ผลยับยั้งของสารสกัดด้วยน้ำของผลมะขามป้อม ต่อรีคอมบิแนนต์เอนไซม์ไซโตโครม พี 450 ของมนุษย์

นางสาว นภาพร อนันต์นฤการ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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INHIBITORY EFFECT OF *PHYLLANTHUS EMBLICA* AQUEOUS EXTRACT ON RECOMBINANT HUMAN CYTOCHROME P450

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	INHIBITORY EFFECT OF *PHYLLANTHUS EMBLICA* AQUEOUS EXTRACT ON RECOMBINANT HUMAN CYTOCHROME P450
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นภาพร อนันต์นฤการ: ผลยับยั้งของสารสกัดด้วยน้ำของผลมะขามป้อม ต่อรีคอมบิแนนต์เอนไซม์ไซโตโครม พี 450 ของมนุษย์ (INHIBITORY EFFECT OF *PHYLLANTHUS EMBLICA* AQUEOUS EXTRACT ON RECOMBINANT HUMAN CYTOCHROME P450) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. พ.ต.ท.หญิง ดร.สมทรง ลาวัณย์ประเสริฐ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ.นวลศรี นิวัติศัยวงศ์, ผศ. ภญ. ดร.วรางคณา วารีสน้อยเจริญ, 98 หน้า.

มะขามป้อม (*Phyllanthus emblica* Linn., Euphorbiaceac) เป็นพืชที่มีการนำส่วนต่างๆ มาใช้ประโยชน์กันมาก เช่น แก้ไอ แก้ปวด ลดไข้ ผลของมะขามป้อมยังเป็นแหล่งที่มีปริมาณวิตามินซีสูงมาก และจากอดีตถึงปัจจุบันยังคงมีการศึกษาฤทธิ์ทางเภสัชวิทยาของมะขามป้อมกันอย่างต่อเนื่อง การศึกษาครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลยับยั้งของสารสกัดด้วยน้ำของผลมะขามป้อม ต่อรีคอมบิแนนต์เอนไซม์ไซโตโครม พี 450 ของมนุษย์ ได้แก่ CYP1A2, CYP2C9, CYP2D6, CYP2E1 และ CYP3A4 แบบนอกกาย ทำการวิเคราะห์หาค่า IC_{50} ของสารสกัดต่อเอนไซม์แต่ละ isoform ศึกษาชนิดของการยับยั้งแบบไม่ผันกลับหรือ mechanism-based inhibition ด้วยการประเมินจากค่า IC_{50} ratio และศึกษาชนิดของการยังยั้งแบบผันกลับโดยใช้ Lineweaver-Burk plot เพื่อวิเคราะห์หาค่า K_i ซึ่งเป็นค่าคงที่ของการยับยั้งของสารที่นำไปใช้ทำนายผลของการยับยั้งของสารสกัดที่อาจส่งผลให้เกิดอันตรกิริยาระหว่างยากับสมุนไพรต่อไปได้ ผลการศึกษาพบว่าสารสกัดมีผลยับยั้งเอนไซม์ CYP1A2, CYP2C9, CYP2D6, CYP2E1 และ CYP3A4 โดยมีค่า $IC_{50} \pm SE$ เท่ากับ 310.28 ± 5.07, 194.72 ± 2.94, 589.52 ± 14.32, 310.27 ± 15.06 และ 325.544 ± 7.44 µg/mL ตามลำดับ นอกจากนี้ยังพบว่าสารสกัดมีผลยับยั้ง CYP3A4 แบบ mechanism-based inhibition โดยมีค่า IC_{50} ratio มากกว่า 1 (1.029) และจากการศึกษาชนิดของการยังยั้งแบบผันกลับ พบว่าสารสกัดมีกลไกในการยับยั้งแบบผันกลับชนิด non competitive ต่อ CYP3A4 โดยมีค่า $K_i \pm SE$ เท่ากับ 117.11 ± 1.55 µg/mL ข้อมูลที่ได้จากการศึกษานี้เป็นประโยชน์ต่อการทำนายโอกาสของการเกิดอันตรกิริยาระหว่างสารสกัดนี้กับยาที่ถูกเปลี่ยนแปลงด้วยเอนไซม์ CYP isoforms เหล่านี้ได้ ควรมีการศึกษาต่อไปถึงผลของสารสกัดด้วยน้ำของผลมะขามป้อมต่อเอนไซม์ CYP แบบในกายเพื่อเป็นข้อมูลยืนยันความปลอดภัยจากอันตรกิริยาระหว่างสมุนไพรและยา ก่อนนำไปใช้ในทางคลินิก

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NAPAPORN ANANNARUKAN: INHIBITORY EFFECT OF *PHYLLANTHUS EMBLICA* AQUEOUS EXTRACT ON RECOMBINANT HUMAN CYTOCHROME P450. THESIS ADVISOR: ASSOC. PROF. POL.LT.COL. SOMSONG LAWANPRASERT, Ph.D., THESIS CO-ADVISORS: ASSOC. PROF. NUANSRI NITIWATTISAIWONG, ASST. PROF. WARANGKANA WARISNOICHAROEN, Ph.D., 98 pp.

Phyllanthus emblica Linn. is a plant in family Euphorbiaceae. Several parts of *P. emblica* are used for antitussive, anti-inflammatory and antipyretic. Its fruits are the rich source of ascorbic acid. From the past to present, investigations regarding pharmacological effects of *P. emblica* are still ongoing. The aim of this study is to investigate the inhibitory effect of *P. emblica* aqueous extract on recombinant human cytochrome P450 such as CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 *in vitro* study. IC_{50} of the extract on each CYP isoform was determined. The mechanism-based inhibition was investigated using the IC_{50} ratio. Then, the type of reversible inhibition was investigated using Lineweaver-Burk plot for determining K_i value, the inhibition constant for predicting of herb-drug interaction. The result showed that the extract exhibited inhibitory effects on CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 with $IC_{50} \pm$ SE of 310.27 ± 5.07, 194.72 ± 2.94, 589.52 ± 14.32, 310.27 ± 15.06 and 325.54 ± 7.44 µg/mL, respectively. Inhibitory effect of the extract on CYP3A4 was shown to be mechanism-based with an IC_{50} ratio of more than 1 (1.029). Also, the extract exhibited CYP3A4 reversibly by the non-competitive type with the $K_i \pm$ SE value of 117.11 ± 1.55µg/mL**.** Results from this study provide preliminary data for predicting the possibility of drug interaction when this extract is co-administered with other medicines that are metabolized by CYP enzymes. Further *in vivo* study is needed so as to assure the safety profile before using the extract clinically without herb-drug interaction.

Department: Pharmacology and Physiology Student's Signature ……..……...…….

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CONTENTS

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Figure **Page**

Figure **Page**

Figure **Page**

LIST OF ABBREVIATIONS

α	alpha
β	beta
γ	gamma
^{o}C	degree celcius
nm	nanometer
nM	nanomolar
µg	microgram
µL	microlitre
µM	micromolar
µg/mL	microgram per milliliter
g	gram
RFU	relative fluorescence unit
et al.	et alii
L	liter
mg	milligram
mL	milliliter
mM	millimolar
pH	potential of hydrogen
SD	standard deviation
SE	standard error of mean
U.S.	United State of America
FDA	Food and Drug Administration
CYP	cytochrome P450
DMSO	dimethylsulfoxide

CHAPTER I

INTRODUCTION

Currently, herbal medicines have become popular as alternative medicines (Eisenberg et al., 1993; Ernst and Cassileth, 1998; Pelletier, Astin and Haskell, 1999; Yamashita et al., 2002; Scott et al., 2005; Zhou et al., 2007). Several studies from the past to present have shown that many plants are interesting candidates in the drug research and development process. As a result, some species of herbal medicine were studied and some of them have already become modern medicines (Joy et al., 2001; Zhou et al., 2007) such as vincristine and vinblastine which were evolved from vinca alkaloids of *Catharanthus roseus*, were used to treat leukemia (Johnson et al., 1963; Verrills et al., 2003). Quinoline alkaloids extracted from bark and root bark of Cinchona *spp*. were developed to be quinine that is one of the anti-malarial agents (White et al., 1996; Druilhe et al., 1998) etc. Therefore, research studies on the pharmacological activities of herbal plants are still an interesting field.

