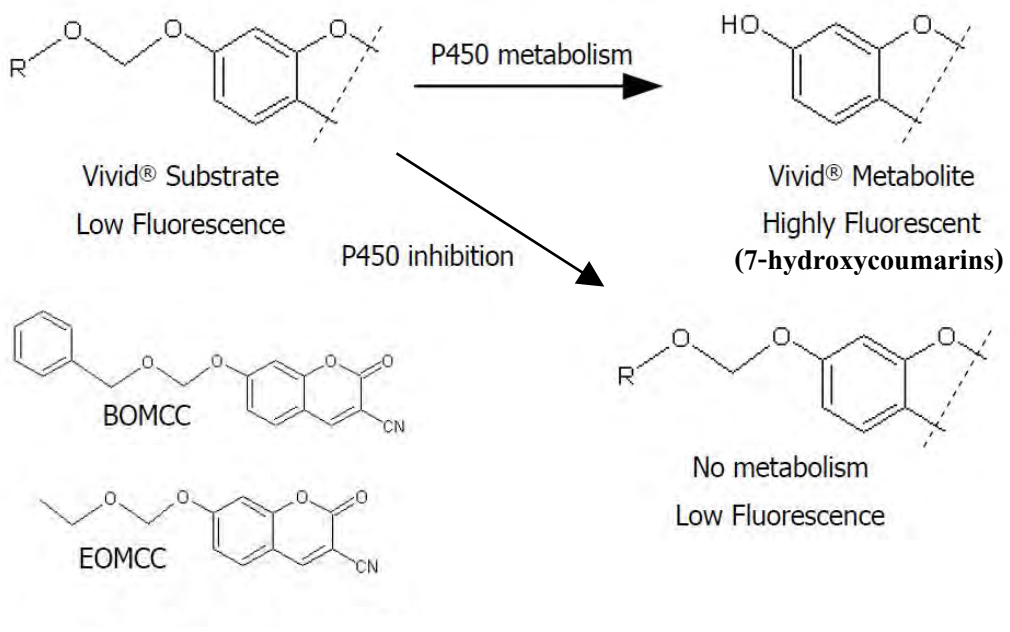


Figure 17 Vivid® assay principle and structure of BOMCC and EOMCC

Reagent

1) Preparation of asiaticoside solution

For studying inhibitory effects of asiaticoside on CYP1A2, CYP2C9, CYP2C19 and CYP2D6, asiaticoside solution was freshly prepared by dissolving 0.00575 g of asiaticoside in 30 µl of DMSO and aliquot 12.5 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (2,500 µM) was then diluted to various final concentrations with 1.25 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (μM)	DMSO in final concentrations (%)
1A2	1, 10, 100 and 1000	0.5
2C9	1, 10, 100 and 1000	0.5
2C19	125, 250, 500, 750, 900 and 1000	0.5
2D6	1, 10, 100 and 1000	0.5

For studying inhibitory effects of asiaticoside on CYP2E1, asiaticoside solution was freshly prepared by dissolving 0.00767 g of asiaticoside in 20 μl of DMSO and aliquot 2.5 μl of the solution to be diluted to 2000 μl with ultrapure water. This stock solution (200 μM) was then diluted to various final concentrations with 0.125 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (μM)	DMSO in final concentrations (%)
2E1	0.2, 2, 20 and 200	0.05

For studying inhibitory effects of asiaticoside on CYP3A4, asiaticoside solution was freshly prepared by dissolving 0.01598 g of asiaticoside in 50 μl of DMSO and aliquot 7.5 μl of the solution to be diluted to 1000 μl with ultrapure water. This stock solution (2,500 μM) was then diluted to various final concentrations with 0.75 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (μM)	DMSO in final concentrations (%)
3A4	25, 50, 125, 250, 500 and 1000	0.3

The final concentrations of asiaticoside solution were limited by asiaticoside solubility and the solvent tolerances of different CYP isoforms reported by the supplier.

2) Preparation of madecassoside solution

Madecassoside stock solution was freshly prepared by dissolving 0.00488 g of madecassoside in 1000 µl of ultrapure water. This stock solution (5,000 µM) was then diluted to various final concentrations with ultrapure water as following.

CYP isoforms	Final concentrations of madecassoside solution (µM)
1A2	1, 10, 100 and 1000
2C9	1, 10, 100 and 1000
2C19	125, 250, 500, 750, 900 and 1000
2D6	1, 10, 100 and 1000
2E1	1, 10, 100 and 1000
3A4	50, 125, 250, 500, 750 and 1000

3) Preparation of Master Pre-Mix for 100 wells

According to the Vivid® CYP450 Screening Kits Protocols, the term "Master Pre-Mix" was prepared by diluting CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer as following. The dilution was done on ice and mixed well by inversion. The solution was kept on ice until used.

CYP isoforms	Vivid® CYP450 Reaction Buffer (µl)	Regeneration System (µl)	CYP450 BACULOSOME® Reagent (µl)
1A2	4850 (buffer I)	100	50
2C9	4800 (buffer II)	100	100
2C19	4850 (buffer II)	100	50
2D6	4800 (buffer I)	100	100

2E1	4850 (buffer III)	100	50
3A4	4850 (buffer I)	100	50

4) Preparation of substrate solution

The Vivid® Substrates were reconstituted by using anhydrous acetonitrile as following. The solutions were kept at room temperature for the immediate use or stored at -20 ºC.

CYP isoforms	Vivid® Substrate (0.1 mg/tube)	Acetronitrile added per tube (µl)
1A2	EOMCC	205
2C9	BOMCC	160
2C19	EOMCC	205
2D6	EOMCC	205
2E1	EOMCC	205
3A4	BOMCC	160

5) Preparation of the pre-mixture of reconstituted substrate and $NADP^+$ solution for 100 wells

Reconstituted substrate, $NADP^+$ solution and Vivid® CYP450 Reaction Buffer were mixed as following. The solutions were prepared on ice and mixed by vortex mixer.

CYP isoforms	Vivid® Substrate	Reconstituted substrate (µl)	$NADP^+$ solution (µl)	Vivid® CYP450 Reaction Buffer (µl)
1A2	EOMCC	15	100	885 (buffer I)
2C9	BOMCC	50	100	850 (buffer II)
2C19	EOMCC	50	100	850 (buffer II)
2D6	EOMCC	50	100	850 (buffer I)
2E1	EOMCC	50	100	850 (buffer III)
3A4	BOMCC	50	100	850 (buffer I)

6) 0.5 M Tris-HCl buffer pH 10.5

Trizma® base of 6.06 g was dissolved and made up to 100 ml with ultrapure water. The solution was adjusted to pH 10.5 with NaOH or HCl.

Procedures

1. Fluorescence intensity of the 96-well black plate was measured before used so as to correct the background fluorescent intensity.

2. In each plate, the experiment was performed in duplicate for each concentration of the test compound, the background of each concentration of the test compound (for correcting the fluorescence intensity due to the test compound), the solvent control (for correcting the inhibitory effects of the solvent using for dissolving the test compound) and the background of the solvent control (for correcting the fluorescence intensity due to the solvent using for dissolving the test compound) (Figure 18).

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="2">Duplicates for solvent control</td><td colspan="8" rowspan="2">Duplicates for test compound at 8 different concentrations</td><td rowspan="2">Duplicates for solvent control</td><td></td><td></td></tr>
<tr><td>B</td><td></td><td></td></tr>
<tr><td>C</td><td rowspan="2">Duplicates for background of the solvent control</td><td colspan="8" rowspan="2">Duplicates for background of the test compound at 8 different concentrations</td><td rowspan="2">Duplicates for background of the solvent control</td><td></td><td></td></tr>
<tr><td>D</td><td></td><td></td></tr>
<tr><td>E</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
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<tr><td>H</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

Figure 18 The plate design for determining the inhibitory effects of asiaticoside or madecassoside

3. Forty microliters of the test compound (asiaticoside or madecassoside solution at different concentrations) and 50 µl of Master Pre-Mix were added into the wells of "the test compound" (2A - 9A and 2B - 9B).

4. Forty microliters of the test compound (asiaticoside or madecassoside solution at different concentrations) and 50 µl of Vivid® Reaction Buffer were added into the wells of "background of the test compound" (2C - 9C and 2D - 9D).

5. Forty microliters of the solvent using for dissolving the test compound and 50 µl of Master Pre-Mix were added into the wells of "solvent control" (1A, 10A, 1B and 10B).

6. Forty microliters of the solvent using for dissolving the test compound and 50 µl of Vivid® Reaction Buffer were added into the wells of "background of the solvent control" (1C, 10C, 1D and 10D).

The solution mixture in the plate was linearly and gently shaken for 30 seconds. The plate was preincubated for 20 minutes at room temperature.

7. The reaction was initiated by an addition of 10 µl of the pre-mixture of reconstituted substrate and $NADP^+$ solution into all wells. The solution mixture in the plate was shaken for 30 seconds as previously described.

8. The plate was covered and protected from light as well as incubated at room temperature for 30 minutes (CYP1A2, CYP2C19, CYP2E1 and CYP3A4), 40 minutes (CYP2C9) or 60 minutes (CYP2D6).

9. At the end of incubation, the reaction was stopped by an addition of 10 µl of 0.5 M Tris-HCl buffer pH 10.5 into all wells and shaken for 30 seconds as previously described.

10. The plate was put into the Fluorescence Reader and the fluorescence intensities were detected by using the excitation wavelength of 390 ± 20 nm (CYP1A2) or 405 ± 10 nm (CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) and emission wavelength of 450 ± 10 nm (CYP1A2) or 460 ± 25 nm (CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4).

11. The experiment from step 1-10 was performed at least 2 times for each CYP isoform.

Calculations

Percent inhibition was calculated for each concentration of the test compound by using the following equation.

$$\% \text{ Inhibition} = \left[1 - \frac{(RFU_{\text{test compound}} - RFU_{\text{background of test compound}})}{(RFU_{\text{solvent control}} - RFU_{\text{background of solvent control}})} \right] \times 100$$

RFU is relative fluorescence unit or fluorescence intensity

The median inhibition concentration (IC_{50}) of the test compound was calculated using Probit analysis of SPSS 16.0.

Verification of the Vivid® CYP450 Screening Kits Protocol

The procedure recommended in the Vivid® CYP450 Screening Kits Protocol was verified by determination of IC_{50} of the known inhibitor of each CYP of interested. The reaction was performed as mentioned above using miconazole (as a known inhibitor of CYP1A2, CYP2C9, CYP2C19 and CYP2D6), imipramine (as a known inhibitor of CYP2E1) and ketoconazole (as a known inhibitor of CYP3A4). In each plate, the experiment was performed in duplicate for each concentration of the known inhibitor, the background of each concentration of the known inhibitor, the solvent control and the background of the solvent control. The experiment was performed at least 2 times for each CYP isoform.

2.2 Assessment of mechanism-based inhibition

Mechanism-based inhibition of CYP activities was determined by modifying from the method of Obach et al. (2007). The test compounds were evaluated for their ability to inhibit the catalytic activity of human CYP enzymes (CYP2C19 and

CYP3A4) by means of the fluorometric inhibition assays conducted in 96-well microplate in the absence and presence of NADPH in the preincubation period. IC_{50} of the test compound in the reaction without or with NADPH preincubation was measured. IC_{50} ratio between IC_{50} of the test compound without NADPH preincubation and IC_{50} of the test compound with NADPH preincubation was calculated. The test compound with an IC_{50} ratio of more than 1 was potentially a mechanism-based inhibitor.

In the reaction, the Vivid® Substrates (EOMCC and DBOMF) were metabolized by a specific CYP enzyme into the products (7-hydroxycoumarin and fluorescein, respectively) that were highly fluorescent in an aqueous solution. EOMCC was used for CYP2C19 and DBOMF was used for CYP3A4 mechanism-based inhibition assessment (Figure 19).

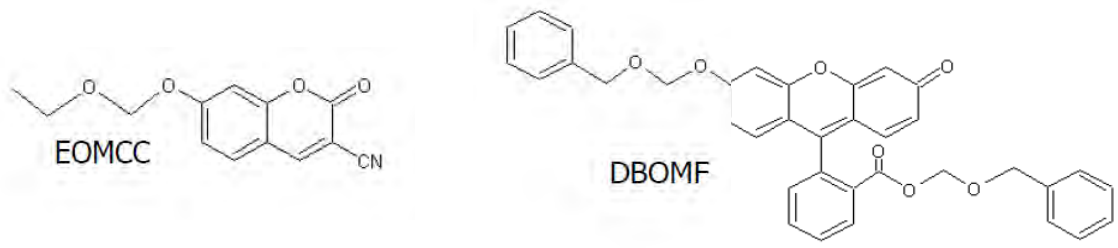

Figure 19 Structures of EOMCC for CYP2C19 and DBOMF for CYP3A4

Reagent

1) Preparation of asiaticoside solutions

Asiaticoside solution for CYP2C19 was freshly prepared by dissolving 0.01439 g of asiaticoside in 50 µl of DMSO and aliquot 15 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (4,500 µM) was then diluted to various final concentrations with 1.5 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (µM)	DMSO in final concentrations (%)
2C19	250, 750, 1000 and 1500	0.5

Asiaticoside solution for CYP3A4 was freshly prepared by dissolving 0.01598 g of asiaticoside in 50 µl of DMSO and aliquot 9 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (3,000 µM) was then diluted to various final concentrations with 0.9 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (µM)	DMSO in final concentrations (%)
3A4	700, 800, 900 and 1000	0.3

2) Preparation of madecassoside solution

Madecassoside solution for each CYP was freshly prepared by dissolving 0.00585 g of madecassoside in 400 µl with ultrapure water. This stock solution (15,000 µM) was then diluted to various final concentrations with ultrapure water as following.

CYP isoforms	Final concentrations of madecassoside solution (µM)
2C19	500, 1000, 2000, 2500, 3000, 4000 and 5000
3A4	1000, 2000, 2500, 3000, 4000 and 5000

3) Preparation of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer for 100 wells

CYP450 BACULOSOME® Reagent and Regeneration System were diluted with Vivid® CYP450 Reaction Buffer as following. The dilution was done on ice and mixed well by inversion. The solutions were kept on ice until used.

CYP isoforms	Vivid® CYP450 Reaction Buffer (µl)	Regeneration System	CYP450 BACULOSOME® Reagent (µl)
2C19	425 (buffer II)	37.5	37.5
3A4	425 (buffer I)	37.5	37.5

4) Preparation of substrate solution

The Vivid® Substrates were reconstituted by using anhydrous acetonitrile as following. The solutions were kept at room temperature for the immediate use or stored at -20 ºC.

CYP isoforms	Vivid® Substrate (0.1 mg/tube)	Acetronitrile added per tube (µl)
2C19	EOMCC	205
3A4	DBOMF	85

5) Preparation of $NADP^+$ solution in Vivid® CYP450 Reaction Buffer for 100 wells

$NADP^+$ solutions in Vivid® CYP450 Reaction Buffer were prepared as following. The solutions were prepared on ice and mixed by vortex mixer.

CYP isoforms	$NADP^+$ solution (µl)	Vivid® CYP450 Reaction Buffer (µl)
2C19	22.5	477.5 (buffer II)
3A4	22.5	477.5 (buffer I)

6) Preparation of the mixture of the reconstituted substrate, $NADP^+$ solution and Regeneration System in Vivid® CYP450 Reaction Buffer for 100 wells

Reconstituted substrate, $NADP^+$ solution, Regeneration System and Vivid® CYP450 Reaction Buffer were mixed as following. The solutions were prepared on ice and mixed by vortex mixer.

CYP isoforms	Vivid® Substrate	Reconstituted substrate (µl)	$NADP^+$ solution (µl)	Regeneration System (µl)	Vivid® CYP450 Reaction Buffer (µl)
2C19	EOMCC	37.5	22.5	93.75	5846.25 (buffer II)
3A4	DBOMF	7.5	22.5	93.75	5876.25(buffer I)

7) 0.5 M Tris-HCl buffer pH 10.5

Procedures

1. Fluorescence intensity of the 96-well black plate was measured before used so as to correct the background fluorescent intensity.

2. In each plate, the experiment was performed in triplicate for each concentration of the test compound either with or without NADPH preincubation as well as the solvent control of the test compound with or without NADPH preincubation. The experiment was performed in duplicate for the background of each concentration of the test compound and the background of the solvent control (Figure 20).

<table>
<tr><th></th><th>1</th><th colspan="8">2 3 4 5 6 7 8 9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="3">Triplicates for solvent control of the test compound with NADPH preincubation</td><td colspan="8" rowspan="3">Triplicates for test compound with NADPH preincubation at 8 different concentrations</td><td rowspan="3">Triplicates for solvent control of the test compound with NADPH preincubation</td><td></td><td></td></tr>
<tr><td>B</td><td></td><td></td></tr>
<tr><td>C</td><td></td><td></td></tr>
<tr><td>D</td><td rowspan="3">Triplicates for solvent control of the test compound without NADPH preincubation</td><td colspan="8" rowspan="3">Triplicates for test compound without NADPH preincubation at 8 different concentrations</td><td rowspan="3">Triplicates for solvent control of the test compound without NADPH preincubation</td><td></td><td></td></tr>
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<tr><td>F</td><td></td><td></td></tr>
<tr><td>G</td><td rowspan="2">Duplicates for background of the solvent control</td><td colspan="8" rowspan="2">Duplicates for Background of the test compound at 8 different concentrations</td><td rowspan="2">Duplicates for background of the solvent control</td><td></td><td></td></tr>
<tr><td>H</td><td></td><td></td></tr>
</table>

Figure 20 The plate design for assessment of mechanism-based inhibition of asiaticoside or madecassoside

3. Wells in rows 2A-9A, 2B-9B and 2C-9C were assigned to be the wells of "Test compound with NADPH preincubation" which were added with these following solutions:

5 µl of asiaticoside or madecassoside solution at various concentrations, the wells in the same column were added with the same concentration of the test compound.

5 µl of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer

5 µl of $NADP^+$ solution in Vivid® CYP450 Reaction Buffer

4. Wells in rows 2D-9D, 2E-9E and 2F-9F were assigned to be the wells of "Test compound without NADPH preincubation" which were added with these following solutions:

5 µl of asiaticoside or madecassoside solution at various concentrations, the wells in the same column were added with the same concentration of the test compound.