One of the large numbers of plants in Euphorbiacea family, *Phyllanthus* is a prominent genus because it consists of a plenty of plant species (Kandavel et al., 2011). *Phyllanthus emblica* Linn. with a botanical synonym name of *Emblica officinalis* Gaertn. has been used for a long time as folk and traditional medicine. Its chemical constituents include alkaloids, benzenoids, flavonoids, hydrolysable tannins, sterol, ascorbic acid or vitamin C, etc (Barthakur et al., 1991; Techadamrongsin and Dechtiwongse Na Ayudhya, 1997; Summanen, 1999; Khan, 2009). *P. emblica* possesses numerous pharmacological activities including antioxidant (Banavaliker, Biyani and Mittal, 2001; Khopde et al., 2001; Liu et al., 2008a; Luo et al., 2009; Khan, 2009), antimicrobial (Ahmad, Mehmood and Mohammad, 1998; Saeed and Tariq, 2007; Rahman et al, 2009), hepatoprotective activity against ethanol (Pramyothin et al., 2006), carbon tetrachloride (Jeena and Kuttan, 2000), *N*-nitrosodiethylamine (Jeena, Joy and Kuttan, 1999), paracetamol (Vidhya Malar and Mettilda Bai, 2009) and diethylnitrosoamine (Sultana, Ahmed and Jahangir, 2008) as well as anti-inflammatory (Muthuraman, Sood and Single, 2010), anti-pyretic (Perianayagama et al., 2004), anti-tussive (Nosál'ová, Mokry´ and Hassan, 2003) anti-proliferative of cancer cells (Zhang et al., 2004, Pinmai et al., 2008), anti-gastric ulcer

(Akhtar, Zaman and Khan, 2004) and hypolipidemic (Mathur et al., 1996; Anila and Vijayalakshmi, 2002), etc.

Regarding toxicity profile, *P. emblica* was shown to be safe in both acute toxicity and sub-acute toxicity test in animal model (Itthipanichpong et al., 1987; นันทวัน บุณยะประภัศร, 2542; สุธรรม อารีกุล, 2551). In addition to toxicity data, drug interaction study is also recommended by the U.S. Food and Drug Administration (FDA) in the process of research and development of a new compound so as to additionally assure the safety before studying in clinic (FDA, 2006). Thus, drug interaction data is needed for *P. emblica* to avoid herb-drug interaction when this plant is developed to be used clinically according to its several pharmacological activities mentioned earlier.

One of the main etiologies of drug interaction is attributed by an effect of interacting compound on metabolism process of the interacted drugs. The major enzyme involved in drug interaction is the enzymes in Phase I metabolism, cytochrome P450 (CYP) (Shou and Dai, 2008; Lu and Huang, 2004). Generally, CYP is the major catalytic enzyme system of drug and xenobiotic metabolism. CYP is found highly remarkable expression in intrahepatic tissues and lower in extrahepatic tissues (Guengerich, 1992; Seliskar and Rozman, 2007). Among 18 families of human CYP, CYPs in families 1, 2 and 3 play an important role in drug and/or xenobiotic metabolism (Parkinson and Ogilvie, 2008; Williams, 2008). CYP can be either induced or inhibited by internal or mostly external factors. Inhibition and induction of any CYP isoforms by any xenobiotics can modulate metabolism of therapeutic agents which are metabolized by the particular CYP isoforms that are affected, resulting in drug interaction (Lynch and Price, 2007; Williams, 2008). Thus, in an attempt to develop *P. emblica* to be used therapeutically, inhibitory and/or inducing effects of *P. emblica* on CYP isoforms which are responsible for drug metabolism should be investigated so as to provide a safety profile regarding drug interaction if this plant is co-administered with other medicines. Thus, the aim of this study is to investigate inhibitory effect of *P. emblica* aqueous extract on human CYP such as CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 using human recombinant CYP by the *in vitro* study.

Hypothesis

P. emblica aqueous extract exhibited an inhibitory effect on recombinant human CYP.

Study design and Processes

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

1. Inhibitory effect of *P. emblica* aqueous extract on the activities of human CYP including CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 were determined.

2. Mechanism-based inhibition studies of *P. emblica* aqueous extract were assessed.

3. Kinetic assay study of *P. emblica* aqueous extract was performed to determine the type of reversible inhibition.

Expected benefits from the study

The information of the inhibitory effects of *P. emblica* aqueous extract on human CYPs would provide the potential possibility of drug interaction between the extract and the prescribing medicines which are the substrates of these CYP isoforms.

CHAPTER II

LITERATURE REVIEWS

Phyllanthus emblica Linn. or *Emblica officinalis* Gaertn. (**Figure 1**), is a plant species in Euphorbiaceae family. It is a deciduous wood, small tree to medium size with an average height of about 5 meters and the average circumference of not exceeded 80 centimeters. Pinnate leaves consist of petiole and a numerous small leaflets which regular arrangement look like a feather. Fleshy fruits of nearly round shape are average of 2 cm in diameter. Raw fruits are green and glossy surface then changed to yellow at the beginning to be ripe fruits (สุธรรม อารีกุล, 2551, Summanen, 1999)

P. emblica can be found in tropical and subtropical country such as China, Cambodia, India, Indonesia, Malaysia, Srilanka and also in Thailand. It is called *yeowkan* or *an mole* in Chinese; *emblica myroblan* or *indian gooseberry* in English, *amla* in Indian, *popok melaka* in Malaysian, *bong ngot* in southern Vietnam, *mak kham pom* in Laos and *ma kham pom* in Thai (Morton, 1987; Khan, 2009).

Figure 1 *Phyllanthus emblica* Linn.

There are many studies indicated that *P. emblica* contains numerous constituents as shown in **Table 1**.

Table1 The chemical constituents of *P. emblica* (modified from Summanen, 1999; Khan, 2009)

Class	Compound	Occurrence	Reference
Alkaloids	phyllanthin	leaves fruits	Majeed et al., 2009
Benzenoid	gallic acid, ellagic acid	leaves	Subramanian et al., 1971
Carbohydrate	glucose	leaves	Zhang et al., 2004
Diterpene	gibberellin A-1, A-3, A-4, A-7 and A-9	fruits	Liu et al., 2008 Zhang et al., 2004
Flavonoid	kaempherol quercetin mucic acid	leaves roots fruits	Anila et al., 2002 Liu et al., 2008 Zhang et al., 2002 Subramanian et al., 1971 Luo et al., 2009 Zhang et al., 2001
Furanolactone	ascorbic acid	leaves fruits	Raghu et al., 2007 Zhang et al., 2002 Jain and Khurdiya, 2004
Hydrolysable tanins	emblicanin A, B	fruits leaves roots	Majeed et al., 2009 Zhang et al., 2004 Techadamrongsin et al., 1997
Sterol	β-sitosterol	leaves roots	Itthipanichpong et al., 1987

P. emblica has been known in its famed source of high ascorbic acid. Many studies reported the content of ascorbic acid in different feature such as ascorbic acid content in a fresh fruit of *P. emblica* was equal to ascorbic acid content in 1 to 2 of orange fruits (Itthipanichpong et al., 1978). The content of ascorbic acid in both fresh

fruits and dried fruits was determined by several methods such as (1) 2,4-Dinitrophenylhydrazine method which determine the oxidation of ascorbic acid to dehydroascorbic acid, (2) indophenols-xylene method which quantitatively determine decoloration of 2,6-dichlorophenol by ascorbic acid. (3) Enzymatic method was used to determine ascorbic acid oxidase reaction and (4) the high performance liquid chromatography (HPLC) was used to determine the quantity of ascorbic acid. The results from those 4 methods studies indicated that *P. emblica* fruits contained ascorbic acid as following: 468.8 ± 36.7, 206.8 ± 32.0, 213.5 ± 39.0 and 236.2 ± 34.3 mg per 100 g of fresh fruits, respectively. In case of dried fruits either sun-dried or shade-dried, ascorbic acid content is reduced as it may resulting from drying processes but ascorbic acid retained estimate to 34 - 38 mg equivalent to 100 g of fresh fruits. Thus, it showed that fresh fruits and dried fruits of *P. emblica* are the good source of ascorbic acid as compared to some other fruit such as guava fruits (Raghu et al., 2007). In an equal amount of fruits, *P. emblica* possesses ascorbic acid 160 times as compared to that in apple (*Malus pumila* Mill.) (Barthakur et al., 1991).

Pharmacological effects

1. Antioxidant activity

The methanol extracts of *P. emblica* fruits from six regions in China were assessed for the antioxidant activity using *in vitro* scavenging assay. The results showed that the extracts exhibited free radical scavenging activity such as 1,1-diphenyl-2-picryhydrazyl (DPPH), superoxide anion and hydroxyl radical. Also, high capability of chelating ferrous ions of the extracts was shown. (Liu et al, 2008a, 2008b; Luo et al, 2009). In the study of Khopde et al (2001), aqueous extract of *P. emblica* fruit exhibited antioxidant activity against γ - radiation-induced lipid peroxidation and attenuated superoxide dismutase activity in rat liver microsome.

2. Antitussive activity

Antitussive activity of *P. emblica* was investigated using mechanical stimulation of laryngopharyngeal and tracheobronchial mucous area of airways in cats. Fresh fruit extract of *P. emblica* exhibited dose-dependent cough suppressive activities. However, the antitussive effect of the extract was less than codeine, the

narcotic antitussive. However, its effect was shown to be more effective than dropropizine which is the non-narcotic antitussive drug (Nosál'ová et al, 2003).

3. Hepatoprotective effect

N - nitrosodiethylamine (NDEA) has been reported in its potential to induce hepatocarcinogenesis. Jeena, Joy and Kuttan (1999) studied the hepatoprotective effect of the extracts of 3 plants, *P. emblica*, *P. amarus* and *Picrorrhiza kurroa.* The results showed that all extracts showed the dose-dependent inhibition effect on hepatocarcinogenesis-induced by NDEA in rats. Level of carcinogen metabolizing enzymes and liver injury markers, for example glutathione *S*-transferase and aniline hygroxylase were shown to be increased in the liver tissue. Serum lipid peroxide, alkaline phosphatase and glutamate pyruvate transminase which are markers of liver injury were shown to be decreased. According to the study of Pramyothin et al. (2006), ethanolic extract of *P. emblica* fresh fruits demonstrated hepatoprotective activity against ethanol induced hepatic injury in rats by decreasing the liver injury markers such as serum aspatate transaminase, alanine transminase, serum triglyceride and IL-1β level. In addition, hepatoprotective activity from acute and chronic CCl_4-induced liver injury was also demonstrated in rats (Jose and Kuttan, 2000).

4. Antimicrobial effect

Agar diffusion technique was used to determine the synergistic effect of antibacterial activity against methicillin-resistant *Staphylococous aureus* (MRSA) activity of crude ethanol extract of either *P. emblica* or *Nymphae odorata* when they were combined with amoxicillin. The results showed that either using the plant extract alone or combined with amoxicillin, effect of *P. emblica* extract was shown to be stronger than those of *Nymphae odorata* extract. The synergistic effect against MRSA was demonstrated when the extracts were combined with amoxicillin (Mandal et al., 2010). Antimicrobial effects of Indian medicinal plants, including *P. emblica*, *Terminalia chebula*, *Terminalia belerica*, *Plumbago zeylanica* and *Holarrhena antidysenterica* against organisms such as *Bacillus subtitis*, *Proteus vulgaris*, *Samonella typhimurium*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococous aureus* were also reported (Ahmad, Mehmood and Mohammad, 1998; Saeed and Tariq, 2007; Rahman et al, 2009).

5. Anti-tumor effect

Pinmai et al. (2008) reported the growth inhibitory effect of the extracts of *P. emblica* and *Terminalia bellrica* on human lung carcinoma (A549) and human hepatocellular carcinoma (HepG2). The sulforhodamine B assay was used to determine growth inhibition of the extracts. The results demonstrated that both plant extracts inhibited both carcinoma cell lines. Then, when each plant extract was combined with known chemotherapeutic drugs such as doxorubicin or cisplatin to evaluate the synergistic inhibitory effect to both carcinoma cell lines. The results indicated that combination of *P. emblica* and *T. bellrica* extract with chemotherapeutic drugs provide beneficial effect by decrease the toxic effect of the chemotherapeutic agents. Zhang et al. (2004) isolated several constituents from *P. emblica* and found antiproliferative effect of the constituents on carcinoma cell lines such as murine melanoma (B16F10) and human uterine carcinoma (HeLa) human gastric adrenocarcinoma. Moreover, antitumor activity of *P. emblica* was found on Daltons lymphoma ascites cells (DLA) and Ehrlich ascites cells (EAC) (Jose, Kuttan and Kuttan, 2001).

6. Anti-inflammatory effect

The free and bound phenolic compounds from *P. emblica* extract were shown to reduce both carrageenan-induced acute inflammation and cotton pellet-induced chronic inflammation in rats. The result showed that the extract decreased the paw volume of rats, reduced the marker of chronic inflammation and the granulomatous tissue as compared to the control (Muthuraman, Sood and Singla, 2010).

7. Hypolipidemic effect

Anti-hyperlipidemic effect of the flavonoids from *P. emblica* and *Mangifera indica* extracts was investigated using biochemical assays. The results showed the effectively decrease of the lipid level including cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL), triglycerides, phospholipids and free fatty acid in serum and tissue of rats which were induced hyperlipidemia by diet (rodent chow with 2% cholesterol and coconut oil). Furthermore, *P. emblica* extract also decreased the activity of HMG CoA reductase, an important enzyme to synthesis cholesterol but this effect was not showed in flavonoid from *Mangifera indica*. (Anila

and Vijayalakshmi, 2002). Matur et al. (1996), demonstrated that fresh fruits juice of *P. emblica* exhibited the lipid lowering and anti-artherosclerotic effect in cholesterol-fed rabbits.

8. Hypotensive effect

Aqueous extract of *P. emblica* dried fruits was used to assess the hypotensive potential in healthy dogs. The investigation was performed by detecting the mean arterial blood pressure, heart rate and respiratory rate. The results exhibited the biphasic action of mean arterial blood pressure with the slightly potential effect to increase at initiation then followed by decrease at longer duration. Increase of rate and depth of respiration as well as decrease of heart rate was shown. Moreover, the extract exhibited the depressor effect on various agonists such as acetylcholine and histamine (Ishaq et al., 2005).

10. Chondroprotective effect

Despite the glucosamine sulphate which is used to improve osteoarthritis (Pavelka´ et al., 2002), *P. emblica* aqueous extract was reported to show the chondroprotective effect in an *in vitro* assay. The results showed that the extract inhibited collagenase type II and hyaluronidase, which were factors contributed significantly to cartilage degradation (Sumantran et al., 2008).