5 µl of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer

5 µl of Vivid® CYP450 Reaction Buffer

5. Wells in rows 2G-9G and 2H-9H were assigned to be the wells of "Background of the test compound" which were added with these following solutions:

5 µl of asiaticoside or madecassoside solution at various concentrations, the wells in the same column were added with the same concentration of the test compound.

5 µl of Vivid® CYP450 Reaction Buffer

5 µl of Vivid® CYP450 Reaction Buffer

6. The wells of 1A, 1B, 1C, 10A, 10B and 10C were assigned to be the wells of "Solvent control of the test compound with NADPH preincubation" which were added with these following solutions:

5 µl of solvent used in asiaticoside or madecassoside solution

5 µl of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer

5 µl of $NADP^+$ solution in Vivid® CYP450 Reaction Buffer

7. The wells of 1D, 1E, 1F, 10D, 10E and 10F were assigned to be the wells of "Solvent control of the test compound without NADPH preincubation" which were added with these following solutions:

5 µl of solvent used in asiaticoside or madecassoside solution

5 µl of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer

5 µl of Vivid® CYP450 Reaction Buffer

8. The wells of 1G, 1H, 10G and 10H were assigned to be the wells of "Background of the solvent control" which were added with these following solutions:

5 µl of solvent used in asiaticoside or madecassoside solution

5 µl of Vivid® CYP450 Reaction Buffer

5 µl of Vivid® CYP450 Reaction Buffer

The solution mixture in the plate was linearly shaken for 30 seconds. The plate was preincubated for 30 minutes for CYP3A4 and 60 minutes for CYP2C19 at room temperature.

9. The reaction was initiated by an addition of 60 µl of the mixture of reconstituted substrate, $NADP^+$ solution and Regeneration System in Vivid® CYP450 Reaction Buffer into all wells. The solution mixture in the plate was shaken for 30 seconds as previously described.

10. The plate was covered and protected from light and incubated at room temperature for 10 minutes for CYP3A4 or 5 minutes for CYP2C19 at room temperature.

11. At the end of incubation, the reaction was stopped by an addition of 25 µl of 0.5 M Tris-HCl buffer pH 10.5 to all wells and shaken for 30 seconds as previously described.

12. The plate was put into the Fluorescent Reader and the fluorescence intensities were detected by using the excitation wavelength of 405 ± 10 nm (CYP2C19) or 485 ±

20 nm (CYP3A4) and emission wavelength of 460 ± 25 nm (CYP2C19) or 535 ± 10 nm (CYP3A4).

Calculations

Percent inhibition and IC_{50} of the test compound were calculated in the same method as mentioned above.

IC_{50} ratio was calculated by using this following equation.

$$IC_{50}\text{ ratio} = \frac{IC_{50}\text{ of the test compound without NADPH preincubation}}{IC_{50}\text{ of the test compound with NADPH preincubation}}$$

Verification of the mechanism-based inhibition screening protocol

The mechanism-based inhibition screening protocol was verified by determination of the IC_{50} ratio of the known mechanism-based inhibitors and the non mechanism-based inhibitors. The reaction was performed as mentioned above using ticlopidine and erythromycin as the known mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively. Miconazole and ketoconazole were used as the non mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively. In each plate, the experiment was performed in triplicates for each concentration of the known inhibitor (the known mechanism-based inhibitor or the non mechanism-based inhibitor). The experiment was performed in triplicate for each concentration of the known inhibitor either with or without NADPH preincubation as well as the solvent control of the known inhibitor with or without NADPH preincubation. The experiment was performed in duplicate for the background of each concentration of the known inhibitor and the background of the solvent control.

2.3 Determination of the type of reversible inhibition using enzymes kinetic study

To determine the type of reversible inhibition and the apparent Ki of asiaticoside and madecassoside, the selective substrate of the approximately concentrations of Km/4, Km/2, Km, and 2Km were used in the reaction with various concentrations of the test compound as described earlier in the IC_{50} determination assay. The kinetic parameters of the reaction (i.e. Vmax and Km) were estimated from Lineweaver–Burk plot which 1/v was plotted along the y-axis and 1/[S] was ploted on the x-axis. The type of inhibition was estimated graphically from Lineweaver–Burk plots. Ki was determined via the second plot of the slopes from Lineweaver–Burk plots *versus* the inhibitor concentrations.

Reagent

1) Preparation of asiaticoside solution

For studying the type of inhibition of asiaticoside on CYP2C19, asiaticoside solution was freshly prepared by dissolving 0.00959 g of asiaticoside in 50 µl of DMSO and aliquot 12.5 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (2,500 µM) was then diluted to various final concentrations with 1.25 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (µM)	DMSO in final concentrations (%)
2C19	125, 750 and 1000	0.5

For studying the type of inhibition of asiaticoside on CYP3A4, asiaticoside solution was freshly prepared by dissolving 0.01199 g of asiaticoside in 50 µl of DMSO and aliquot 7.5 µl of the solution to be diluted to 1000 µl with ultrapure water.

This stock solution (1,875 μM) was then diluted to various final concentrations with 0.75 % DMSO as following.

CYP isoforms	Final concentrations of asiaticoside solution (μM)	DMSO in final concentrations (%)
3A4	300, 450 and 750	0.3

The final concentrations of asiaticoside solution were limited by the solubility of asiaticoside and the solvent tolerance of the individual CYP isoform reported by the supplier.

2) Preparation of madecassoside solution

Madecassoside stock solution was freshly prepared by dissolving 0.00488 g of madecassoside in 2000 μl of ultrapure water. This stock solution (containing 2,500 μM of madecassoside) was then diluted to various final concentrations with ultrapure water as following.

CYP isoforms	Final concentrations of madecassoside solution (μM)
2C19	125, 250, 500, 750 and 1000
3A4	125, 250 and 500

3) Preparation of Master Pre-Mix for 100 wells

Master Pre-Mix was prepared by diluting CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer as following. The dilution was done on ice and mixed well by inversion. The solutions were kept on ice until used.

CYP isoforms	Vivid® CYP450 Reaction Buffer (μl)	Regeneration System	CYP450 BACULOSOME® Reagent (μl)
2C19	4850 (buffer II)	100	50
3A4	4850 (buffer I)	100	50

4) Preparation of substrate solution

The Vivid® Substrates were reconstituted by using anhydrous acetonitrile as following. The solutions were kept at room temperature for the immediate use or stored at -20 ºC.

CYP isoforms	Vivid® Substrate (0.1 mg/tube)	Acetonitrile added per tube (µl)
2C19	EOMCC	205
3A4	BOMCC	160

5) Preparation of pre-mixture of reconstituted substrate and $NADP^+$ solution for 100 wells

Reconstituted substrate, $NADP^+$ solution and Vivid® CYP450 Reaction Buffer were mixed as following. The solutions were prepared on ice and mixed by vortex mixer.

CYP isoforms	Vivid® Substrate	Concentration of substrate	Reconstituted substrate (µl)	$NADP^+$ solution (µl)	Vivid® CYP450 Reaction Buffer (µl)
2C19	EOMCC (Km = 4 µM)	2.5	12.5	100	887.5
		5	25	100	875
		10	50	100	850
		20	100	100	800
3A4	BOMCC (Km = 10 µM)	2.5	12.5	100	887.5
		5	25	100	875
		10	50	100	850
		20	100	100	800

6) Preparation of the fluorescence standard solution

Fluorescence standard was reconstituted using DMSO as following. The solutions were kept at room temperature for used or stored at -20 ºC.

CYP isoforms	Fluorescence standard	µmol per tube [X]	DMSO added per tube (µl) [X x 10000]
2C19	Blue standard	0.1	1000
3A4	Blue standard	0.1	1000

Procedures

1. Preparation of the standard curve

1.1. Reaction Buffer (2X) was diluted with water to prepare 1X Reaction Buffer for preparing of the standard curve. Using a 96-well black plate, one standard curve containing 8 concentrations of the standard could be done with 8 wells using totally of the 1 ml of Reaction Buffer. Each concentration of the standard curve was performed in duplicate.

1.2. Beginning at the first well of the first row, 195 µl of 1X Reaction Buffer was added.

1.3. Reaction Buffer (1X) of 100 µl was added to each of the remaining wells in the row.

1.4. Five microliters of the reconstituted fluorescent standard solution was added to the first well containing 195 µl of 1X Reaction Buffer to achieve a starting concentration of 2.5 µM of the standard. The solution was mixed well by pipetting.

1.5. One hundred microliters of the solution in 1.4 was transfered to the next well containing 100 µl of 1X Reaction Buffer I/II and the solution was mixed by pipetting.

1.6. The dilution was continued in the same way as 1.5 except the last well of the row which was a standard blank containing only 1X Reaction Buffer I/II. Thus the resulting fluorescent standard concentrations were: 2,500, 1,250, 625, 312.5, 156.25, 78.125, 39.063 and 0 nM.

1.7. The experiment from step 1.1-1.6 was repeated so as to obtain the duplicated data.

1.8. The experiment from step 1.1-1.7 was performed to achieve a standard curve for each CYP isoform because Reaction Buffer of CYP2C19 (Reaction Buffer II) and CYP3A4 (Reaction Buffer I) were different in concentration.

2. Fluorescence intensity of the 96-well black plate was measured before used so as to correct the background fluorescent intensity.

3. In each plate, the experiment was performed in triplicate for each of the test compound and the solvent control (for correcting the reaction without the test compound) (Figure 21).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Triplicates for solvent control	Triplicates for the test compound at 5 different concentrations										
B												
C												
D												
E												
F												
G												
H												

Figure 21 The plate design for assessment of the type of reversible inhibition of asiaticoside and madecassoside

4. Forty microliters of the test compound (asiaticoside or madecassoside solution at different concentrations) and 50 µl of Master Pre-Mix were added into the wells of "The test compound" (2A-6A, 2B-6B and 2C-6C). The wells in the same column were added with the same concentration of the test compound.

5. Forty microliters of the solvent using for dissolving the test compound and 50 µl of Master Pre-Mix were added into the wells of "Solvent control" (1A, 1B and 1C).

The solution mixture in the plate was linearly and gently shaken for 30 seconds. The plate was preincubated for 20 minutes at room temperature.

6. The reaction was initiated by an addition of 10 µl of the pre-mixture of reconstituted substrate and $NADP^+$ solution into all wells. The solution mixture in the plate was shaken for 30 seconds as previously described.

7. The fluorescent intensities were measured every minute for 20 minutes using the excitation wavelength of 405 ± 10 nm and emission wavelength of 460 ± 25 nm for both CYP2C19 and CYP3A4.

8. The experiment from step 1-7 was repeated using various concentrations of reconstituted substrate in the pre-mixture for each CYP isoform.

Calculations

The amount of fluorescent product (in nmol) in the reaction was calculated by using the standard curve. Initial velocity was determined by dividing the nmol of the product by the reaction time.

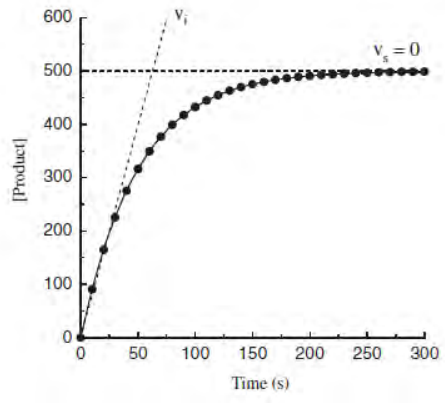


Figure 22 Typical enzyme reaction progress curve showed the initial velocity region (V_i) and the terminal velocity (V_s) (Robert, 2005).

Verification of the Vivid® CYP450 Screening Kits Protocol for enzyme kinetic study

Before assaying the test compound, the Vivid® CYP450 Screening Kits Protocol was verified for enzyme kinetic study using the particular known inhibitors. The reaction was performed as mentioned above using miconazole for verification the method for CYP2C19 and ketoconazole for CYP3A4.

CHAPTER IV

RESULTS

1. Verification of the Vivid® CYP450 Screening Kits Protocol

The procedure was verified by determination the IC_{50} of the known inhibitors of each CYP. Miconazole was used as a known inhibitor of CYP1A2, CYP2C9, CYP2C19 and CYP2D6. Imipramine was used as a known inhibitor of CYP2E1 whereas ketoconazole was used as a known inhibitor of CYP3A4. The inhibition curves of probit unit of percent inhibition of each CYP activity versus logarithm of concentration of the known inhibitor were shown in Figure 23.

The IC_{50} and 95% confidence intervals of the inhibitors on each CYP were summarized in Table 3.

Table 3 Verification of the protocol by using the known inhibitors

CYP isoforms	Known inhibitors	IC_{50}(μM)	95% Confidence intervals (μM)
1A2	miconazole	3.57	3.07-4.10
2C9	miconazole	3.74	3.17-4.28
2C19	miconazole	0.09	0.03-0.16
2D6	miconazole	1.14	0.98-1.32
2E1	imipramine	163.77	92.22-242.80
3A4	ketoconazole	0.08	0.04-0.13

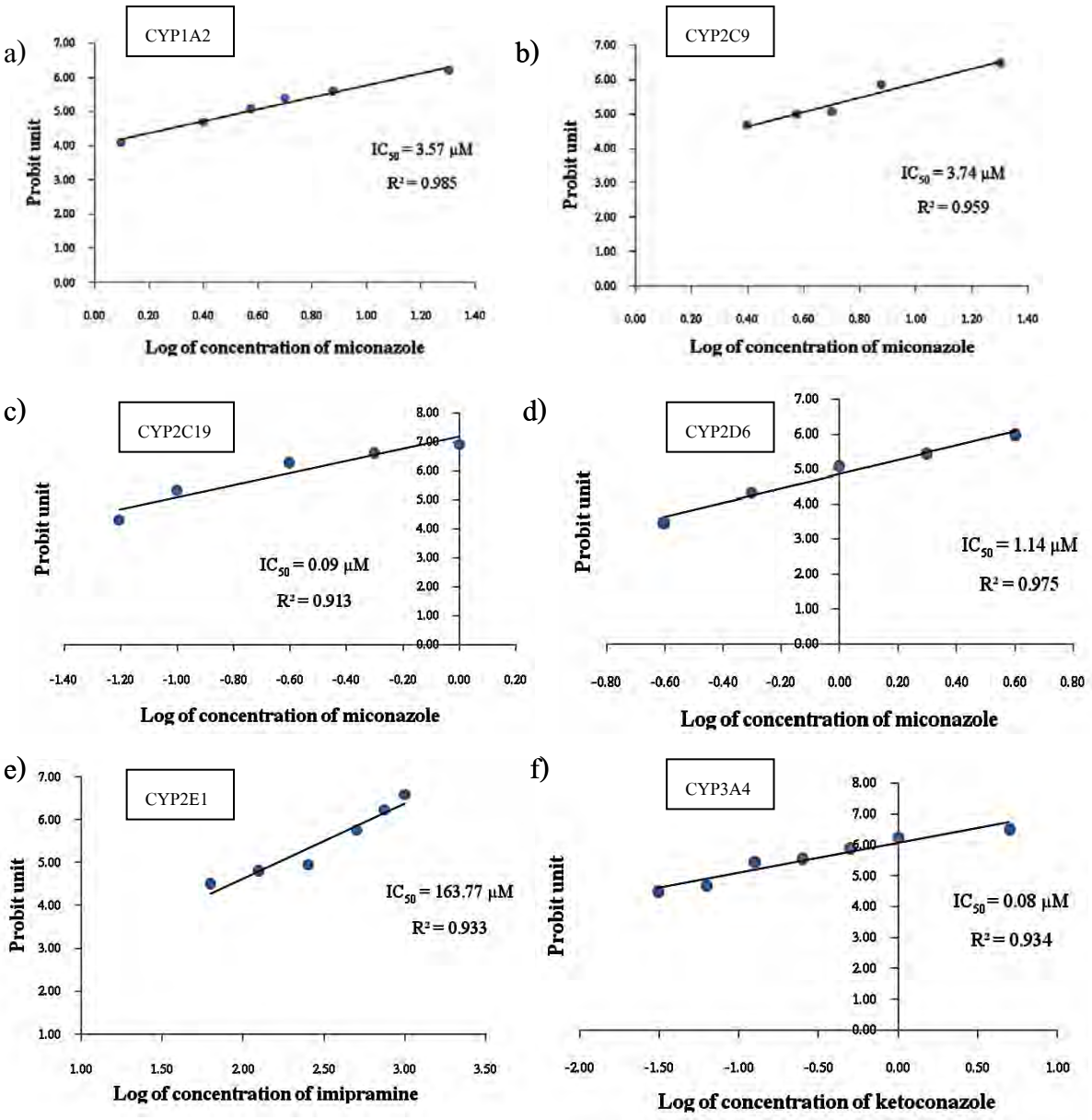


Figure 23 The inhibition curves of the known inhibitors of CYP1A2 (a), CYP2C9 (b), CYP2C19 (c), CYP2D6 (d), CYP2E1 (e) and CYP3A4 (f)

2. IC_{50} determination of asiaticoside on human CYP

2.1) IC_{50} determination of asiaticoside on CYP1A2 activity

IC_{50} of asiaticoside on CYP1A2 could not be determined at the concentration of asiaticoside up to 1,000 µM. At 1,000 µM, asiaticoside demonstrated only 13.76% inhibitory effect on CYP1A2 activites.

2.2) IC_{50} determination of asiaticoside on CYP2C9 activity

IC_{50} of asiaticoside on CYP2C9 could not be determined at the concentration of asiaticoside up to 1,000 μM. At 1,000 μM, asiaticoside demonstrated only 25.41% inhibitory effect on CYP2C9 activites.

2.3) IC_{50} determination of asiaticoside on CYP2C19 activity

Inhibitory effect of asiaticoside on CYP2C19 activity was determined at the concentration of asiaticoside between 125 to 1,000 μM. The inhibition curve of probit unit of percent inhibition of CYP2C19 activity versus logarithm of concentration of asiaticoside was shown in Figure 24. IC_{50} of asiaticoside on CYP2C19 activity was 412.68 μM with 95% confidence interval of 331.43-502.90 μM.