11. Collagen production

Effect of *P. emblica* extract on the production of procollagen, matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) which were associated to the tension of skin was demonstrated (Fujii et al., 2008). In that study, mitochondrial activity of human skin fibroblasts was determined to evaluate cell proliferation. The result showed that *P. emblica* extract increased the mitochondrial activity in fibroblasts in a concentration-dependent manner. Moreover, the extract exhibited production of procollagen in a concentration - and time - dependent manner as well as increase of TIMP-1 production, which is impede the actions of MMPs in the dermis. The effect on MMP-1, which damaged the collagen in skin was shown to be decreased.

12. Cyto-protective and immunomodulating properties

Cyto-protective and immunomodulating properties of of *P. emblica* ethanol extract were reported by Ram et al. (2002). They used lymphocytes which were isolated from splenocytes of Sprague-Dawley rats to investigate the chromium (Cr)-induced cytotoxicity and apoptosis of cell culture. The results showed that *P. emblica* fruit extract inhibited cytotoxicity, apoptosis and increased proliferation effect of lymphocytes as compared to control. Moreover, the hall mark of apoptosis, DNA ladder was not found on the agarose gel electrophoresis in the extract treatment.

13. Snake venom neutralization

Methanol root extracts of *Vitex negundo* and *P. emblica* were used to determine the neutralized effects on *Vipera russelli* and *Naja Kaouthia* venoms, the well-known venom-induced lethality. The *in vitro* results showed that both extracts exhibited protective effect at various doses of venom toxicity (Alama and Gomes, 2003).

14. Antiulcer effects

Antiulcer effects of *P. emblica* fruits aqueous extract and its purified fraction were performed in rats which were indomethacin-induced gastric ulcer. The results showed that *P. emblica* aqueous extract and its purified fraction reduced gastric pH and exhibited inhibitory effect on gastric lesions as compared to the control group. (Akhtar et al., 2004)

15. Memory enhancing

Vasudevan and Parle (2007) investigated effect of *P. emblica* on memory enhancement in mice using the behavior model. The results showed that *P. emblica* extract improved short-term memory of mice by the reduction of transfer latency and increased the step-down latency which represented for long-term memory as compared to the control group and drug-induced amnesia groups. Moreover, the extract decreased activity of cholinesterase in mice brain and decreased serum cholesterol levels in both young and aged mice as compared to the control (Vasudevan and Parle, 2007).

Biotransformation

Biotransformation, is an important process to alter physiochemical properties of foreign chemicals, is termed “xenobiotics” (chemicals, drugs, foods, pollutants, etc.) from lipophilicity to hydrophilicity and then eliminate from the body (Parkinson and Ogilvie, 2008; Williams, 2008). Biotransformation of xenobiotics is divided into 2 phases, phase I and phase II metabolism. Phase of metabolism can be described as following.

Phase I metabolism

The major role of phase I metabolism is the addition of functional groups to the molecule of xenobiotics to more polar molecules before continuing through phase II metabolism. Phase I metabolism includes several reactions such as oxidation, reduction, hydrolysis, etc.

Phase II metabolism

Phase II metabolism includes the reactions which conjugate the endogeneous substances with functional groups on the molecules of xenobiotics to converse the xenobiotics or their metabolites from phase I metabolism to yield soluble products before excreting from the body in bile or urine (Gibson and Skett, 2001).

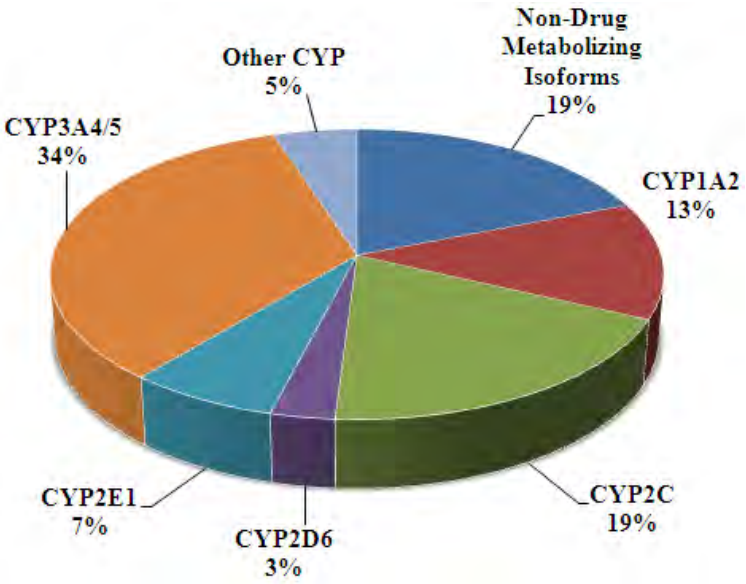
Cytochrome P450 (CYP)

CYP, an important enzyme in phase I metabolism, is one of the most etiology of drug-drug interaction, food-drug-interaction and herbal-drug interaction (Manzi and Shannon, 2005). Drug interaction can occur throughout the pharmacokinetic process including absorption, distribution, metabolism and excretion.

CYP is the superfamily of enzymes which found abundant in human liver and a few found in extrahepatic tissues such as intestine, brain, placenta, kidney, bone marrow, etc (Anzenbacher and Anzenbacherova, 2001; Williams, 2008). Each isoform of CYP is different by its amino acid sequence. CYP in family 1, 2 and 3 exhibited the major role to metabolize most prescribing medicines (Copeland, 2005; Williams, 2008). The percentage of each CYP isoform and the percentage

of prescribing medicines that are metabolized by each CYP isoform are shown in **Figure 2.**

(a)



(b)

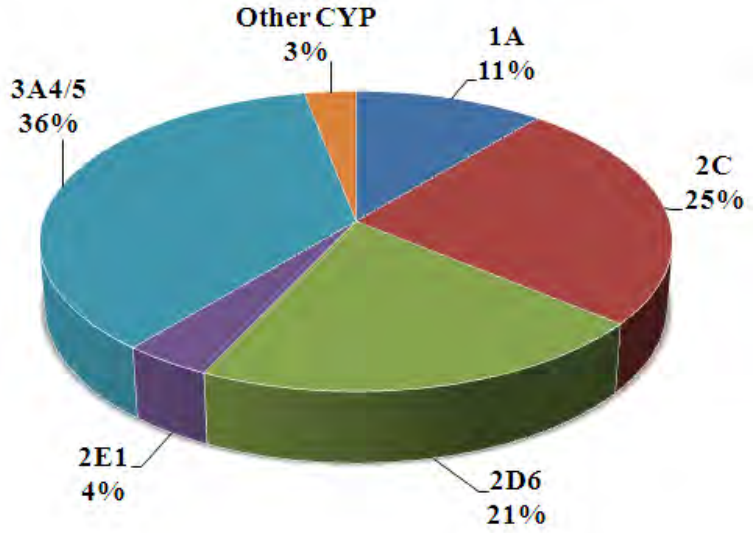


Figure 2 Demonstration of the percentage of amount of CYP in human liver (a). The percentage of prescribing drugs that are metabolized by CYP (b). (modified from: Anzenbacher and Anzenbacherova, 2001; Eddershaw and Dickins, 2004; Williams, 2008)

Examples of human CYP responsible to xenobiotic metabolism (Goshman, Fish and Roller, 1999; Coleman, 2005; Manzi and Shannon, 2005; Williams, 2008) are described as following:

CYP1A2

CYP1A2 exhibits for about 13% in human liver. It plays a major role in the metabolism of polycyclic aromatic hydrocarbon and benzene compounds such as naphthoflavone, benzo (a) pyrene. About 11% of prescribing drugs are metabolized by CYP1A2 such as theophylline, caffeine, tricyclic antidepressants (TCAs) and warfarin. Inhibitors of CYP1A2 are cimetidine, erythromycin and ticlopidine. Inducers of CYP1A2 are β-naphthoflavone, carbamazepine, omeprazole, rifampicin and verapamil.