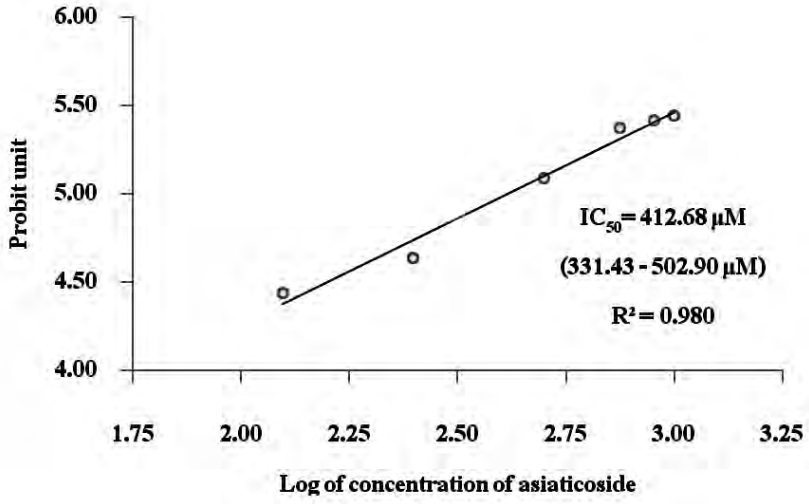


Figure 24 Inhibitory effect of asiaticoside on CYP2C19 activity. Each point presented the mean of quadruplicate experiment.

2.4) IC_{50} determination of asiaticoside on CYP2D6 activity

IC_{50} of asiaticoside on CYP2D6 could not be determined at the concentration of asiaticoside up to 1,000 μM. At 1,000 μM, asiaticoside demonstrated only 27.77% inhibitory effect on CYP2D6 activites.

2.5) IC_{50} determination of asiaticoside on CYP2E1 activity

Asiaticoside did not cause inhibitory effect on CYP2E1 activity at the concentration up to 200 μM of asiaticoside. Assessment of the inhibitory effect of

asiaticoside on CYP2E1 was limited by the solvent tolerance of the enzyme and the solubility of asiaticoside which was maximal at 200 μM.

2.6) IC_{50} determination of asiaticoside on CYP3A4 activity

Inhibitory effect of asiaticoside on CYP3A4 activity was determined at the concentration of asiaticoside between 25 to 1,000 μM. The inhibition curve of probit unit of percent inhibition of CYP3A4 activity versus logarithm concentration of asiaticoside was shown in Figure 25. IC_{50} of asiaticoside on CYP3A4 activity was 343.35 μM with 95% confidence interval of 236.75-562.67 μM.

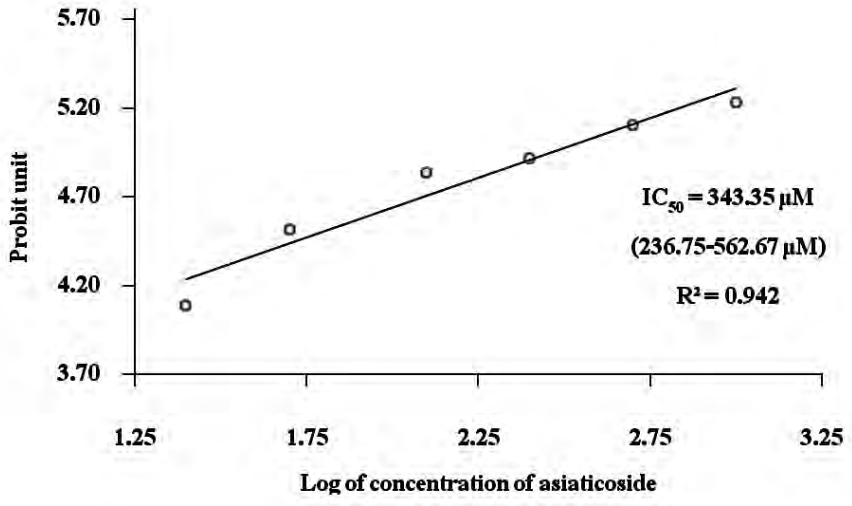


Figure 25 Inhibitory effect of asiaticoside on CYP3A4 activity. Each point presented the mean of quadruplicate experiment.

3. IC_{50} determination of madecassoside on human CYP

3.1) IC_{50} determination of madecassoside on CYP1A2 activity

Madecassoside did not exhibit any inhibitory effect on CYP1A2 activity at the concentration up to 1,000 μM of madecassoside.

3.2) IC_{50} determination of madecassoside on CYP2C9 activity

IC_{50} of madecassoside on CYP2C9 could not be determined at the concentration of madecassoside up to 1,000 μM. At 1,000 μM, madecassoside demonstrated only 41.65% inhibitory effect on CYP2C9 activites.

3.3) IC_{50} determination of madecassoside on CYP2C19 activity

Inhibitory effect of madecassoside on CYP2C19 activity was determined at the concentration of madecasoside between 125 to 1,000 μM. The inhibition curve of probit unit of percent inhibition of CYP2C19 activity versus logarithm concentration of madecassoside was shown in Figure 26. IC_{50} of madecassoside on CYP2C19 activity was 539.04 μM with 95% confidence interval of 467.40-624.86 μM.

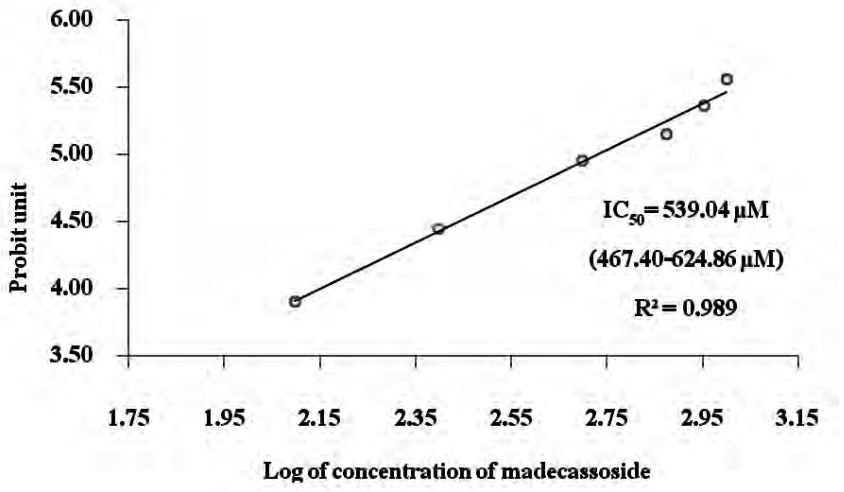


Figure 26 Inhibitory effect of madecassoside on CYP2C19 activity. Each point presented the mean of quadruplicate experiment.

3.4) IC_{50} determination of madecassoside on CYP2D6 activity

Madecassoside did not exhibit any inhibitory effect on CYP2D6 activity at the concentration up to 1,000 μM of madecassoside.

3.5) IC_{50} determination of madecassoside on CYP2E1 activity

IC_{50} of madecassoside on CYP2E1 could not be determined at the concentration of madecassoside up to 1,000 μM. At 1,000 μM, madecassoside demonstrated only 16.77% inhibitory effect on CYP2E1 activites.

3.6) IC_{50} determination of madecassoside on CYP3A4 activity

Inhibitory effect of madecassoside on CYP3A4 activity was determined at the concentration of madecassoside between 50 to 1,000 μM. The inhibition curve of probit

unit of percent inhibition of CYP3A4 activity versus logarithm concentration of madecassoside was shown in Figure 27. IC_{50} of madecassoside on CYP3A4 activity was 453.32 μM with 95% confidence interval of 311.59-748.41 μM

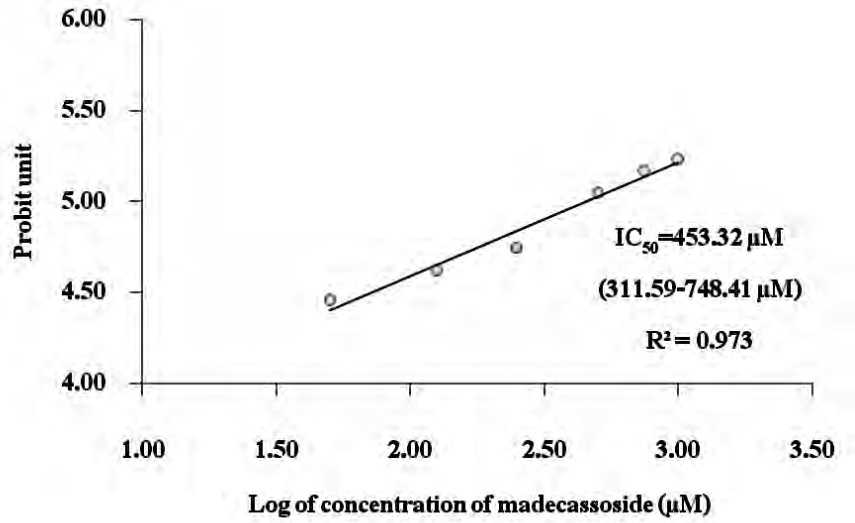


Figure 27 Inhibitory effect of madecassoside on CYP3A4 activity. Each point presented the mean of quadruplicate experiment.

4. Verification of the mechanism-based inhibition screening protocol

The procedure was verified by determination of the IC_{50} ratio of the known mechanism-based inhibitors in the absence of NADPH preincubation and in the presence of NADPH preincubation. The IC_{50} ratio of the non mechanism-based inhibitors was also determined in the same manners. Ticlopidine and erythromycin were used as known mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively. Miconazole and ketoconazole were used as non mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively. The inhibition curves of probit unit of percent inhibition of each CYP activity versus logarithm of concentration of the known mechanism-based inhibitors and non mechanism-based inhibitors were shown in Figure 28 and 29. In addition, the IC_{50} ratios of the inhibitors of each CYP were shown in Table 4.

The IC_{50} ratio of ticlopidine, the mechanism-based inhibitor of CYP2C19 was 1.14 (more than 1) whereas the IC_{50} ratio of miconazole, the non mechanism-based

inhibitors of CYP2C19 was 0.86 (less than 1). Likewise, the IC_{50} ratio of erythromycin, the mechanism-based inhibitor of CYP3A4 was 1.60 (more than 1) whereas the IC_{50} ratio of ketoconazole, the non mechanism-based inhibitors of CYP3A4 was 0.78 (less than 1).

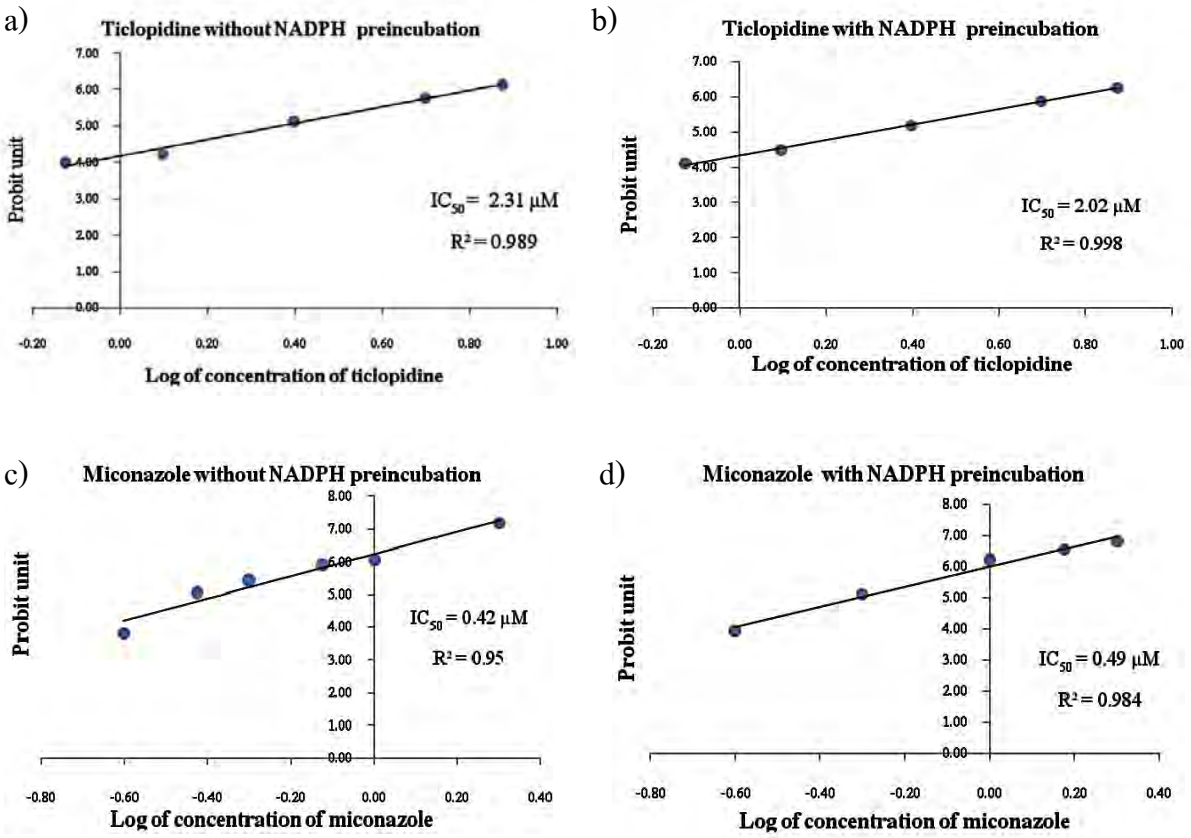


Figure 28 The inhibition curves of known mechanism-based and non mechanism-based inhibitors of CYP2C19. Ticlopidine, a mechanism-based inhibitor of CYP2C19, was used in the reaction without NADPH preincubation (a) and with NADPH preincubation (b). Miconazole, a non mechanism-based inhibitor of CYP2C19, was used in the reaction without NADPH preincubation (c) and with NADPH preincubation (d).

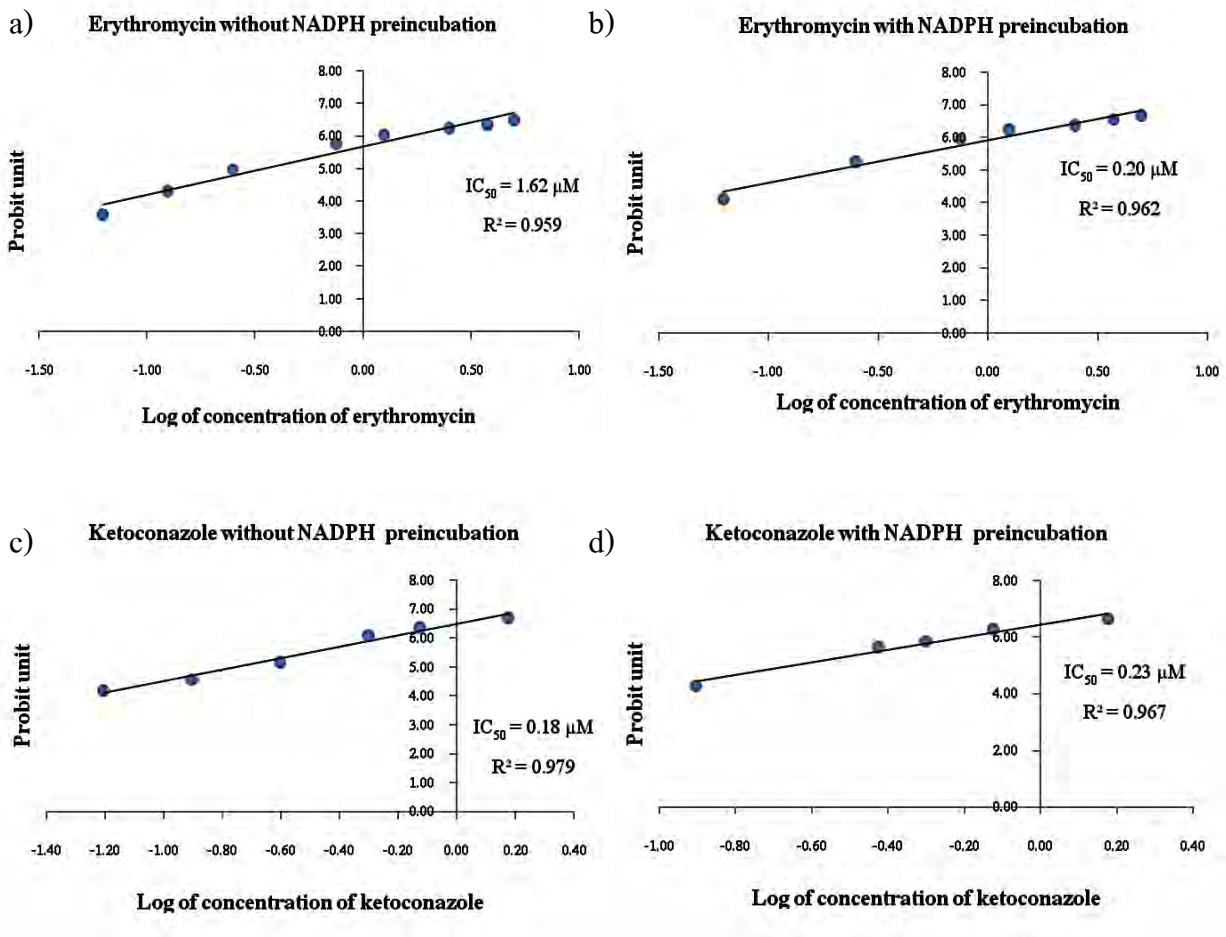


Figure 29 The inhibition curves of known mechanism-based and non mechanism-based inhibitors of CYP3A4. Erythromycin, a mechanism-based inhibitor of CYP3A4, was used in the reaction without NADPH preincubation (a) and with NADPH preincubation (b). Ketoconazole, a non mechanism-based inhibitor of CYP3A4, was used in the reaction without NADPH preincubation (c) and with NADPH preincubation (d).