CYP2C9

CYP2C9 represents about 19% in human liver. About 25% of prescribing drugs were metabolized by CYP2C9 such as diclofenac, ibuprofen, phenytoin and warfarin. Inhibitors of CYP2C9 are sulfamethoxazole-trimethoprim, fluconazole and isoniazid. Inducers of CYP2C9 are carbamazepine, ethanol, rifampicin and phenobarbital.

CYP2D6

Although CYP2D6 exhibits for a few about 3% in human liver, its major role in drug metabolism is about 21%. Prescribing drugs metabolized by CYP2D6 are β-blockers, haloperidol, codeine and TCAs. Inhibitors of CYP2D6 are bupropion, celecoxib, cocaine and haloperidol. Inducers of CYP2D6 are not reported but this CYP isoform is increasing in pregnancy.

CYP2E1

CYP2E1 exhibits for about 7% in human liver. The percentage of prescribing drugs metabolized by CYP2E1 is about 4%. Its role in xenobiotic metabolism is found

in several chemicals such as carbontetrachloride, ethanol, nitrosamine and also acetaminophen. Inhibitors of CYP2E1 are disulfiram, dietyl dithiocarbamate, and imipramine. Inducers of CYP2E1 are isoniazid, tobacco, ritronavir and the major in alcoholism.

CYP3A4

CYP3A4 is the major enzyme in drugs metabolism in human and the amount of this enzyme is about 34% in human liver. About 36% of prescribing drugs are metabolized by CYP3A4 such as cyclosporine, atorvastatin and carbamazepine. Inhibitors of CYP3A4 are omeprazole, ketoconazole, protease inhibitors, fluconazole and grapefruit juice. Inducers of CYP3A4 are prednisolone, dexamethasone, rifampicin, carbamazepine, efavirenze and St. John's Wort.

Summary of the substrates, inhibitors and inducers of CYP isoforms mentioned above are shown in **Table 2.**

Table 2 Substrates, inhibitors and inducers of human CYP enzymes (modified from Eddershaw and Dickins, 2004; Badyal and Dadhich, 2001; Manzi and Shannon, 2005)

CYP isoform	Substrate	Inhibitor	Inducer
CYP1A2	Acetaminophen, Amitriptyline, Caffeine, Clozapine, Estradiol, Haloperidol, Naproxen, Propanolol, Theophylline	Fluvoxamine, Fluoxetine, Ciprofloxacin, Grapefruit juice, Verapamil, Omeprazole	Tobacco, Carbamazepine, Omeprazole, Phenobarbital
CYP2C9	Amitriptyline, Celecoxib, Diclofenac, Ibuprofen, Phenytoin, Tamoxifen	Amiodarone, Ketoconazole, Metronidazole, Fluconazole	Carbamazepine, Phenytoin, Ethanol, Rifampicin

CYP isoform	Substrate	Inhibitor	Inducer
CYP2D6	Amitriptyline, Chlorpheniramine, Clozapine, Dextromethrophan, Fluoxetine, Paroxetine, Propanolol	Amiodarone, Cimetidine, Fluoxetine, Quinidine	Pregnancy
CYP2E1	Acetaminophen, Caffeine, Ethanol, Dapsone, Halothane, Isoniazid, Theophylline	Disulfiram, Imipramine, Methylpyrazole	Acetone, Chronic ethanol, Isoniazid, Ritronavir, Tobacco
CYP3A4	Alprazolam, Amiodarone, Bupropion, Calcium, Carbamazepine, Cocaine, Codeine, Diazepam, Doxycycline, Erythromycin, Nicardipine, Ritronavir, Simvastatin, Tamoxifen	Itraconazole, Ketoconazole, Fluconazole Erythromycin, Cimetidine Clarithromycin , Fluoxetine, Paroxetine Verapamil, Grapefruit juice	Carbamazepine, Efavirenz, Phenobarbitone, Phenytoin, Prednisolone, Rifampicin, St. John’s Wort

Modulation of CYP either increasing or decreasing of CYP functions changes the metabolism of xenobiotics and/or drugs. Inhibition of CYP is one of the most occurrence drug interactions (Parkinson and Ogilvie, 2008).

Mechanism of CYP inhibition

The characteristic mechanism of CYP inhibition can be classified into 2 major types as following.

Irreversible inhibition or mechanism-based inhibition

Mechanism-based inhibition is an important type of CYP inhibition. The characteristic of this type of inhibition is the inhibitor is oxidized by CYP generating the intermediate-metabolite that binds irreversibly to CYP resulting in dysfunction of CYP. Thus, this manner was often called suicide inhibition. Mechanism-based inhibitor of CYP is chloramphenicol, cyclophosphamide, 17α-ethinyl estradiol, grape fruit juice, norethisterone, spironolactone, serotonin reuptake inhibitors, etc (Coleman, 2005; Williams, 2008). The schematic of this type of inhibition is shown in **Figure 3**.

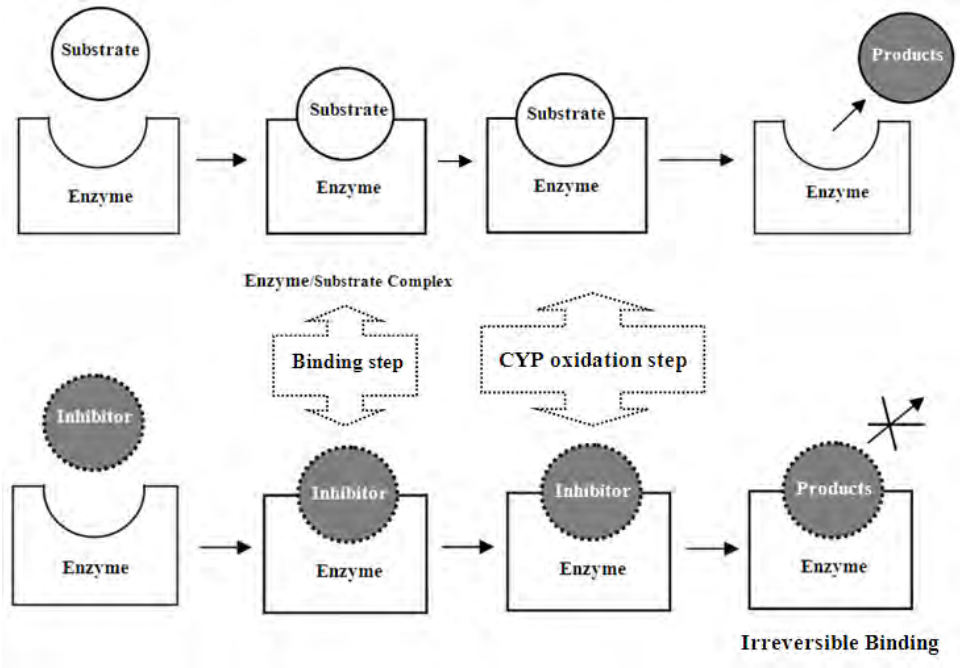


Figure 3 The schematic explanation of irreversible inhibition or mechanism-based inhibition (modified from: Coleman, 2005)

Reversible inhibition

This type of inhibition is catalytic independent. Reversible inhibition is divided into 3 types, including competitive, non competitive and un-competitive inhibition. Each type of reversible inhibition is different in mechanism as following.

Competitive inhibition

The characteristic of competitive inhibition is that the inhibitor competes with the substrate to bind to the active site of the enzyme. Thus, the substrate is unable to bind to the enzyme and product cannot to be produced. This inhibition type is the most observed type of enzyme inhibition (Tracy, 2008). Competitive inhibitor of CYP including cimetidine, diltiazem, fluconazole, itraconazole, ketoconazole, quinidine, etc (Coleman, 2005; Williams, 2008). The schematic of this type of inhibition is shown in **Figure 4**.