Table 4 IC_{50} ratio of the mechanism-based inhibitors and non mechanism-based inhibitors of CYP2C19 and CYP3A4

CYP isoforms	Known inhibitors		Without NADPH preincubatiion IC_{50} (μM)	With NADPH preincubatiion IC_{50} (μM)	IC_{50} ratio
2C19	mechanism-based inhibitor	Ticlopidine	2.31	2.02	1.14
	non mechanism-based inhibitor	Miconazole	0.42	0.49	0.86
3A4	mechanism-based inhibitor	Erythromycin	0.32	0.20	1.60
	non mechanism-based inhibitor	Ketoconazole	0.18	0.23	0.78

5. Assessment of mechanism-based inhibition of asiaticoside

5.1) Assessment of mechanism-based inhibition of asiaticoside on CYP2C19 activity

Due to the limition of asiaticoside solubility, IC_{50} of asiaticoside could not be achieved by the protocol of mechanism-based inhibition assessment either in the absence or in the presence of NADPH preincubation. Thus, asiaticoside could not be assessed whether it is potentially a mechanism-based inhibitor of CYP2C19.

5.2) Assessment of mechanism-based inhibition of asiaticoside on CYP3A4 activity

Due to the limition of asiaticoside solubility, IC_{50} of asiaticoside could not be achieved by the protocol of mechanism-based inhibition assessment either in the absence or the presence of NADPH preincubation. Thus, asiaticoside could not be assessed whether it is potentially a mechanism-based inhibitor of CYP3A4.

6. Assessment of mechanism-based inhibition of madecassoside

6.1) Assessment of mechanism-based inhibition of madecassoside on CYP2C19 activity

IC_{50} of madecassoside on CYP2C19 activity in the absence of NADPH preincubation was 1,783 μM. IC_{50} of madecassoside on CYP2C19 activity in the presence of NADPH preincubation was 1,581 μM. Thus, IC_{50} ratio was 1.13. This result demonstrated the possibility that madecassoside was probably a mechanism-based inhibitor of CYP2C19. The inhibition curves were shown in Figure 30.

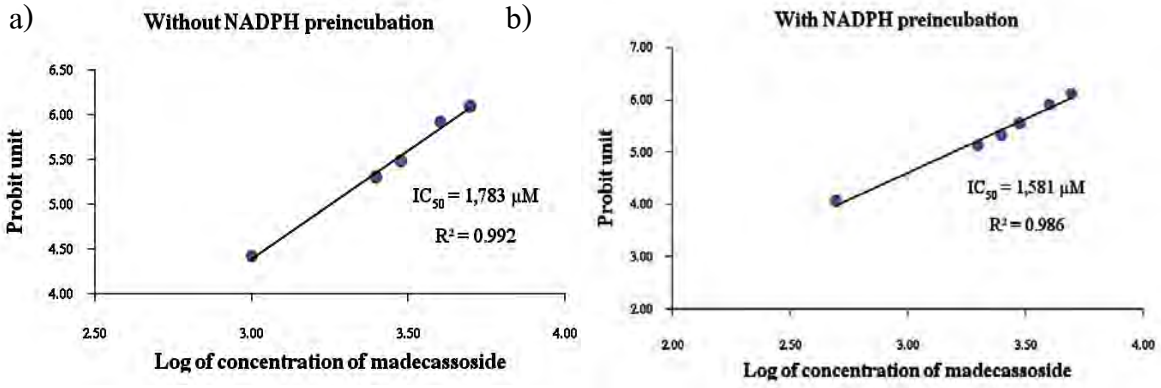


Figure 30 The inhibition curves of madecassoside for mechanism-based inhibition screening assay on CYP2C19.

6.2) Assessment of mechanism-based inhibition of madecassoside on CYP3A4 activity

IC_{50} of madecassoside on CYP3A4 activity in the absence of NADPH preincubation was 4,255 μM. IC_{50} of madecassoside on CYP3A4 activity in the presence of NADPH preincubation was 4,003 μM. Thus, IC_{50} ratio was 1.06. This result demonstrated the possibility that madecassoside was probably a mechanism-based inhibitor of CYP3A4. The inhibition curves were shown in Figure 31.

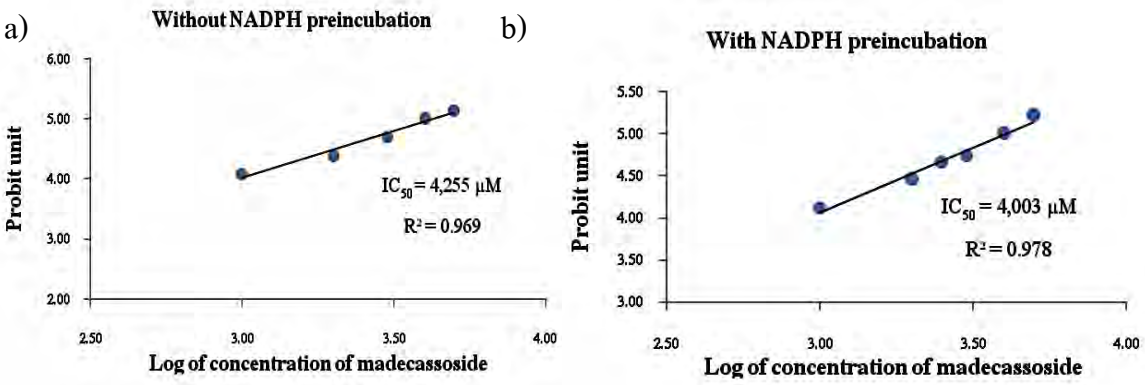


Figure 31 The inhibition curves of madecassoside for mechanism-based inhibition screening assay on CYP3A4.

7. Preparation of the standard curve for determination of the type of reversible inhibition using enzymes kinetic study

7.1) Standard curve for studying the type of inhibition on CYP2C19

The standard curve was constructed by performing a serial dilution of the reconstituted Fluorescent Blue Standard in Vivid® CYP450 Reaction Buffer (Reaction Buffer II) and measured the RFU. The standard curve of RFU versus Fluorescent standard concentrations and the linear regression equation of the standard curve were shown in Figure 32

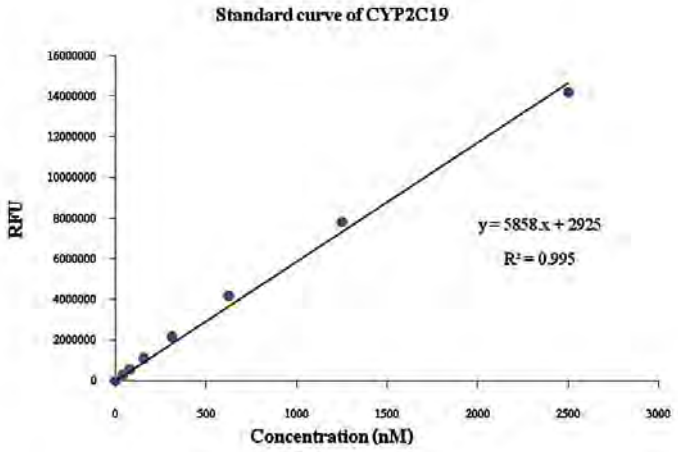


Figure 32 Standard curve of the fluorescent Blue standard for studying the type of inhibition on CYP2C19

7.2) Standard curve for studying the type of inhibition on CYP3A4

The standard curve was constructed by performing a serial dilution of the reconstituted Fluorescent Blue Standard in Vivid® CYP450 Reaction Buffer (Reaction Buffer I) and measured the RFU. The standard curve of RFU versus Fluorescent standard concentrations and the linear regression equation of the standard curve were shown in Figure 33.

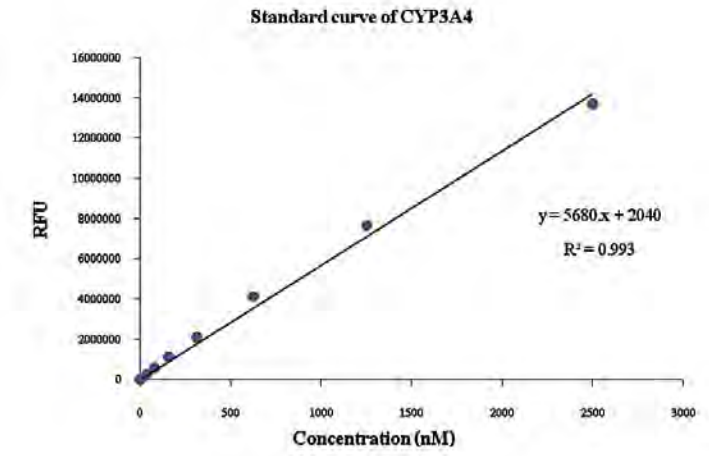


Figure 33 Standard curve of the fluorescent Blue standard for studying the type of inhibition on CYP3A4

8. Verification of the Vivid® CYP450 Screening Kits Protocol for enzyme kinetic study

The procedure of studying the type of reversible inhibition was verified by determination of the kinetic parameters and the type of inhibition of the known inhibitors of each type of inhibition. The reaction was performed as mentioned in Materials and Methods. Miconazole was used as a competitive inhibitor of CYP2C19 whereas ketoconazole was used as a noncompetitive inhibitor of CYP3A4. Lineweaver-Burk plots and the second plot of these inhibitors were shown in Figure 34. The results showed that miconazole exhibited the pattern of competitive inhibition on CYP2C19 with a Ki value of 0.27 μM. Ketoconazole demonstrated a pattern of noncompetitive inhibition on CYP3A4 with a Ki value of 0.04 μM.

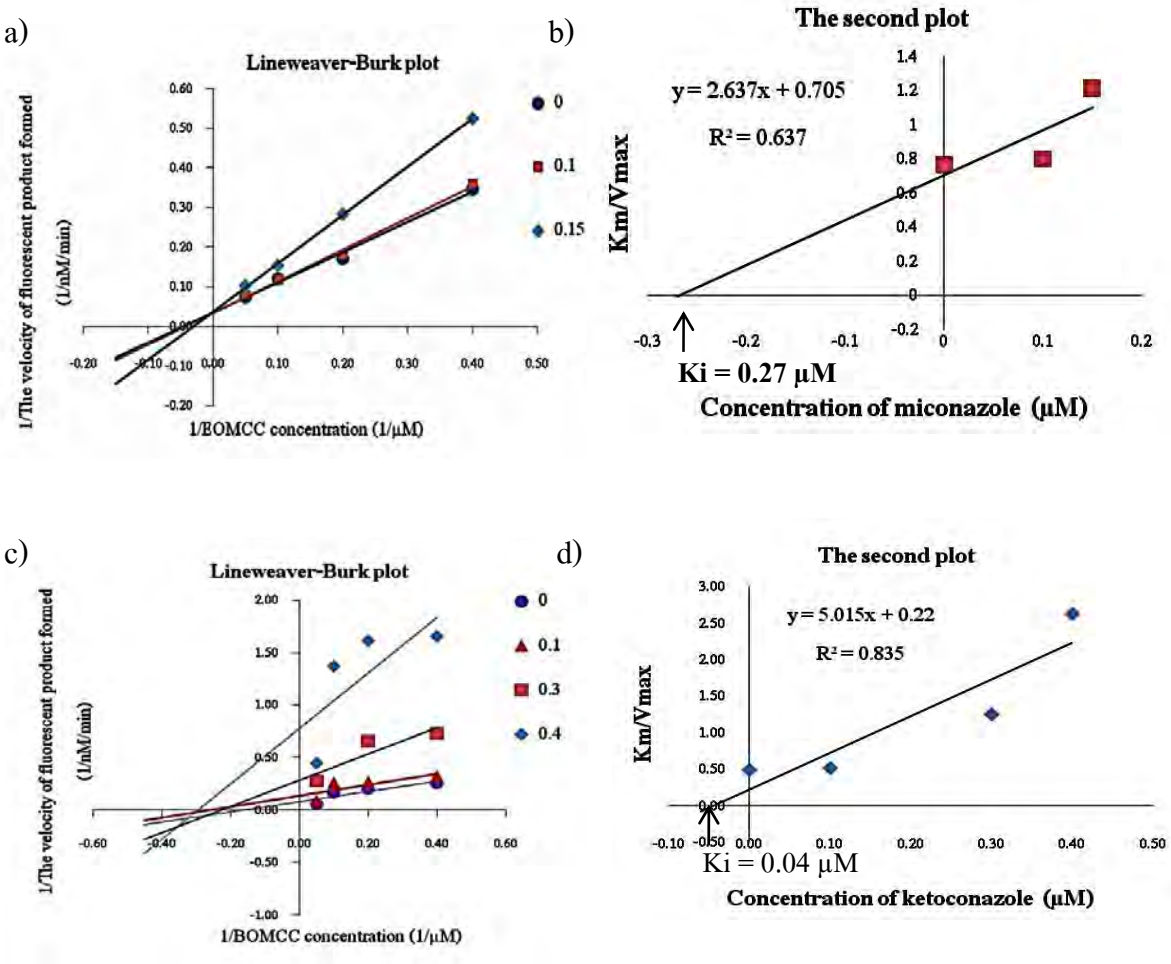
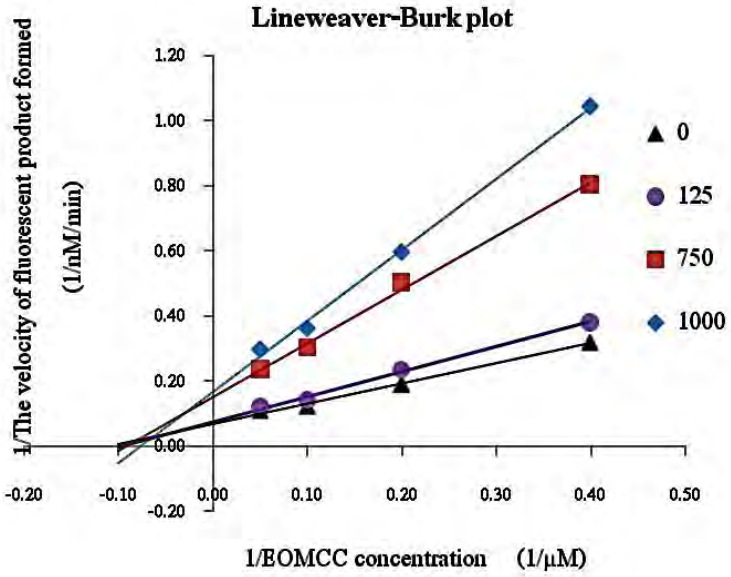


Figure 34 Lineweaver-Burk plots for the inhibition of CYP2C19 by miconazole (a), the second plot of miconazole (b), the inhibition of CYP3A4 by ketoconazole (c) and the second plot of ketoconazole (d)

9. Determination of the type of reversible inhibition on CYP2C19 by asiaticoside

To characterize the type of reversible inhibition on CYP2C19 by asiaticoside, the enzyme kinetic assay was conducted by varying the concentrations of asiaticoside and varying the concentrations of the selective substrates. Lineweaver-Burk plots and the second plot of the reaction were shown in Figure 35. Based on the linear regression analysis of the enzyme kinetic data, the type of inhibition of asiaticoside on CYP2C19 was probably a noncompetitive inhibition with a Ki value of 588 μM.

a)



b)

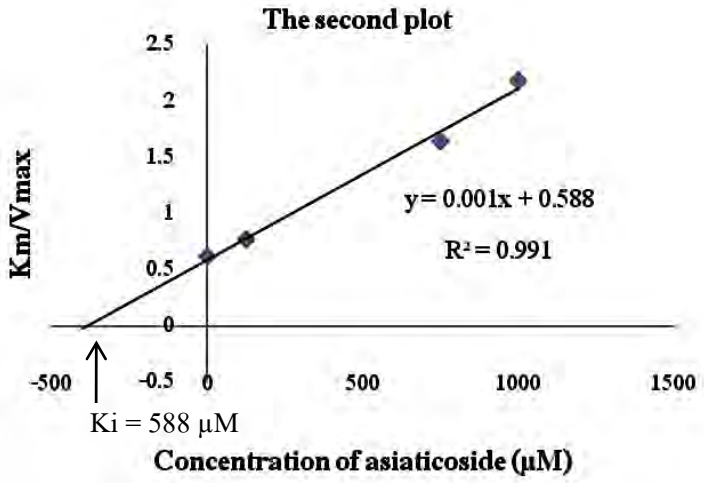
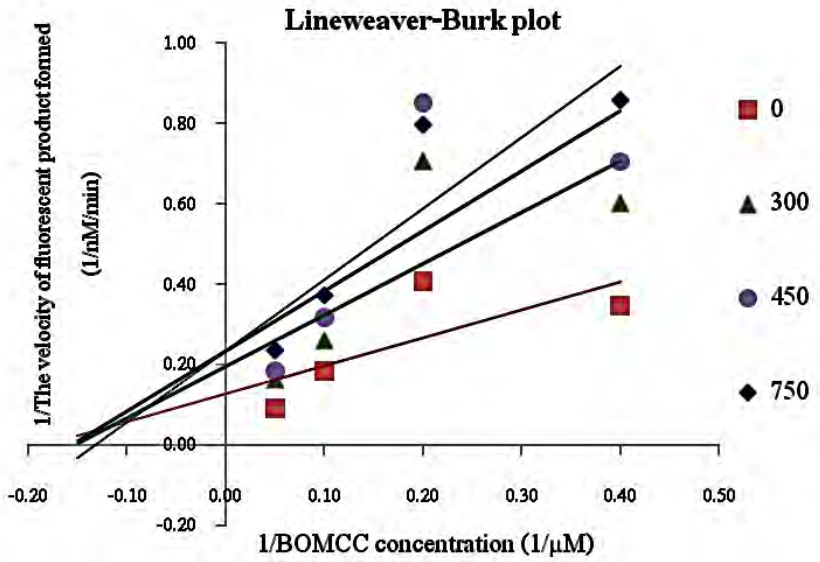


Figure 35 Lineweaver-Burk plots for the inhibition of CYP2C19 by asiaticoside (a) and the second plot of asiaticoside (b)

10. Determination of the type of reversible inhibition on CYP3A4 by asiaticoside

The type of reversible inhibition of asiaticoside on CYP3A4 was determined. As shown in Figure 36, asiaticoside potentially exhibited the pattern of noncompetitive inhibition on CYP3A4 with a Ki value of 769 µM.