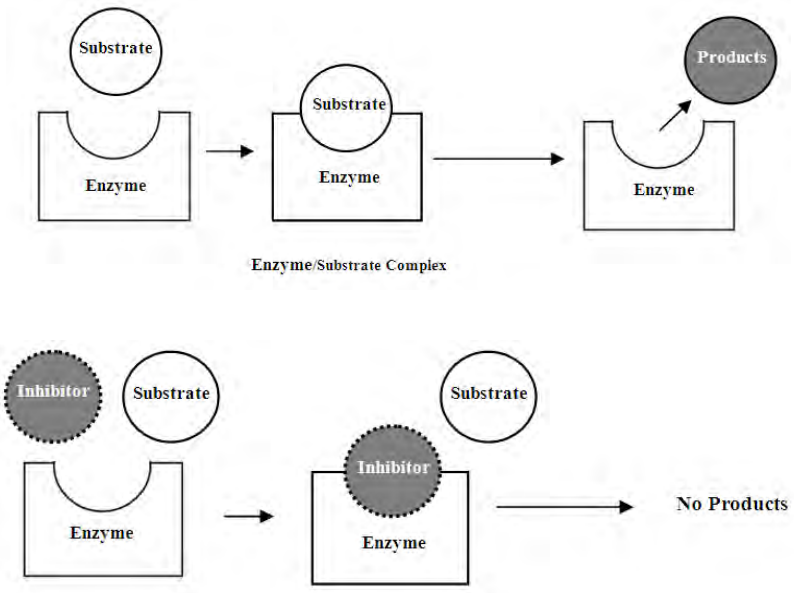


Figure 4 The schematic explanation of competitive inhibition (modified from: Coleman, 2005)

Non-competitive inhibition

Non-competitive inhibition is different from the competitive inhibition because the inhibitor dose not competes with the substrate to bind to the active site of the enzyme. Instead, inhibitor binding to other site of the enzyme causes the conformation change of the active site thus, the substrate is unable to bind to the enzyme and the product is not occurred. The schematic of this type of inhibition is shown in **Figure 5**.

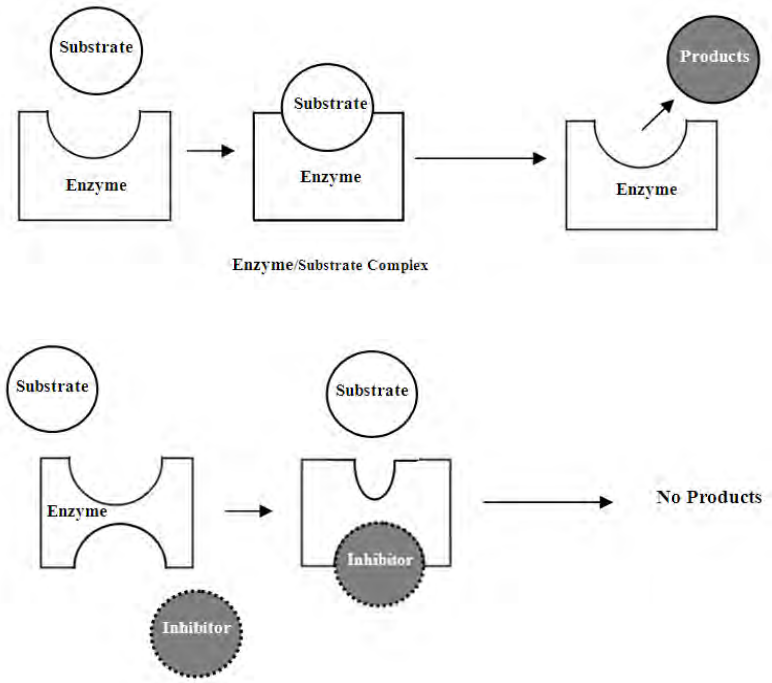


Figure 5 The schematic explanation of non-competitive inhibition (modified from: Coleman, 2005)

Un-competitive inhibition

Un-competitive inhibition is explained by binding of inhibitor and substrate at the same active site. Then, the complex of enzyme-substrate-inhibitor was dysfunction. Thus, the product is not produced. The schematic of this type of inhibition is shown in **Figure 6.**

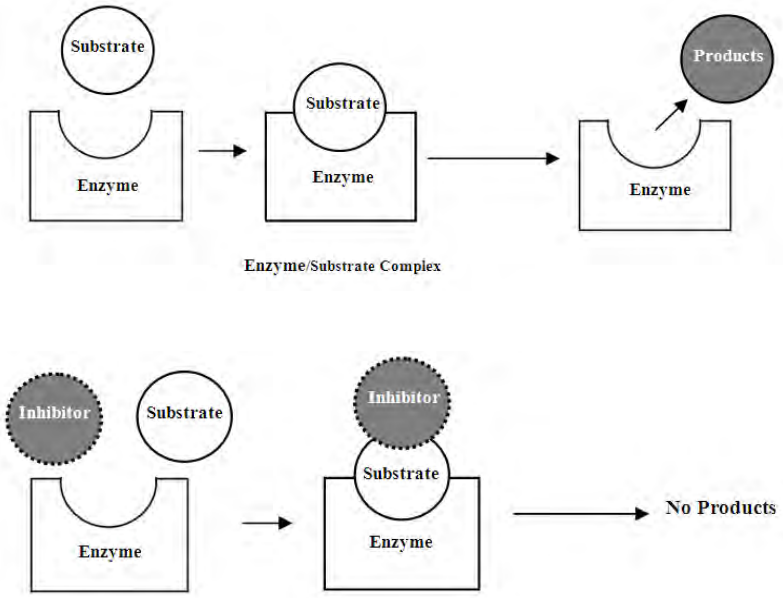


Figure 6 The schematic explanation of un-competitive inhibition (modified from: Coleman, 2005)

Kinetic of enzyme

The characteristic of enzyme kinetic is often explained by the Michaelis-Menten equation as following:

$$v = \frac{V_{max}\,[S]}{K_m + [S]} \qquad \text{..... (1)}$$

The term *v* is the initial velocity of the reaction, V_{max} refers to the maximum velocity of the reaction which obtaines from the excess substrate concentration of the reaction, [S] is the substrate concentration and K_m, which is commonly refer to as the Michaelis constant, is the constant of the substrate concentration that yield a velocity

equal to half of the maximal velocity of the reaction. The initial velocity derived from the slope of the enzyme-product progress curve, is plotting between the disappearance of substrate (S) and the appearance of product (P) in the enzyme-catalyzed reaction which showes in **Figure 7.**

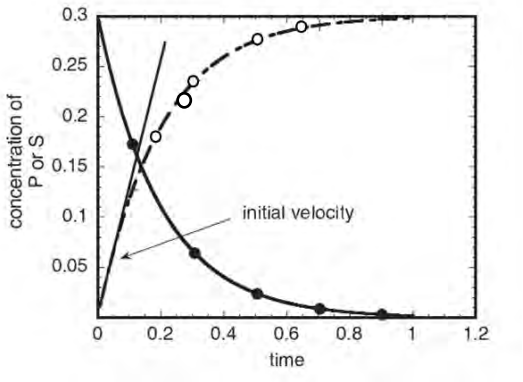


Figure 7 The enzyme product progress curves, the time course for the disappearance of substrate (●) and the appearance of product (○) in the enzyme-catalyzed reaction (modified from: Lee and Berdis, 2005)

The kinetic parameters, V_{max} and K_m obtain from the plotting between the velocity of enzyme reactions versus [S] in y-axis and x-axis, respectively. The plot showes as the hyperbolic curve in **Figure 8.**

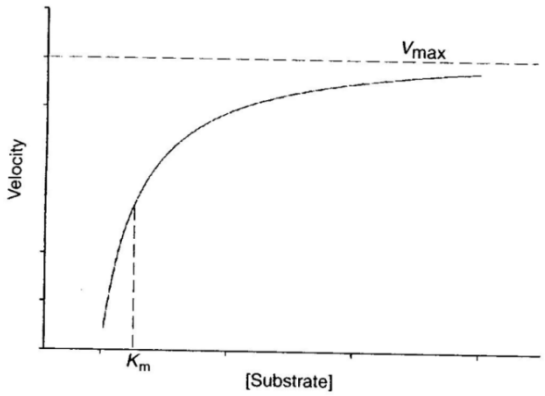


Figure 8 The hyperbolic curve of enzyme reaction following Michaelis-Menten equation (Tracy, 2008)

Determination of the type of reversible inhibition is commonly estimated from the double-reciprocal plot, Lineweaver-Burk plot, which performed by linearity plotting between $1/v$ versus 1/[S] in y-axis and x-axis, respectively. The slope from

this plotting is K_m/V_{max}. The characteristic of Lineweaver-Burk plot is shown in **Figure 9**.