a)



b)

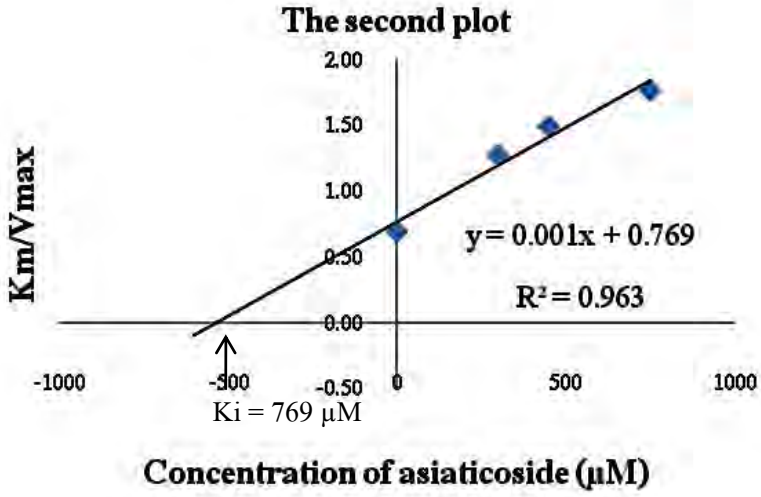
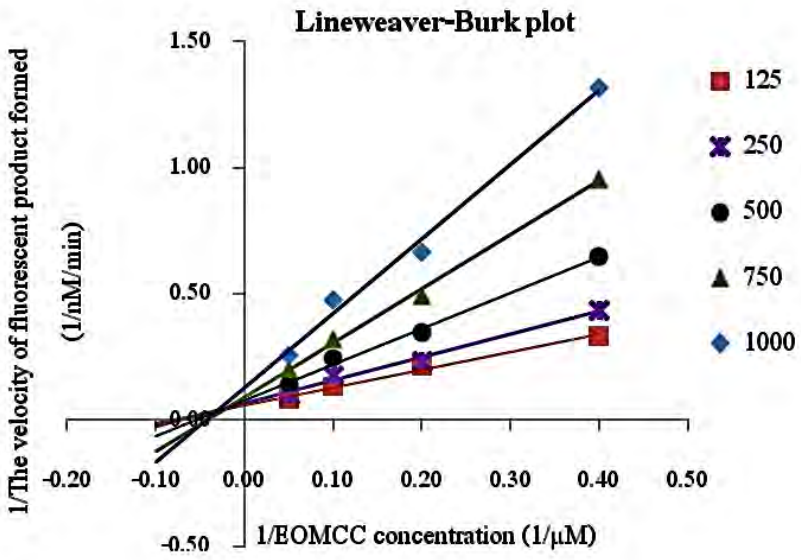


Figure 36 Lineweaver-Burk plots for the inhibition of CYP3A4 by asiaticoside (a) and the second plot of asiaticoside (b)

11. Determination of the type of reversible inhibition on CYP2C19 by madecassoside

As shown in Figure 37, Lineweaver-Burk plots suggested that madecassoside potentially exhibited the pattern of noncompetitive inhibition on CYP2C19 with a Ki value of 140 µM.

a)



b)

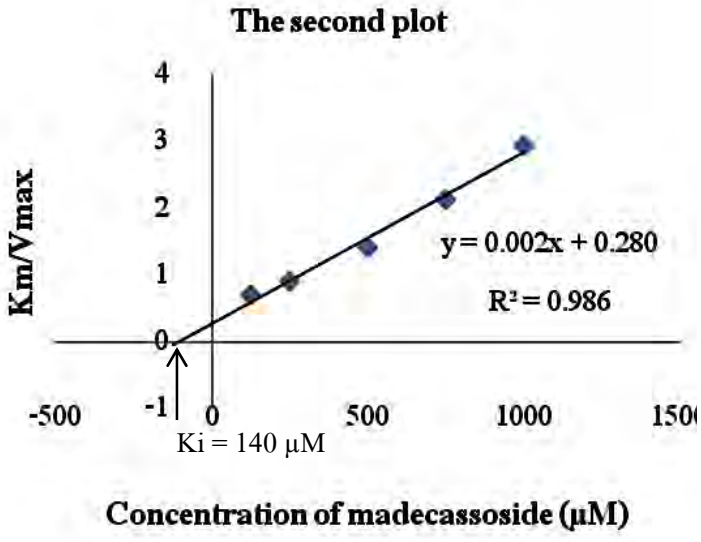
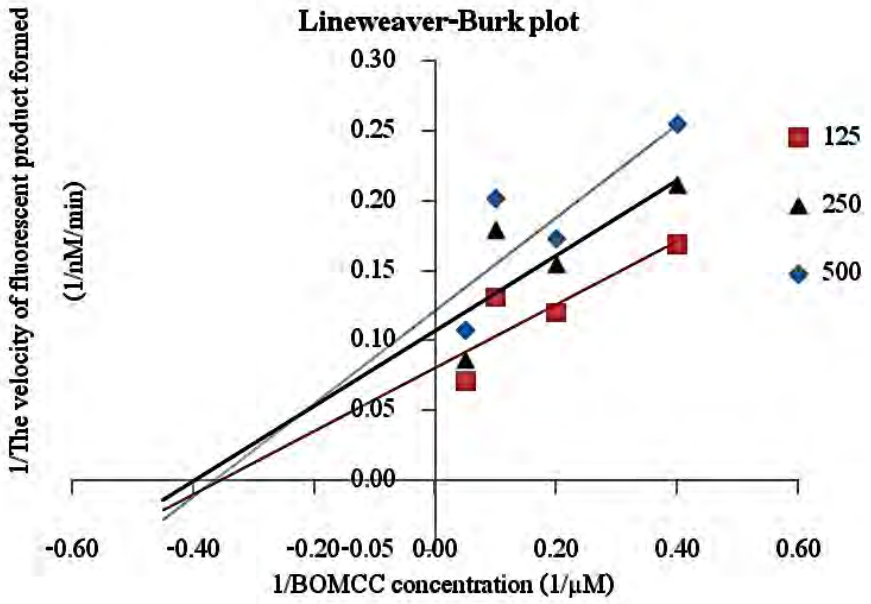


Figure 37 Lineweaver-Burk plots for the inhibition of CYP2C19 by madecassoside (a) and the second plot of madecassoside (b)

12. Determination of the type of reversible inhibition on CYP3A4 by madecassoside

The type of reversible inhibition of madecassoside on CYP3A4 was determined. As shown in Figure 38, madecassoside potentially exhibited the pattern of noncompetitive inhibition of CYP3A4 with a Ki value of 692.86 µM.

a)



b)

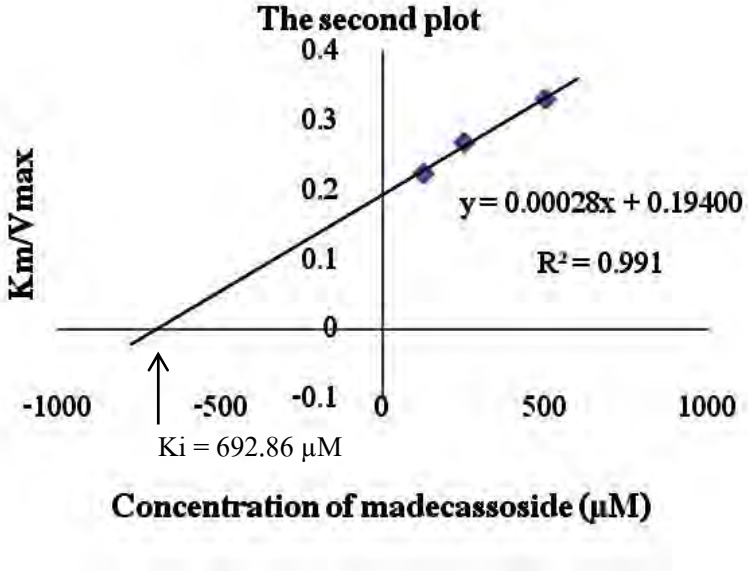


Figure 38 Lineweaver-Burk plots for the inhibition of CYP3A4 by madecassoside (a) and the second plot of madecassoside (b)

CHAPTER V

DISCUSSION AND CONCLUSION

The aim of this study was to investigate inhibitory effects and types of inhibition of asiaticoside and madecassoside on human CYPs by using recombinant human CYPs in an *in vitro* study. Asiaticoside and madecassoside are the major components in ECa233. From the previous study, ECa233 showed inhibitory effects on CYP2B6, CYP2C19 and CYP3A4 (Pitchayapa Seeka, 2008). However, asiaticoside and madecassoside which are also good candidates to be developed as alternative medicines due to their several beneficial pharmacological findings have not been reported their effects on human CYPs. The results from this study would provide preliminary information of asiaticoside and madecassoside in terms of drug-drug interaction potential if both compounds are co-administrated with other currently used medicines. In addition, the results from this study would support the effects of ECa233 on human CYPs which were reported earlier by Pitchayapa (2008) whether the effects found in ECa233 were attributed from asiaticoside and madecassoside. This study included 3 parts: IC_{50} determination of asiaticoside and madecassoside on human CYP, assessment of mechanism-based inhibition and determination of the type of inhibition using enzymes kinetic study.

In this study, recombinant human CYP enzymes were used to determine the inhibitory effect and the type of inhibition. The Vivid® CYP450 Screening Kit comprised CYP450 BACULOSOME® Reagent which are microsomes prepared from insect cells expressing the particular human CYP isozyme and rabbit NADPH CYP reductase. The Vivid® fluorogenic substrates (BOMCC, EOMCC and DBOMF) are normally metabolized by the specific CYP isoforms into products that are highly fluorescent in aqueous solutions. In the IC_{50} determination study, BOMCC was used for CYP2C9 and CYP3A4 whereas EOMCC was used for CYP1A2, CYP2C19,

CYP2D6 and CYP2E1 according to supplier's protocol (www.invitrogen.com). Before using the protocol to determine the IC_{50} of asiaticoside and madecassoside on each CYP, the protocol was verified by using a selective inhibitor of each CYP isoforms. Imipramine and ketoconazole were used as selective inhibitors for CYP2E1 and CYP3A4, respectively whereas miconazole was used as inhibitor for CYP1A2, CYP2C9, CYP2C19 and CYP2D6 (Bryan and Brad, 2008; Bryan et al., 2008). Table 5 showed the IC_{50} of each known inhibitor of each CYP found in this study compared to the IC_{50} reported by other studies. Some minor differences of the IC_{50} value were shown between this study and other previous studies which could be occurred due to many factors among studies such as differences of the substrate, temperature, variation among the lot number of the reagent kits, the instrument and the analysists.

Table 5 IC_{50} of each known inhibitor found in this study compared to the IC_{50} reported by other studies.

CYP isoforms	**Known inhibitor**	**IC_{50} (95% Confidence limits), (μM) in this study**	**IC_{50} (μM) from other studies***
1A2	miconazole	3.57 (3.07-4.10)	2.8
2C9	miconazole	3.74 (3.17-4.28)	2.2
2C19	miconazole	0.09 (0.03-0.16)	0.069
2D6	miconazole	1.14 (0.98-1.32)	0.43
2E1	imipramine	163.77 (92.22-242.80)	380
3A4	ketoconazole	0.08 (0.04-0.13)	0.13

(*) IC_{50} data from Bryan and Brad (2008) and Bryan et al. (2008).

In the mechanism-based inhibition study, DBOMF was used as a selective substrate for CYP3A4 and EOMCC was used for CYP2C19 according to supplier's protocol. Mechanism-based inhibition protocol was modified from the method of Obach et al. (2007). In this method, the test compound was performed in the reaction without NADPH preincubation and with NADPH preincubation. If the test compound

was a mechanism-based inhibitor, the curve between percent of CYP inhibition and the concentration of the test compound would shift to the left when the test compound was preincubated with NADPH as compared to the curve constructed from the reaction which was not preincubated with NADPH. Thus, the IC_{50} ratio between IC_{50} of the test compound without NADPH preincubation and IC_{50} of the test compound with NADPH preincubation would be more than one. Before using this protocol, the protocol was verified by using selective mechanism-based inhibitor of each CYP isoform. Mechanism-based inhibition was assessed only on CYP2C19 and CYP3A4 that had been found to be inhibited by asiaticoside and madecassoside in the IC_{50} determination assay. Ticlopidine and erythromycin were used as the known mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively (Obach et al., 2007). In addition, miconazole and ketoconazole were also used as the non mechanism-based inhibitors of CYP2C19 and CYP3A4, respectively (Wenjiang et al., 2002; David, 2002). As expected, the results of protocol verification showed that the IC_{50} ratio of ticlopidine, which was known to be a mechanism-based inhibitor of CYP2C19 was more than one. In contrast, the IC_{50} ratio of miconazole which was known to be a non mechanism-based inhibitor of CYP2C19 was less than one. Likewise, the IC_{50} ratio of erythromycin which was known to be a mechanism-based inhibitor of CYP3A4 was more than one. In contrast, the IC_{50} ratio of ketoconazole which was known to be a non mechanism-based inhibitor of CYP3A4 was less than one.

Enzyme kinetic study was performed using the protocol suggested by the supplier (www.invitrogen.com) to determine the type of reversible inhibition and the Ki value of asiaticoside and madecassoside on CYP isoforms that had been found to be inhibited in the IC_{50} determination screening. EOMCC and BOMCC were used as selective substrates of CYP2C19 and CYP3A4, respectively. As mention earlier in the Materials and Methods, the enzyme kinetic assay was performed by varying the concentrations of substrate and the test compounds. Initial velocity of each reaction was calculated by using the slope of the curve that constructed from concentration of

the product formed and the reaction time. Km and Vmax were obtained from the Lineweaver-Burk Plot and the type of inhibition was determined from the pattern of the curves of Lineweaver-Burk Plot constructed from different concentrations of the test compound of which each concentration was incubated with different concentrations of the substrates. Changes of Km and Vmax values would characterize the type of inhibition. Ki value which was obtained from the second plot (constructed from Km/Vmax versus concentrations of the inhibitor or the test compound) would provide the information whether the test compound would potentially to cause drug-drug interaction *in vivo* (Zhang et al., 2008).

Results from this study showed that asiaticoside and madecassoside demonstrated concentration-related inhibitory effects on the activities of CYP2C19 and CYP3A4. Asiaticoside inhibited CYP2C19 and CYP3A4 with the IC_{50} of 412.68 μM and 343.35 μM, respectively. Madecassoside inhibited CYP2C19 and CYP3A4 with the IC_{50} of 539.04 μM and 453.32 μM, respectively. Both asiaticoside and madecassoside did not inhibit or exhibited very negligible inhibitory effect on CYP1A2, CYP2C9, CYP2D6 at the concentrations of both compounds in the reaction mixture up to 1,000 μM. Except for CYP2E1, assessment of the inhibitory effect of asiaticoside on this enzyme was limited by the solubility of asiaticoside and the limitation of DMSO that could be used in the reaction which could not be more than 0.1% in the reaction mixture. And at the maximal concentration of 200 μM of asiaticoside, inhibitory effect of this compound on CYP2E1 was not shown. For madecassoside, no effect or only negligible inhibitory effect of this compound on CYP2E1 was shown at the concentration up to 1000 μM.

No effect or a very negligible inhibitory effect of asiaticoside and madecassoside on CYP1A2, CYP2C9, CYP2D6 and CYP2E1, provided screening information that both compounds did not have a potential effect to cause drug-drug interaction with the currently used medicines that were metabolized by these CYP isoforms. As asisticoside and madecassoside were the major compounds accounted for

more than 80% of the total constituents in ECa233, the result from this study would give an information whether the effect of ECa233 on CYP activities were attributed from these 2 compounds. In the previous study of Kornphimol et al. (2009), ECa233 did not demonstrate any inhibitory effect on CYP1A2 and CYP2E1 both *in vivo* in rats and *in vitro* study using rat liver microsomes. Moreover, Pitchayapa (2008) demonstrated that ECa233 did not show any inhibitory effect on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 in an *in vitro* study using recombinant human CYPs. Thus, effects of ECa233 on human CYP1A2, CYP2C9, CYP2D6 and CYP2E1 were consistent to and possibly attributed from the effects of asiaticoside and madecassoside on these CYP isoforms.

Regarding CYP2C19 and CYP3A4, asiaticoside and madecassoside demonstrated inhibitory effects on these CYP isoforms. These results also supported the previous study of Pitchayapa (2008). In that study, ECa233 showed inhibitory effect on CYP2C19 and CYP3A4 with IC_{50} of 365.18 and 210.98 μg/ml, respectively. Thus, inhibitory effects of ECa233 on CYP2C19 and CYP3A4 were likely attributed from the effects of asiaticoside and madecassoside. Regarding CYP3A4, results of this present study and the study of Pitchayapa (2008) were not consistent to the previous study conducted by Kornphimol et al. (2009), which demonstrated that ECa233 did not inhibit CYP3A4 activity *in vivo* in rats. This could be simply explained by the possibility that the sensitivity of the screening kit used in this present study and the study of Pitchayapa (2008) was higher than the sensitivity of erythromycin N-demethylation assay used in the study of Kornphimol et al. (2009) which might not be sufficiently sensitive to detect the small inhibition. Even though the inhibitory effect of asiaticoside and madecassoside on CYP2C19 and CYP3A4 were found, these effects seemed to be small as compared to the effect of the selective inhibitors which were used to verify the method. Table 6 showed the IC_{50} of asiaticoside, madecassoside, ECa233 on CYP2C19 and CYP3A4 compared to the IC_{50} of the corresponding selective inhibitors.

Table 6 IC_{50} of asiaticoside, madecassoside and ECa233 compared to the IC_{50} of the corresponding selective inhibitors.