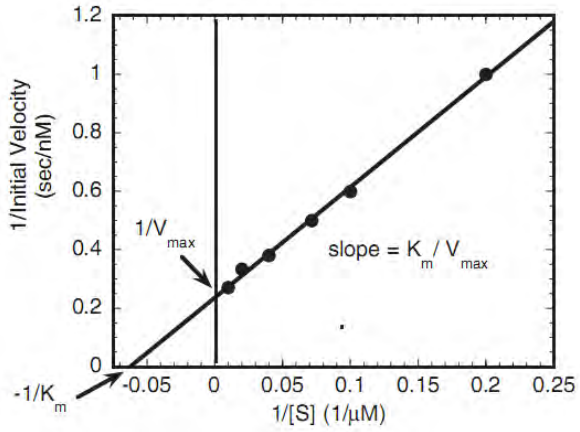
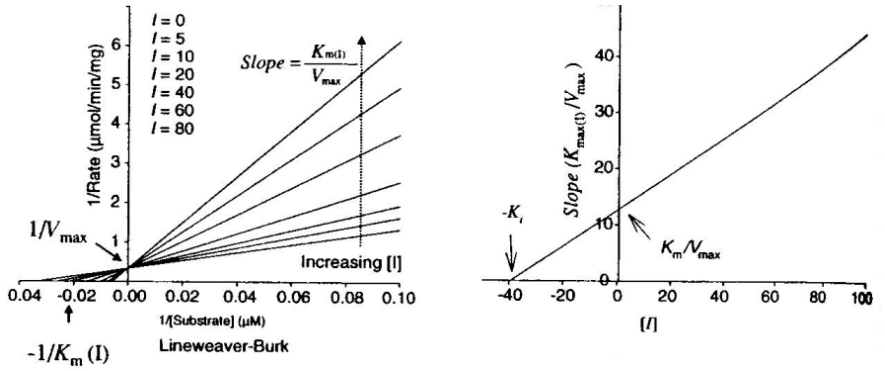


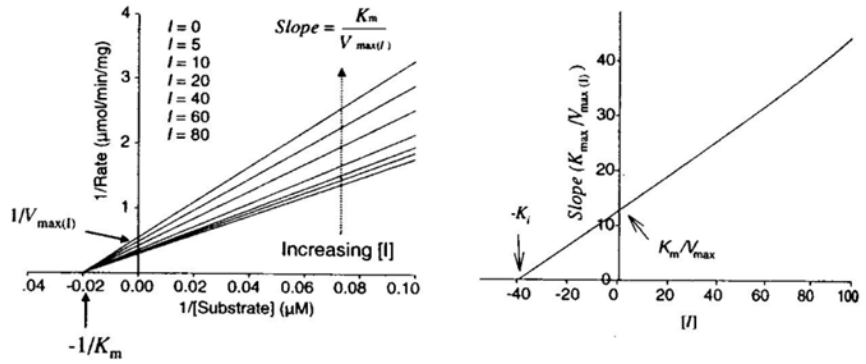
Figure 9 The characteristic of Lineweaver-Burk plot following the Michaelis-Menten kinetics (Lee and Berdis, 2005)

The kinetic evaluation of inhibition type from Lineweaver-Burk plot for competitive mechanism, has no effect on V_{max} , but the slope of the double-reciprocal plot changes (increase in K_m), whereas, in the non-competitive mechanism, decrease in V_{max} with no change in K_m and un-competitive mechanism, decrease in V_{max} but has no effect on slope (K_m/V_{max}). In case of the competitive inhibition and non competitive inhibition, the second plot performed by plotting between K_m/V_{max} versus [I] on the y-axis and x-axis, respectively, whereas the uncompetitive inhibition, the plotting was performed between $1/V_{max}$ versus [I] on the y-axis and x-axis, respectively. The kinetic parameter, K_m/V_{max} is derived from slope of the Lineweaver-Burk plot, $1/V_{max}$ is derived from the y-intercept of the Lineweaver-Burk plot and [I] is the inhibitor concentrations. The extrapolation of the line to the intercept with the x-axis yields the value of the inhibition constants (K_i) (Stojan, 2005). K_i value represents the dissociation constant of the enzyme-inhibitor complex. The characteristic of Lineweaver-Burk plot and the second plot of each type of reversible inhibition are shown in **Figure 10.**

(a)



(b)



(c)

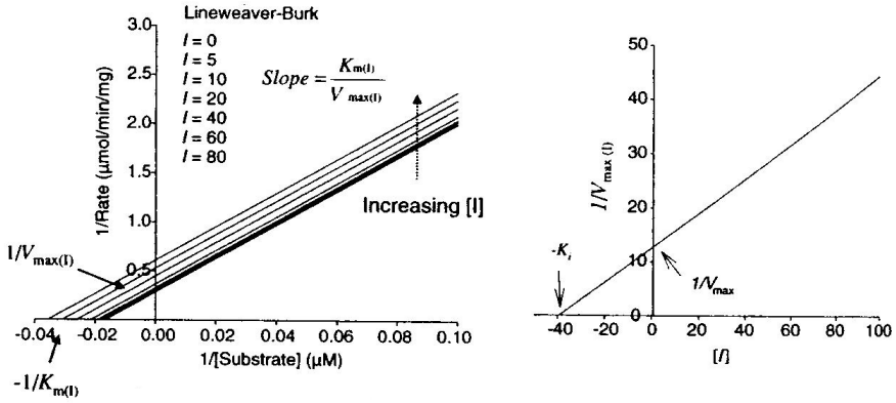


Figure 10 The characteristic of Lineweaver-Burk plot (left) and the second plot (right) of each type of reversible inhibition. Competitive inhibition (a) Non competitive inhibition (b) Uncompetitive (c) (Shou and Dai, 2008)

CHAPTER III

MATERIALS AND METHODS

1. Materials

1. 1 Equipments

The following instruments were used in this study.

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

2) Autopipette tips 200 and 1000 µl (Gibson, France)

3) Fluorescence, Microplate reader VICTOR^3 V (Perkin Elmer, USA)

4) Multi-channel pipette 20 and 200 µl (Rainin Instrument, USA)

5) Multi-channel pipette tips 20 and 200 µl (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 consist of five components as following:

1) CYP450 BACULOSOME® Reagent which consists of recombinant human cytochrome P450 (CYP1A2, CYP2C9, CYP2D6, CYP2E1 or CYP3A4) and rabbit NADPH CYP reductase. CYP2E1 also contains human cytochrome b_5.

2) Vivid® Substrates include 7-benzyloxymethyloxy-3-yanocoumarin (BOMCC) for CYP2C9 and CYP3A4, ethoxymethyloxy-3-cyanocoumarin

(EOMCC) for CYP1A2, CYP2D6 and CYP2E1, dibenzyloxymethylfluorescein (DBOMF) for CYP3A4

3) Vivid® CYP450 Reaction Buffers include either Buffer I (200 mM potassium phosphate buffer pH 8.0), Buffer II (100 mM potassium phosphate buffer pH 8.0) or Buffer III (400 mM potassium phosphate buffer pH 8.0) depending on CYP isoforms.