CYP isoforms	IC_{50} values						
	Asiaticoside		Madecassoside		ECa233 (μg/ml)*	Selective inhibitors Miconazole (CYP2C19) Ketoconazole (CYP3A4)	
	(μM)	(μg/ml)	(μM)	(μg/ml)		(μM)	(μg/ml)
CYP2C19	412.68	395.81	539.04	525.63	365.18	0.09	0.04
CYP3A4	343.35	329.31	453.32	442.04	210.98	0.08	0.04

(*) IC_{50} data from Pitchayapa (2008)

As asiaticoside and madecassoside demonstrated inhibitory effects on CYP2C19 and CYP3A4, further studies on the types of inhibition on both CYPs were performed. Types of inhibition were investigated for mechanism-based inhibition and reversible inhibition of various types (competitive, noncompetitive and uncompetitive). Regarding the mechanism-based inhibition study, madecassoside was shown to probably be a mechanism-based inhibitor of CYP2C19 (Figure 39, Appendix G) and CYP3A4 (Figure 40, Appendix G) which was consistent to the characteristic of ECa233 on both CYP isoforms. Unfortunately, asiaticoside which was limited by its solubility, assessment of its mechanism-based inhibition potential could not be performed. The mechanism-based inhibition of madecassoside and ECa233 on CYP3A4 was consistent to a previous study of Dumrongsakunchai et al. (2007). In that study, they found that aqueous extract of *C. asiatica* significantly decreased CYP3A4 activity when the extract was preincubated with NADPH (before starting the usual reaction) in the time-dependent manner. Normally, mechanism-based inhibition is characterized as an irreversible inhibition. This type of inhibition is catalytic-dependent. The inhibitor is metabolized by CYP to yield reactive metabolite intermediate that can inactivate that CYP irreversibly as so called suicide inhibition. In

the other way, the inhibitor is metabolized by CYP to yield metabolite-intermediate complex of which the metabolite bind tightly to the heme of CYP that causes the catalytic site inert. The irreversible inhibitory effect persists for long time even after withdrawal of the inhibitors. Because the new enzyme will be produced by gene encoding that may take several days or several hours to recover the enzyme activity to the normal level. At this time, the CYP activity is smaller than usual that may cause the potential toxicity of the used drug that are detoxified by these CYPs (Zhang et al., 2008).

CYP3A4 metabolizes more than 120 currently used medicines and comprises around 28 percent of total human CYPs in the liver. CYP2C19 also metabolizes many drugs such as omeprazole, the proton pump inhibitor, R-warfarin, barbiturates, mephenytoin, etc. In addition, CYP2C19 has polymorphisms and there is high incidence of poor metabolize phenotypes in Asians account for about 23 percent. Thus, the compound in *C.asiatica*, madecassoside inhibit both CYPs via mechanism-based inhibition, if the effect is strong, serious drug interaction between these compound and drugs which are metabolized via CYP2C19 or CYP3A4 is concerned.

From the kinetic assay study, it was shown that asiaticoside and madecassoside were potentially noncompetitive inhibitors of CYP2C19 and CYP3A4. Asiaticoside exhibited a pattern of noncompetitive inhibition of CYP2C19 and CYP3A4 with Ki value of 588 and 769 μM, respectively. Madecassoside also exhibited a pattern of noncompetitive inhibition of CYP2C19 and CYP3A4 with Ki value of 140 and 692.86 μM, respectively. These results were in agreement with the result of ECa233 which was shown to be noncompetitive inhibitor of CYP3A4 (Figure 41, Appendix A).

From this study, asiaticoside and madecassoside exhibited both mechanism-based inhibition and reversible inhibition of noncompetitive type. This characteristic can always be found that some parent drugs themselves exhibit some degree of competitive or noncompetitive inhibition. However, preincubation of the inhibitor with liver homogenates results in a substantial increase in the inhibitory action of these

drugs (Gibson and Skett, 2001). In such cases, irreversible inactivation is preceded by a reversible step, similar to the classical reversible inhibition (Smith and Simons, 2005).

Use of the *in vitro* data to prospectively predict the *in vivo* drug-drug interactions were performed by using [I]/Ki ratio. A ratio of [I]/Ki > 1 suggests that an interaction is highly likely, whereas ratios of 0.1 < [I]/Ki < 1 or [I]/Ki < 0.1 indicate that an interaction is likely or not likely, respectively. Where [I] is the inhibitor concentration at the CYP enzyme and Ki is the inhibition constant that can be experimentally determined *in vitro* (Zhang et al., 2008). For example, the Ki of ketoconazole in the kinetic assay in this study was 0.04 μM. Generally, the serum concentration of ketoconazole is approximately 50 μg/ml or 94 μM (Corstiaan et al, 1982). [I]/Ki ratio of ketoconazole was thus equal to 94/0.04 or 2,350 which was much more than one. So, ketoconazole is highly likely to cause drug-drug interaction *in vivo*. Thus, the Ki values of asiaticoside and madecassoside obtained from this study were useful to predict possibility of drug-drug interaction *in vivo* if the concentrations of both compounds in serum are known. In addition, Ki value and the type of reversible inhibition are used in quantitative predictions of *in vivo* CYP inhibition-mediated drug-drug interactions and are necessary variables in general equation to determine the ratio between area under the curve in the presence and absence of an inhibitor (Zhang et al., 2008).

In conclusion, asiaticoside and madecassoside appeared to inhibit CYP2C19 and CYP3A4 whereas CYP1A2, CYP2C9, CYP2D6 and CYP2E1 were not affected. The inhibition of both compounds on CYP2C19 and CYP3A4 are mechanism-based inhibition and reversible inhibition of noncompetitive type. However, the inhibitory effect of both asiaticoside and madecassoside on both CYPs was very small as compared to the effect of the selective inhibitor of each CYP. Results from this study provided the potential information that both asiaticoside and madecassoside are probable to cause drug–drug interactions with medicines that are metabolized by these

CYPs. Further investigation is needed to evaluate whether these inhibitory effects of both compounds on both CYP isoforms are clinically significant.

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APPENDICES

Appendix A

- Assessment of mechanism-based inhibition of ECa233 on CYP2C19 and CYP3A4 activities
- Determination of the type of reversible inhibition of ECa233 on CYP3A4 activity

To obtain additional information for the discussion part of this thesis, mechanism of inhibition of ECa233 was investigated. The study was performed to investigate for the mechanism-based inhibition as well as the type of reversible inhibition in the same manner as performed for asiaticoside and madecassoside as following:

1. Assessment of mechanism-based inhibition of ECa233 on CYP2C19 and CYP3A4 activities

1.1) Preparation of ECa233 solution

ECa233 solution for CYP2C19 was freshly prepared by dissolving 0.01400 g of ECa233 in 20 µl of DMSO and aliquot 15 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (10,500 µg/ml) was then diluted to various final concentrations with 1.5 % DMSO as following.

CYP isoforms	Final concentrations of ECa233 solution (µg/ml)	DMSO in final concentrations (%)
2C19	1000, 2000, 2500, 2750, 3000 and 3500	0.5

ECa233 solution for CYP3A4 was freshly prepared by dissolving 0.03333 g of Eca233 in 50 µl of DMSO and aliquot 9 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (6,000 µg/ml) was then diluted to various final concentrations with 0.9 % DMSO as following.

CYP isoforms	Final concentrations of ECa233 solution (µg/ml)	DMSO in final concentrations (%)
3A4	125, 250, 500, 750, 1000, 1500, 1750 and 2000	0.3

1.2) Assessment of mechanism-based inhibition of ECa233 on CYP2C19 activity

The study was performed in the same manner as asiaticoside and madecassoside, as mentioned in CHAPTER III. The result showed that IC_{50} of ECa233 on CYP2C19 activity in the absence of NADPH preincubation was 2,188 µg/ml. IC_{50} of ECa233 on CYP2C19 activity in the presence of NADPH preincubation was 1,899 µg/ml. Thus, IC_{50} ratio was 1.15. This result demonstrated the possibility that ECa233 was probably a mechanism-based inhibitor of CYP2C19. The inhibition curves were shown in Figure 39. The raw data of the result in Figure 39 (a) were shown in Table G1 whereas those of result in Figure 39 (b) were shown in Table G2.

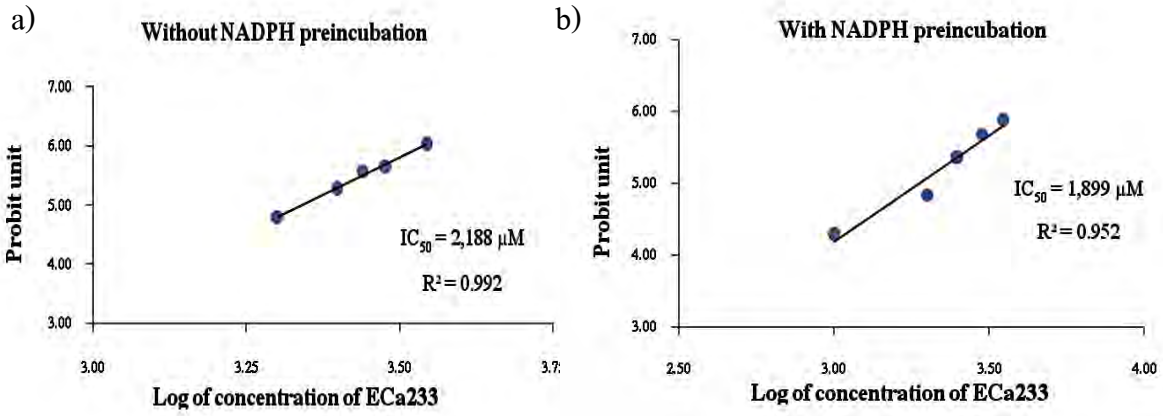


Figure 39 The inhibition curves of ECa233 for mechanism-based inhibition screening assay on CYP2C19.

Assessment of mechanism-based inhibition of ECa233 on CYP3A4 activity

The study was performed in the same manner as asiaticoside and madecassoside, as mentioned in CHAPTER III. The result showed that IC_{50} of ECa233 on CYP3A4 activity in the absence of NADPH preincubation was 2,311 µg/ml. IC_{50} of ECa233 on CYP3A4 activity in the presence of NADPH preincubation was 1,872 µg/ml. Thus, IC_{50} ratio was 1.23. This demonstrated the possibility that ECa233 was probably a mechanism-based inhibitor of CYP3A4. The inhibition curves were shown in Figure 40. The raw data of the result in Figure 40 (a) were shown in Table G3 whereas those of result in Figure 40 (b) were shown in Table G4.

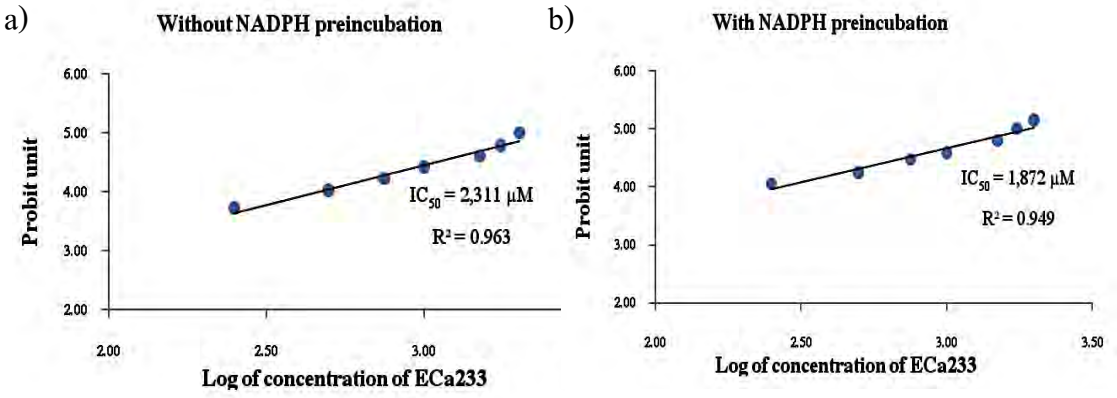


Figure 40 The inhibition curves of ECa233 for mechanism-based inhibition screening assay on CYP3A4.

2. Determination of the type of reversible inhibition of ECa233 on CYP3A4 activity

2.1) Preparation of ECa233 solution

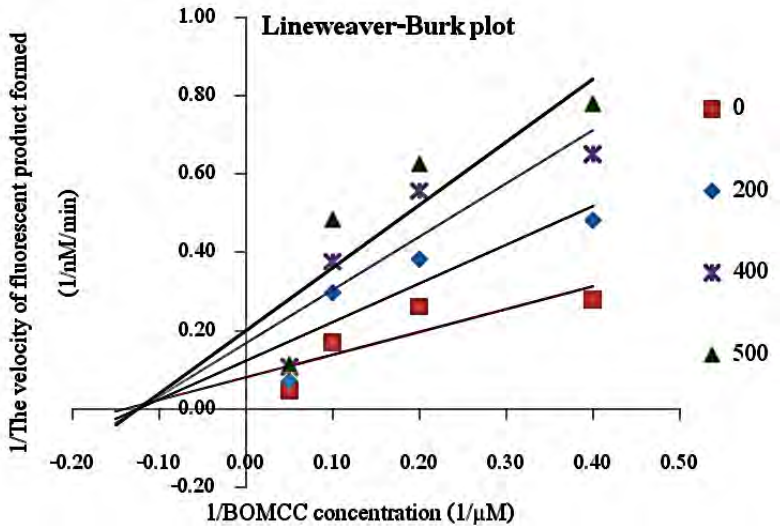
ECa233 solution for CYP3A4 was freshly prepared by dissolving 0.01667 g of Eca233 in 50 µl of DMSO and aliquot 3.75 µl of the solution to be diluted to 1000 µl with ultrapure water. This stock solution (1,250 µg/ml) was then diluted to various final concentrations with 0.375 % DMSO as following.

CYP isoforms	Final concentrations of ECa233 solution (µg/ml)	DMSO in final concentrations (%)
3A4	200, 400 and 500	0.15

2.2) Determination of the type of reversible inhibition on CYP3A4 by ECa233

The type of reversible inhibition of Eca233 on CYP3A4 was determined in the same manner as asiaticoside and madecassoside as mentioned in CHAPTER III. As shown in Figure 41, ECa233 potentially exhibited the pattern of noncompetitive inhibition on CYP3A4 with a Ki value of 290 µM. The raw data of the result in Figure 41 (a) were shown in Table G5.

a)



b)

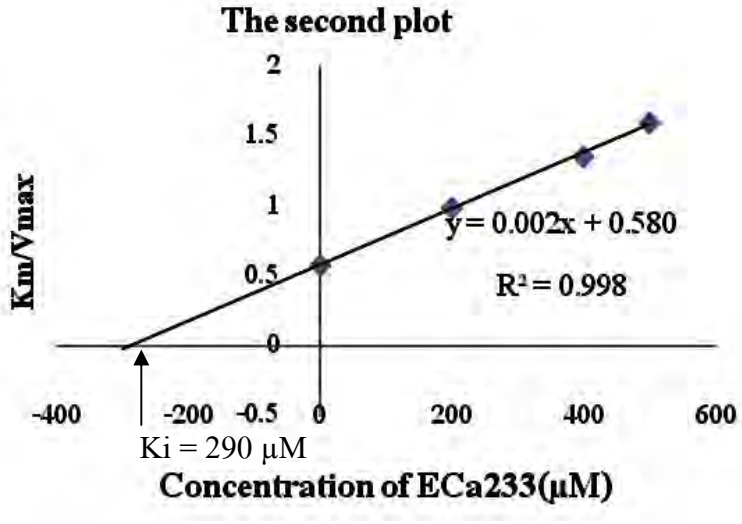


Figure 41 Lineweaver-Burk plots for the inhibition of CYP3A4 by ECa233 (a) and the second plot of ECa233 (b)

Appendix B

Vivid CYP450 Screening Kits Protocol

invitrogen™

Vivid® CYP450 Screening Kits Protocol

Cat. no. P2856, P2857, P2858, P2859, P2860, P2861, P2862, P2863, P2864, P2968, P2969, P2970, P2971, P2972, P3019, P3020 and P3021

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1.0 INTRODUCTION

Vivid® CYP450 Screening Kits enable rapid measurement of interactions between drug candidates and cytochrome P450 enzymes using a simple "mix-and-read" fluorescent assay that is designed for high-throughput screening in multiwell plates. These kits will allow investigators to rapidly identify compound-CYP450 interactions, eliminating unsuitable compounds early in the drug discovery process. Vivid® CYP450 Screening Kits can also be used to generate predictive structure-activity relationship models to guide medicinal chemists in their design of compounds.

Test compounds are analyzed by their capacity to inhibit the production of a fluorescent signal in reactions using recombinant CYP450 isozymes and specific Vivid® CYP450 Substrates. The availability of more than one structurally unrelated fluorogenic Vivid® CYP450 Substrate for CYP3A4, CYP3A5, CYP2C9, CYP2B6 and CYP2D6 reduces the potential for false negatives (and false positives) that could result from substrate-dependent interactions.

2.0 MATERIALS SUPPLIED

Vivid® CYP450 Screening Kit	Description	Cat. no.	Quantity	Storage
Vivid® CYP1A2 Blue (P2863)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP1A2 BACULOSOMES® Reagent	P2792	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Blue (P3019)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80 °C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2B6 Cyan (P3020)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2B6 BACULOSOMES® Reagent	P3028	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Blue (P2861)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Green (P2860)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® BOMF Substrate	P2869	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP2C9 Red (P2859)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C9 BACULOSOMES® Reagent	P2378	0.5 nmol	-80°C
	Vivid® OOMR Substrate	P2868	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP2C19 Blue (P2864)	Vivid® CYP450 Reaction Buffer II	P2913	50 ml	RT
	CYP2C19 BACULOSOMES® Reagent	P2570	0.5 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Blue (P2972)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP2D6 Cyan (P2862)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP2D6 BACULOSOMES® Reagent	P2283	0.5 nmol x 2	-80°C
	Vivid® MOBFC Substrate	P2871	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP2E1 Blue (P3021)	Vivid® CYP450 Reaction Buffer III	P2949	50 ml	RT
	CYP2E1 BACULOSOMES® Reagent	P2948	1.0 nmol	-80°C
	Vivid® EOMCC Substrate	P3024	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Blue (P2858)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Cyan (P2968)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Green (P2857)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected
Vivid® CYP3A4 Red (P2856)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A4 BACULOSOMES® Reagent	P2377	0.5 nmol	-80°C
	Vivid® BOMR Substrate	P2865	0.1 mg	-20°C, light protected
	Vivid® Red Fluorescent Standard	P2874	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Blue (P2970)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMCC Substrate	P2975	0.1 mg	-20°C, light protected
	Vivid® Blue Fluorescent Standard	P2876	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Cyan (P2971)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® BOMFC Substrate	P2976	0.1 mg	-20°C, light protected
	Vivid® Cyan Fluorescent Standard	P2877	0.1 µmol	-20°C, light protected
Vivid® CYP3A5 Green (P2969)	Vivid® CYP450 Reaction Buffer I	P2881	50 ml	RT
	CYP3A5 BACULOSOMES® Reagent	P2512	0.5 nmol	-80°C
	Vivid® DBOMF Substrate	P2974	0.1 mg	-20°C, light protected
	Vivid® Green Fluorescent Standard	P2875	0.1 µmol	-20°C, light protected

All kits also contain 0.5 ml Regeneration System, 100X (P2878, 333 mM Glucose-6-phosphate and 30 U/ml Glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate pH 8.0) and 0.5 ml NADP+ (P2879, 10 mM NADP+ in 100 mM potassium phosphate pH 8.0). Store both components at -80°C.

- The Vivid® CYP450 Reaction Buffers are 200 mM (Reaction buffer I), 100 mM (Reaction buffer II), or 400 mM (Reaction buffer III) potassium phosphate pH 8.0 .
- CYP450 BACULOSOMES® Reagents consist of recombinant human Cytochrome P450 (1 µM) and rabbit NADPH P450 Reductase.
- The Vivid® Substrates and Standards are supplied as a dried film. Reconstitution is necessary before use.

2.1 Materials Required but not Supplied

- Multiwell black plates suitable for fluorescence measurements (Note: black-walled, clear bottom plates are needed for bottom-read fluorescent microplate readers). Invitrogen recommends using Costar #3915 non-treated plates
- Fluorescence plate reader with filters as described in Table 6
- Pipeting devices
- Reagent reservoir(s)
- Acetonitrile, anhydrous
- DMSO, reagent grade
- Deionized water
- Stop Reagent (CYP450 isozyme specific inhibitor) if performing an endpoint assay or in kinetic mode for the positive control of inhibition. For more information on inhibitors, see Section 7.0.

3.0 STORAGE AND STABILITY

Vivid® CYP450 Substrates and Fluorescent Standards are stable for at least six months when stored desiccated and protected from light at –20°C. For short-term storage, acetonitrile- or DMSO-based stock solutions should be stored in a desiccator at 4°C. Long-term storage requires that organic solutions be kept desiccated at –20°C. DMSO solutions are hygroscopic, and cold vials should be warmed to ambient temperature before opening. After opening, they should be capped promptly to avoid reagent dilution by absorbed moisture. The CYP450 BACULOSOMES® Reagent should be stored at -80°C. No significant decrease in activity (see enclosed Certificate of Analysis) was observed after 5 freeze/thaw cycles except for CYP2D6 which showed a 5% decrease. The Regeneration System should be stored at -80°C. Upon first thaw, aliquot into single use vials as the reagent should not be subjected to additional freeze/thaw cycles. The $NADP^+$ should be stored at -80°C and is stable for at least 10 freeze/thaw cycles. Store protected from light. The Vivid® CYP450 Reaction Buffer (2X) can be stored at 4°C or room temperature.

4.0 ASSAY THEORY

Vivid® CYP450 Screening Kits are designed to assess metabolism and inhibition of the predominant human P450 isozymes involved in hepatic drug metabolism: CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. The kits employ Vivid® CYP450 Substrates and CYP450 BACULOSOMES® Reagents. The CYP450 BACULOSOMES® Reagents are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase (CYP2E1 also contains human cytochrome b_5). CYP450 BACULOSOMES® Reagents offer a distinct advantage over human liver microsomes in that only one CYP450 enzyme is expressed, thereby preventing metabolism by other CYP450s. The Vivid® Substrates are metabolized by a specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions. Figure 1 schematically depicts the metabolism of a Vivid® CYP450 Substrate into a fluorescent metabolite. Note that the Vivid® Substrates have two potential sites for metabolism (indicated by arrows in Figure 1) and that oxidation at either site releases the highly fluorescent metabolite.

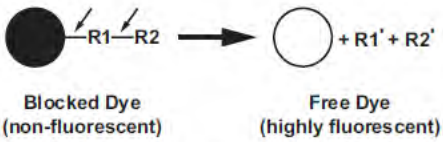

Figure 1. Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

The fluorescent metabolites are excited in the visible light spectrum, which minimizes interference caused by the background fluorescence of UV-excitable compounds and NADPH. The excellent reaction kinetics and optical properties of the Vivid® Substrates allow their use at concentrations at or below their K_m value in a reaction with P450 isozymes, assuring detection of even weak CYP450 inhibitors and providing the convenience of room temperature or 37°C incubations. The Vivid® CYP450 Assay may be run in a kinetic or endpoint mode (which is illustrated in Figure 2).

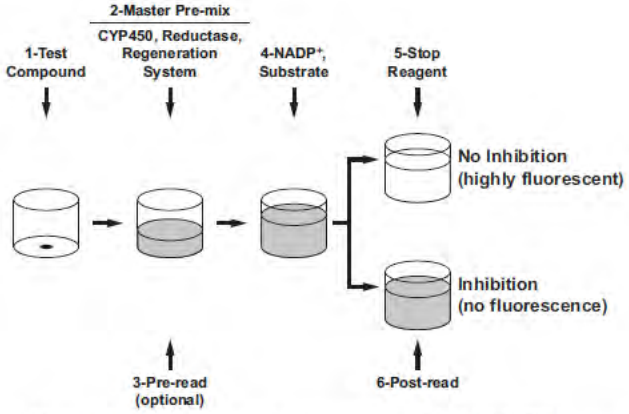

Figure 2. A schematic representation of an endpoint Vivid® CYP450 Assay

In end point (Section 5.1.9.2) mode, the test compounds (Step 1) are first combined with the Master Pre-mix (Step 2), consisting of CYP450 BACULOSOMES® Reagents and the Regeneration System (consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The Regeneration System converts $NADP^+$ into NADPH, which is required to start the CYP450 reaction. After a brief pre-incubation, the background fluorescence of the test compound and Master Pre-mix is measured (Step 3, pre-read). The enzymatic reaction is initiated by the addition of a mix of $NADP^+$ and the appropriate Vivid® Substrate (Step 4) and plate is incubated for the desired reaction time. After the addition of a Stop Reagent (Step 5), the fluorescence is measured in Step 6.

In kinetic mode (Section 5.1.9.1), the fluorescence is measured continuously starting after Step 4 (and eliminating Steps 5 and 6). Standard curves, constructed from the supplied Fluorescent Standard, can be used to calculate reaction rates from the observed fluorescence intensities in both assay formats. Assay parameters for isozymes CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 are listed in Tables 4 and 5.

5.0 VIVID® CYP450 HIGH-THROUGHPUT SCREENING ASSAY PROTOCOL

Each complete reaction must contain CYP450 BACULOSOMES® Reagent, Vivid® CYP450 Substrate, NADP+ and Regeneration System, all in the appropriate Vivid® CYP450 Reaction Buffer (supplied with each kit as a 2X solution). There are two possible modes for this assay: kinetic and endpoint. The method you choose will depend on your analytical needs and the equipment available. The kinetic mode is useful for analysis of one multiwell plate at a time and does not require the addition of the stop reagent. In endpoint mode, after an appropriate incubation time, the reaction is stopped by the addition of the CYP450 isozyme-specific inhibitor. Running in endpoint mode allows the reaction to be performed in several multiwell plates simultaneously.

Note: The following protocol is configured for use with one 96-well plate and 100 µl reactions. However, the protocol can be modified to accommodate several different plate formats by adjusting the calculations for the number of wells (and volume per well) in your experiment. See Trubetsoy *et al.* (2005) (see Section 9.0 for a complete list of references) for use of Vivid® kits in 1536-well plate formats. Each kit supplies enough reagents for at least 300 x 100 µl reactions.

5.1 Assay Procedure

5.1.1 Thaw Reagents

1. Thaw the P450 BACULOSOMES®, Regeneration System, and NADP+ on ice until ready to use. Do not vortex P450 BACULOSOMES® or Regeneration System.
2. Suggested assay conditions for screening with Vivid® kits are described in Table 1.

Table 1. Assay Conditions

Condition	Purpose	Dispensing
Test Compound	Screen for inhibition by compound of interest	40 µl 2.5X test compound 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Positive Inhibition Control	Inhibit the reaction with a known P450 inhibitor	40 µl 2.5X positive inhibition control (see Section 7.0) 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Solvent Control (No inhibitor)	Accounts for possible solvent inhibition caused by introduction of test compounds originally dissolved in an organic solvent such as DMSO	40 µl 2.5X solvent control 50 µl Master Pre-Mix 10 µl Vivid Substrate and NADP+
Background	Enables subtraction of background fluorescence during data analysis	40 µl 2.5X solvent control 50 µl Vivid® CYP450 Reaction Buffer 10 µl Vivid Substrate and NADP+

5.1.2 *Reconstitution of Vivid® Substrate and Fluorescent Standard*

1. Reconstitute the Vivid® Standard using anhydrous acetonitrile and Fluorescent Standard using DMSO (see Tables 2 and 3).
2. Keep these solutions at room temperature for immediate use, or store at -20°C.

Table 2. Reconstitution of the Vivid® CYP450 substrates

Isozyme Type	Vivid® CYP450 Substrate	Molecular weight	mg per tube	µmol per tube	µl acetonitrile added per tube	[stock solution] (mM)	[screening concentration] (µM)
1A2	Vivid® EOMCC	245.2	0.1	0.41	205	2	3
2B6	Vivid® BOMCC	307.3	0.1	0.32	160	2	5
	Vivid® BOMFC	350.3	0.1	0.28	140	2	2
2C9	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMF	452.5	0.1	0.22	110	2	2
	Vivid® OOMR[i]	355.4	0.1	0.28	140	2	2
2C19	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
2D6	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
	Vivid® MOBFC	350.3	0.1	0.28	140	2	5
2E1	Vivid® EOMCC	245.2	0.1	0.41	205	2	10
3A4	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2
	Vivid® BOMR	333.3	0.1	0.30	150	2	3
3A5	Vivid® BOMCC	307.3	0.1	0.32	160	2	10
	Vivid® BOMFC	350.3	0.1	0.28	140	2	5
	Vivid® DBOMF	572.6	0.1	0.17	85	2	2

[i] Heat at 70°C for 3-5 minutes and vortex to reconstitute.

Table 3. Reconstitution of the Fluorescent Standard. Use the blank cells in the table for your calculations. The value [X] is the amount of Assay Standard listed on the tube label.

Assay Standard	µmol per tube [X]	Reconstitution Solvent	µl Reconstitution Solvent added per tube [X x 10000]	[Fluorescent Standard] after Reconstitution, µM
Example	0.11	DMSO	1100 µl	100
Red Standard		DMSO/water (1:1)		100
Green Standard		DMSO		100
Blue Standard		DMSO		100
Cyan Standard		DMSO		100

5.1.3 *Prepare Standard Curve (Optional)*

1. With room temperature water, dilute enough Reaction Buffer (2X) to prepare enough 1X Reaction Buffer for your standard curve. In a 96-well plate, one standard curve can be run in 8 wells using 1 ml of Reaction Buffer. We recommend that at least six points (in addition to the blank) be used for the standard curve and that it be performed in duplicate.
2. To the first well of the column add 195 µl 1X Reaction Buffer.
3. Add 100 µl of 1X Reaction Buffer to each of the remaining wells in the column.
4. Add 5 µl of Fluorescent Standard (Table 3) to the first well containing 195 µl of buffer to achieve a starting concentration of 2.5 µM. Mix well.
5. Transfer 100 µl from this well into the next well containing 100 µl 1X Reaction Buffer and mix by pipetting. This is a two-fold dilution.
6. Repeat this dilution step, leaving the last well as an assay blank containing 1X Reaction Buffer only and no standard. The resulting Fluorescent Standard concentrations are: 2.5 µM, 1.25 µM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, 39.063 nM and 0 nM.

Note: These are suggested initial concentrations for the standard curve. More or less may be appropriate depending on you experimental needs.

Note: The assay can be performed simply using fluorescence values instead of converting to concentration of product formed.

5.1.4 *Prepare Test Compounds, Positive Inhibition Control, and Solvent Control*

1. Prepare 2.5X Test Compounds by dilution into deionized water. (For IC_{50} determination, a serial dilution of the test compound is required.)
2. Prepare a 2.5X solution of a known P450 Inhibitor in deionized water for positive control of inhibition (optional).

   Note: We recommend use of the inhibitors listed in Section 7.0.
3. Prepare a solution of the solvent used to dissolve the test compounds and known P450 inhibitor at 2.5X final concentration.

   Note: See Section 8.0 for information about particular solvents and tolerances.

5.1.5 *Dispense Test Compounds, Positive Inhibition Control, and Solvent Control*

1. Add 40 µl of the 2.5X solutions prepared in Section 5.1.4 to desired wells of the plate.
2. We recommend at least three replicates for the Positive Inhibition Control and Solvent Control.

5.1.6 *Prepare and Dispense Master Pre-Mix*

1. Prepare the Master Pre-Mix by diluting P450 BACULOSOMES® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer (2X) on ice (see Table 4). Mix by inversion.
2. Dispense 50 µl of Master Pre-Mix to each well. Mix.

   Note: To account for background fluorescence in the absence of CYP450 activity, dispense 50 µl of Vivid® CYP450 Reaction Buffer without P450 BACULOSOMES® to desired wells of the plate.

Table 4. Master Pre-mix (pre-mix of CYP450 BACULOSOMES® Reagents and Regeneration System). Keep on ice until ready to use

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Regeneration System (100X) added	µl of CYP450 BACULOSOMES® added	Concentration of CYP450 in Master Pre-mix (2X), nM	Screening concentration of CYP450, nM¶
1A2	Vivid® EOMCC	4850 (Buffer I)	100	50	10	5
2B6	Vivid® BOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® BOMFC	4880 (Buffer I)	100	20	4	2
2C9	Vivid® BOMCC	4800 (Buffer II)	100	100	20	10
	Vivid® BOMF	4800 (Buffer II)	100	100	20	10
	Vivid® OOMR	4800 (Buffer II)	100	100	20	10
2C19	Vivid® EOMCC	4850 (Buffer II)	100	50	10	5
2D6	Vivid® EOMCC	4800 (Buffer I)	100	100	20	10
	Vivid® MOBFC	4700 (Buffer I)	100	200	40	20
2E1	Vivid® EOMCC	4850 (Buffer III)	100	50	10	5
3A4	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5
	Vivid® BOMR	4850 (Buffer I)	100	50	10	5
3A5	Vivid® BOMCC	4850 (Buffer I)	100	50	10	5
	Vivid® BOMFC	4850 (Buffer I)	100	50	10	5
	Vivid® DBOMF	4850 (Buffer I)	100	50	10	5

¶ For your first experiment, we suggest these concentrations of the CYP450 enzyme. Based on your results, you may find more or less enzyme is necessary.

5.1.7 *Pre-Incubate*

1 Incubate the plate for 20 minutes at room temperature to allow the compounds to interact with the CYP450 in the absence of enzyme turnover.

2. During this pre-incubation, prepare the pre-mixture of Vivid® Substrate and NADP+ (see Table 5).

3. You may also wish to include a pre-read at this point to determine if your compounds are fluorescent.

Isozyme Type	Vivid® CYP450 Substrate	µl of Vivid® CYP450 Reaction Buffer (2X) added	µl of Reconstituted Substrate added (Section 5.1.2)	µl of NADP+ (100X) added	Final % ACN from substrate
1A2	Vivid® EOMCC	885 (Buffer I)	15	100	0.15
2B6	Vivid® BOMCC	875 (Buffer I)	25	100	0.25
	Vivid® BOMFC	960 (Buffer I)	10	30	0.10
2C9	Vivid® BOMCC	850 (Buffer II)	50	100	0.50
	Vivid® BOMF	890 (Buffer II)	10	100	0.10
	Vivid® OOMR	890 (Buffer II)	10	100	0.10
2C19	Vivid® EOMCC	850 (Buffer II)	50	100	0.50
2D6	Vivid® EOMCC	850 (Buffer I)	50	100	0.50
	Vivid® MOBFC	945 (Buffer I)	25	30	0.25
2E1	Vivid® EOMCC	850 (Buffer III)	50	100	0.50
3A4	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10
	Vivid® BOMR	885 (Buffer I)	15	100	0.15
3A5	Vivid® BOMCC	850 (Buffer I)	50	100	0.50
	Vivid® BOMFC	945 (Buffer I)	25	30	0.25
	Vivid® DBOMF	890 (Buffer I)	10	100	0.10

5.1.8 *Start Reaction*

1. Start the reaction by adding 10 µl per well of the Vivid® Substrate and NADP+ mixture prepared in Step 5.1.7 and mix.

5.1.9 *Measure Fluorescence*

1. **Kinetic Assay Mode (recommended):** Immediately (less than 2 minutes) transfer the plate into the fluorescent plate reader and monitor fluorescence over time at excitation and emission wavelengths listed in Table 6.

2. **Endpoint Assay Mode:** Incubate the plate for the desired amount of time, then add 10 µl of recommended stop reagent (see Section 7.0) to each well to quench the reaction. Measure fluorescence in the fluorescent plate reader at excitation and emission wavelengths listed in Table 6.

   Note: Appropriate reaction times will vary by kit and experimental conditions. We recommend that you determine the linear activity range for the assay under the conditions you wish to use. Typically, such reaction times will fall within 5 to 60 minutes.

3. Proceed to Section 6.0 for data analysis.

Table 6. Recommended excitation and emission wavelengths and filter sets

		Vivid® Fluorescent Standard							
		Red		Blue		Green		Cyan	
Fluorescence Plate Readers	Excitation/ Emission	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width	center (nm)	Band width
with monochromators	excitation	530	–	409	–	485	–	400	–
	emission	585	–	460	–	530	–	502	–
using filters	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
with dichroic mirror	excitation	530	25	405	20	485	20	405	40
	emission	605	55	460	40	530	25	490	40
	dichroic	555	–	425	–	505	–	435	–

Red Standard is sodium salt of resorufin. Blue Standard is 3-cyano-7-hydroxycoumarin. Cyan Standard is 7-hydroxy-4-trifluoromethylcoumarin. We recommend exciting this dye off-peak at 400 nm (its excitation maximum is 385 nm) to minimize background from NADPH fluorescence. Green Standard is fluorescein.

For optimal signal to noise, filters must be blocked to OD of 6 outside their transparency range (UV and red blockage) and be free of pinholes. Filters may be purchased from:

Chroma Technology Corp.
72 Cotton Mill Hill, Unit A-9
Brattleboro, VT 05301
Phone: (800) 824-7662 or (802) 257-1800
Fax: (802) 257-9400.
www.chroma.com

6.0 SUGGESTED PROTOCOL FOR THE ANALYSIS OF RESULTS

6.1 Kinetic Assay Mode

1. Obtain reaction rates by calculating the change in fluorescence per unit time.
2. Calculate the percent inhibition due to presence of test compound or positive inhibition control using the equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{rate in presence of test compound or positive inhibition control}}{\text{rate in absence of test compound or positive inhibition control}} \right) \text{X } 100\%$$

6.2 Endpoint Assay Mode

1. Subtract background fluorescence.
2. Calculate percent inhibition due to presence of test compound or positive inhibition using the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{RFU in presence of test compound or positive inhibition control}}{\text{RFU in absence of test compound or positive inhibition control}} \right) \text{X } 100\%$$

Optional: Both types of data analysis above can be performed using a standard curve as described in Section 5.1.3 in order to calculate reaction rates as nmol product formed per unit time.

7.0 SUGGESTED CYP450 INHIBITORS (STOP REAGENT)

Table 7. Suggested CYP450 Inhibitors

Enzyme	Inhibitor (Stop Reagent)	Sigma-Aldrich Cat. no.	Suggested Final Concentration**
CYP1A2	α-naphthoflavone	N5757	3 µM
CYP2B6	miconazole	M3512	30 µM
CYP2C9	sulfaphenazole	S0758	10 µM
CYP2C19	miconazole	M3512	30 µM
CYP2D6	quinidine	Q3625	1 µM
CYP2E1	diethyldithiocarbamate	228680	100 µM
CYP3A4	ketoconazole	K1003	10 µM
CYP3A5	ketoconazole	K1003	30 µM

** To stop the reaction, the suggested final inhibitor concentration in the assay to produce inhibition of 90% or better is indicated in the above table. For an endpoint assay the volume of the added Stop Reagent should not exceed 10% of the final reaction volume [*e.g.*, 10 µl will be added per 100 µl reaction volume. This 10% increase in the volume of an endpoint reaction does not have a significant effect on the reaction (or the calculations)].

8.0 SOLVENT TOLERANCES

P450 activity can be inhibited by solvents commonly used to dissolve test compounds. While we always recommend including a solvent control in your experimental design, the following sample data is intended as a guide for the selection and use of organic solvents. Table values are percent inhibition at the indicated solvent concentration. Values preceded by a "+" indicate an increase in activity. Dashed lines indicate inhibition not detected. Note that lower concentrations are listed for 2E1 Blue; this isozyme is particularly sensitive to the presence of organic solvents.

Table 8. Solvent Tolerances

Vivid® Kit	Solvent concentration (%)	DMSO (% Inhibition)	Acetonitrile (% Inhibition)	Methanol (% Inhibition)	Ethanol (% Inhibition)
1A2 Blue	1	7	–	–	–
	0.1	–	–	–	–
	0.01	–	–	–	–
2B6 Blue	1	16	7	20	32
	0.1	–	–	–	–
	0.01	–	–	–	–
2B6 Cyan	1	–	–	–	9
	0.1	–	–	–	–
	0.01	–	–	–	–
2C9 Blue	1	55	9	46	61
	0.1	25	–	7	11
	0.01	–	–	–	–
2C9 Green	1	–	–	30	38
	0.1	–	–	–	–
	0.01	–	–	–	–
2C9 Red	1	21	5	45	53
	0.1	8	–	9	9
	0.01	5	–	–	–
2C19 Blue	1	23	–	21	42
	0.1	–	–	–	5
	0.01	–	–	–	–
2D6 Blue	1	58	–	37	56
	0.1	16	–	–	10
	0.01	4	–	–	–
2D6 Cyan	1	21	6	38	49
	0.1	–	–	4	5
	0.01	–	–	–	–
2E1 Blue	0.1	85	36	26	98
	0.01	35	15	3	75
	0.001	20	7	8	25
3A4 Blue	1	68	–	12	10
	0.1	25	–	4	–
	0.01	6	–	–	–
3A4 Cyan	1	68	4	20	11
	0.1	29	–	6	–
	0.01	7	–	–	–
3A4 Green	1	47	–	–	–
	0.1	9	–	–	–
	0.01	–	–	–	–
3A4 Red	1	48	–	6	5
	0.1	13	–	–	–
	0.01	–	–	–	–
3A5 Blue	1	75	5	29	32
	0.1	34	–	14	8
	0.01	8	–	5	6
3A5 Cyan	1	71	6	21	23
	0.1	30	–	8	6
	0.01	–	–	5	6
3A5 Green	1	15	–	–	–
	0.1	–	–	–	–
	0.01	–	–	–	–

9.0 REFERENCES

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Marks, B.D, *et al.* (2004) High-throughput screening assays for the assessment of CYP2C9*1, CYP2C9*2, and CYP2C9*3 metabolism using fluorogenic Vivid® substrates. *J. Biomol. Screen.* **9:**439-49.

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For structures of the Vivid Substrates and poster presentations containing additional details and applications of Vivid CYP450 Screening Kits, please visit us online at: www.invitrogen.com/drugdiscovery.

10.0 PURCHASER NOTIFICATION

Limited Use Label License No. 162: Cytochrome P450 enzymes, assays and substrates

This product is the subject of one or more of US Patent 5,891,696, 6,143,492, and 6,420,130. The purchase of this product conveys to the buyer the non-transferrable right to use the purchased amount o the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using the product or its components for Commercial Purposes. The buyer may transfer information or materials made through use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patent claiming this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California, 92008. Tel: (760) 603-7200. Fax: (760) 602-6500.

Appendix C

Publication

Effects of Asiaticoside and Madecassoside on Human Cytochrome P450

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Abstract

Introduction: Asiaticoside and madecassoside are the major triterpenoid compounds from *Centella asiatica* (Linn.) Urban. Asiaticoside was reported to possess wound healing activity, anti-inflammatory, protection of gastric ulcer and free radical scavenger. Madecassoside exhibited anti-inflammatory, antioxidant and rheumatoid arthritis healing activities.

Objective: The purpose of this study was to investigate the effects of asiaticoside and madecassoside on the activities of human cytochrome P450 (CYP) including CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 by using recombinant human CYP.

Materials and methods: Inhibition of CYP activities was determined by using the Vivid® CYP450 Screening Kit Protocol. The Vivid® Substrates were metabolized by specific CYP enzymes into products that were highly fluorescent in aqueous solutions.

Results: The results demonstrated that asiaticoside and madecassoside possessed inhibitory effects on CYP3A4 and CYP2C19 while the inhibitory effects of both compounds on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 were not shown.

Discussion and Conclusion: The inhibitory effect of asiaticoside and madecassoside on CYP3A4 and CYP2C19 activities suggested that these compounds were needed to be further investigated whether they would cause drug-drug interaction with medicines that are metabolizes by these enzymes.

Introduction

Asiaticoside and madecassoside (Figure 1) are the major triterpenoid compounds from *Centella asiatica* (Linn.) Urban. *C.asiatica* is a pantropical plant in Thailand that is normally used as food, beverage and traditional medicine. There are many preclinical studies of asiaticoside and madecassoside for the pharmacological activity. Asiaticoside was reported to possess the effects of anticancer, wound healing activity, anti-inflammatory, protection of gastric ulcer and free radical scavenge. Madecassoside exhibited anti-inflammatory, antioxidant and rheumatoid arthritis healing activity in mice.

During the research and development process of any new compounds, metabolism/drug-drug interaction studies are required. Effect of any compounds on CYP is a beneficial information regarding drug-drug interaction and the possibility of the compound to increase/decrease risks of xenobiotic-induced toxicity/mutagenesis/carcinogenesis. In 2007, Seeka P. investigated the effect of the standard extract of *C. asiatica* (ECa 233) on human CYP by using recombinant human CYP. The results showed that ECa233 inhibited CYP2B6, CYP3A4 and CYP2C19 with IC_{50} less than 1,000 µg/ml.

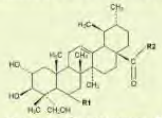

Figure 1 Chemical structure of pentacyclic triterpenes. R1 = H (for asiaticoside) or OH (for madecassoside), R2 = glucoseglucose-rhamnose.

Objective

The aim of this study was to investigate effects of asiaticoside and madecassoside on human CYPs by using recombinant human CYPs in an *in vitro* study.

Experiment

Inhibitions of CYP activities were determined according to the Vivid® CYP450 Screening Kits Protocol (www.invitrogen.com). The Vivid® Substrates were metabolized by a specific CYP enzyme into a product that was highly fluorescent in aqueous solutions (Figure 2).

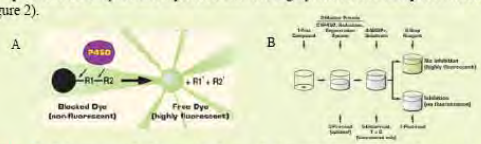

Figure 2 Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite (A) and a schematic representation of an endpoint Vivid® CYP450 Assay (B).

Results

The results demonstrated that asiaticoside and madecassoside possessed inhibitory effects on CYP3A4 (Figure 3A and 3B) and CYP2C19 (Figure 4A and 4B). However, no inhibition effects of asiaticoside and madecassoside on CYP1A2,CYP2C9, CYP2D6 and CYP2E1 were observed.

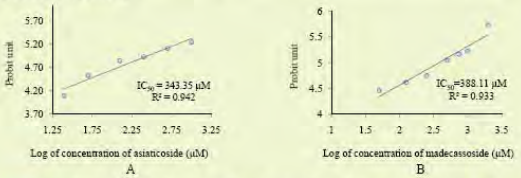

Figure 3 Inhibition effect of asiaticoside (A) and madecassoside (B) on CYP3A4. Each point presented the mean of n= 4.

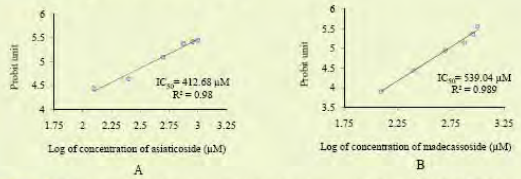

Figure 4 Inhibition effect of asiaticoside (A) and madecassoside (B) on CYP2C19. Each point presented the mean of n= 4.

Discussion and Conclusion

Inhibitory effects of asiaticoside and madecassoside on CYP3A4 and CYP2C19 activities suggested the possibility of these compounds regarding drug-drug interaction if these compounds were administrated concomitantly with other medicines that are metabolizes by these enzymes. Further study should be investigated whether this inhibitory effect would be clinically significant.

Acknowledgements

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Original article **P21**

Effects of Asiaticoside and Madecassoside on Human Cytochrome P450

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Abstract

Introduction: Asiaticoside and madecassoside are the major triterpenoid compounds from *Centella asiatica* (Linn.) Urban. Asiaticoside was reported to possess wound healing activity, anti-inflammatory, protection of gastric ulcer and free radical scavenger. Madecassoside exhibited anti-inflammatory, antioxidant and rheumatoid arthritis healing activities.
Objective: The purpose of this study was to investigate the effects of asiaticoside and madecassoside on the activities of human cytochrome P450 (CYP) including CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 by using recombinant human CYP.
Materials and methods: Inhibition of CYP450 activities was determined by using the Vivid® CYP450 Screening Kits Protocol. The Vivid® Substrates were metabolized by specific CYP enzymes into products that were highly fluorescent in aqueous solutions.
Results: The results demonstrated that asiaticoside and madecassoside possessed inhibitory effects on CYP3A4 and CYP2C19 while the inhibitory effects of both compounds on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 were not shown.
Discussion and Conclusion: The inhibitory effect of asiaticoside and madecassoside on CYP3A4 and CYP2C19 activities suggested that these compounds were needed to be further investigated whether they would cause drug-drug interaction with medicines that are metabolizes by these enzymes.

Key words: Asiaticoside, Madecassoside, Cytochrome P450

Introduction

Asiaticoside and madecassoside are the major triterpenoid compounds from *Centella asiatica* (Linn.) Urban. *C.asiatica* is a pantropical plant in Thailand that is normally used as food, beverage and traditional medicine (1, 2). There are many preclinical studies of asiaticoside and madecassoside for the pharmacological activity. Asiaticoside was reported to possess the effects of anticancer (3), wound healing activity, anti-inflammatory, protection of gastric ulcer and free radical scavenger (4). Madecassoside exhibited anti-inflammatory (5), antioxidant (6) and rheumatoid arthritis healing activity in mice (7).

During the research and development process of any new compounds, metabolism/ drug-drug interaction studies are required. Effect of any compounds on CYP is a beneficial information regarding drug-drug interaction and the possibility of the compound to increase/decrease risks of xenobiotic-induced toxicity/mutagenesis/carcinogenesis. In 2007, Seeka P. investigated the effect of the standard extract of *Centella asiatica* (ECa 233) on human

CYP by using recombinant human CYP (8). The results showed that ECa233 inhibited CYP2B6, CYP3A4 and CYP2C19 with IC_{50} less than 1,000 µg/ml. As asiaticoside and madecassoside are the major constituents in ECa233, the aim of this study was to investigate effects of both compounds on human CYPs by using recombinant human CYPs in an *in vitro* study.

Materials and Methods

1. Materials

Dimethylsulfoxide (DMSO) and Trizma® base were purchased from Sigma Chemical Co. Ltd., USA. Acetonitrile anhydrous were purchased from Labscan Asia Co. Ltd., Thailand. Ethanol and hydrochloric acid (HCl) were purchased from Merck, Germany. Asiaticoside and madecassoside were kindly provided by Associate Professor Dr. Chamnan Patarapanich, Faculty of Pharmaceuical Sciences, Chulalongkorn University. The Vivid® CYP450 Screening Kit was purchased from Invitrogen Drug Discovery Solutions, USA. It consists of five components as following:

- CYP450 BACULOSOME® Reagents consisted of recombinant human cytochrome P450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4) and rabbit NADPH P450 reductase
- Vivid® Substrates comprised either 7-benzyloxymethyloxy-3-cyanocoumarin (BOMCC) or ethoxymethyloxy-3-cyanocoumarin (EOMCC) depending upon the individual CYP isoforms.
- Vivid® CYP450 Reaction Buffers were potassium phosphate pH 8.0 in the different concentrations depending upon the individual CYP isoforms.
- Regeneration System consisted of Glucose-6-phosphate (G6P) and Glucose -6-phosphate dehydrogenase (G6PD) in potassium phosphate pH 8.0
- $NADP^+$ in potassium phosphate pH 8.0

2. Enzymes assay

Inhibitions of CYP activities were determined according to the Vivid® CYP450 Screening Kits Protocol (www.invitrogen.com). The Vivid® Substrates (BOMCC and EOMCC) were metabolized by a specific CYP enzyme into a product that was highly fluorescent in aqueous solutions.

Briefly, in 96 black well plate, add 40 µl of the compound solution per well and then add 50 µl of the mixture of CYP450 BACULOSOME® Reagents and Regeneration System in Vivid® CYP450 Reaction Buffers or Master Pre-mix. The mixtures were preincubated for 20 min at room temperature. Then the reaction was started by adding 10 µl of the mixture of reconstituted substrate and $NADP^+$ in Vivid® CYP450 Reaction Buffers and incubated for 30-60 min at room temperature. At the end of incubation, 10 µl of 0.5 M Tris-HCl buffer, pH 10.5 was added to quench the reaction. The fluorescence of product was measured by Fluorescence, Absorbance and Luminescence Reader VICTOR3V (Perkin Elmer, USA).

3. Statistical analysis.

Percent inhibition was calculated for each concentration of the test compounds by using the following equation.

$$\% \text{ inhibition} = \left[\frac{1 - (RFU_{\text{test compound}} - RFU_{\text{background of test compound}})}{(RFU_{\text{solvent control}} - RFU_{\text{background of solvent control}})} \right] \times 100$$

RFU – relative fluorescence unit or fluorescence intensity

The median inhibition concentration (IC_{50}) was calculated for the test compounds by using Probit analysis of SPSS 16.0 software.

Results

The results showed that asiaticoside inhibited activities of CYP3A4 and CYP2C19 with IC_{50} of 343.35 µM and 412.68 µM, respectively. Madecassoside inhibited activities of CYP3A4 and CYP2C19 with IC_{50} of 388.11 µM and 539.04 µM, respectively. However, no effects of asiaticoside and madecassoside on CYP1A2, CYP2C9, CYP2D6 and CYP2E1 were observed (Table 1).

Table 1: IC_{50} values of asiaticoside and madecassoside on human CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 activities

	IC_{50} values (µM)					
	CYP1A2	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
Asiaticoside	>1000	>1000	412.68 (331.43-502.90)	>1000	>200*	343.35 (236.75-562.67)
Madecassoside	>1000	>1000	539.04 (467.40-624.86)	>1000	>1000	388.11 (286.50-526.08)

Data presented IC_{50} values with 95% confidence interval from four experiments (n=4).

* The data was limited by the solubility of asiaticoside and the amount of DMSO.

Discussion and Conclusion

Inhibitory effects of asiaticoside and madecassoside on CYP3A4 and CYP2C19 activities suggested the possibility of these compounds regarding drug-drug interaction if these compounds were administrated concomitantly with other medicines that are metabolizes by these enzymes. Further study should be investigated whether this inhibitory effect would be clinically significant.

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BIOGRAPHY

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