4) Regeneration System consists 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 standard blue

The crude of *P. emblica* extract was purchased from Merck Chemical, Germany. The extract was a pale brown powder extracted from fruits of *P. emblica*. It contains hydrolysable tannoids which are the active ingredients not least 50 %.

These following chemicals were used in this study.

1) Acetonitrile, anhydrous (Labscan Asia, Thailand)

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

3) Erythromycin ethylsuccinate (Siam Pharmaceutical, Thailand)

4) Ethanol (Merck, Germany)

5) Hydrochloric acid (HCl) (Merck, Germany)

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

7) Ketoconazole (Siam Pharmaceutical, Thailand)

8) Methanol (Merck, Germany)

9) Miconazole (The Government Pharmaceutical Organization, Thailand)

10) α-Naphthoflavone (Sigma Chemical Co., Ltd., USA)

11) Sodium hydroxide (NaOH) (Merck, Germany)

12) Sulfaphenazole (Sigma Chemical Co., Ltd., USA)

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

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

2. Methods

2.1 Determining the inhibitory effect of *P. emblica* aqueous extract on human CYP

The Vivid® CYP450 Screening Kits Protocol (www.invitrogen.com) was used to investigate the inhibitory effect of *P. emblica* aqueous extract on CYP activities.

The principal of this study is the transition of the substrate or Vivid® substrate from non-fluorescent to highly fluorescent metabolite by specific enzyme CYP. Example of Vivid® substrate includes BOMCC, EOMCC and DBOMF. The principal of the transition of Vivid® substrate by CYP450 is shown in **Figure 11** and structures of the substrate are shown in **Figure 12**.

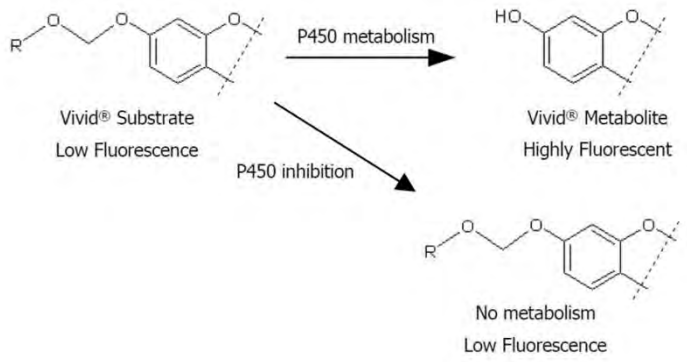


Figure 11 Schematic demonstrates the principal of the transition of the Vivid® substrate by CYP

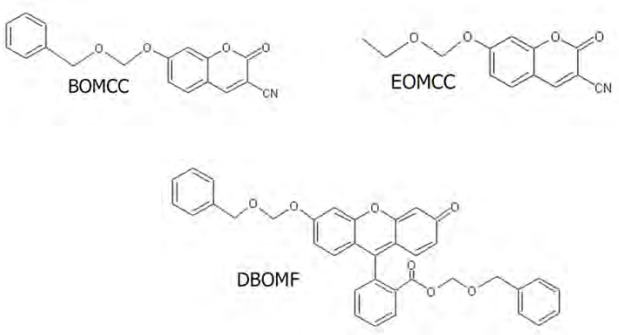


Figure 12 The chemical structure of Vivid® substrates

Reagents

1. Preparation of *P. emblica* aqueous extract solution

P. emblica aqueous extract solution was freshly prepared on each day of the experiment. Ten milligram of *P. emblica* aqueous extract was dissolved in 1000 µL of ultra-pure water to make a concentration of 10 mg/mL of stock solution. Then a serial dilution was prepared to various concentrations with ultra-pure water depending upon each assay of CYP as following.

CYP isoform	Final concentrations of *P. emblica* aqueous extract (µg/mL)
1A2	100, 200, 250, 300, 400, 500
2C9	80, 100, 150, 200, 300, 400
2D6	400, 500, 800, 1200, 1500, 2000
2E1	120, 150, 250, 350, 500, 1000
3A4	200, 250, 350, 400, 500, 800, 1200

2. Preparation of Master Pre-mix solution for 100 wells

According to the Vivid® CYP450 Screening Kits Protocols, Master Pre-Mix was prepared by diluting CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer as following.

CYP isoform	Vivid® CYP450 Reaction Buffer (µL)	CYP450 BACULOSOME® Reagent (µL)	Regeneration system (µL)
1A2	4850 (Buffer I)	50	100
2C9	4800 (Buffer II)	100	100
2D6	4800 (Buffer I)	100	100
2E1	4850 (Buffer III)	50	100
3A4	4850 (Buffer I)	50	100

Throughout the dilution processes, the solution was strictly prepared on ice and mixed well by gentle inversion. Master Pre-mix solution was kept on ice during the preparation and the experimentation.

3. Preparation of substrate solution

Substrates solution was prepared according to the Vivid® CYP450 Screening Kit Protocols as following.

CYP isoform	Vivid® Substrate (0.1 mg/tube)	Anhydrous acetonitrile added per tube (µL)
1A2	EOMCC	205
2C9	BOMCC	160
2D6	EOMCC	205
2E1	EOMCC	205
3A4	BOMCC	160

Anhydrous acetonitrile was used to reconstitute Vivid® Substrate. The solution was kept at room temperature for the immediate use or stored at -20ºC.

4. Preparation of Pre-mixture of reconstituted Vivid® Substrate and $NADP^+$ solution for 100 wells

Pre-mixture of reconstitute Vivid® Substrate and $NADP^+$ solution was prepared in Vivid® CYP450 Reaction Buffer as following.

CYP isoform	Vivid® Substrate	Reconstitute Vivid® Substrate (µL)	$NADP^+$ solution (µL)	Vivid® CYP450 Reaction Buffer (µL)
1A2	EOMCC	15	100	885 (Buffer I)
2C9	BOMCC	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)

Then the mixture was mixed well by vortex mixer and kept on ice.

5. Preparation of 0.5M Tris-HCl buffer pH 10.5

Tris-HCl buffer pH 10.5 was used to stop the reaction in the experiment. It was prepared by dissolving 30.3 g of Trizma® base, dissolved in ultra-pure water, adjusted the pH to 10.5 using HCl and NaOH and adjusted the volume to 500 mL with ultra-pure water.

Procedure

1. Background fluorescent intensity of the 96-well black plate was measured before using in the experiment.

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 of fluorescence intensity from the test compound), the solvent control (for correcting the inhibitory effect of the solvent which dissolved the test compound) and the background of the solvent control (for correcting the fluorescence intensity from solvent which dissolved the test compound) (**Figure 13**).

<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">Solvent control</td><td colspan="8">Test compound (various concentrations)</td><td rowspan="2">Solvent control</td><td></td><td></td></tr>
<tr><td>B</td><td colspan="8">Test compound (various concentrations)</td><td></td><td></td></tr>
<tr><td>C</td><td rowspan="2">Background of the solvent control</td><td colspan="8">Background of the test compound</td><td rowspan="2">Background of the solvent control</td><td></td><td></td></tr>
<tr><td>D</td><td colspan="8">Background of the test compound</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 13 The well assignment of 96-well black plate for determining the inhibitory effect of *P. emblica* aqueous extract

3. Forty microliters of the test compound (*P. emblica* aqueous extract solution of various concentrations) and 50 µL of Master Pre-mix were added into each well of **"test compound"** (row A, between 2A to 9A and row B, between 2B to 9B).

4. Forty microliters of the test compound (*P. emblica* aqueous extract solution of various concentrations) and 50 µL of Vivid® Reaction Buffer were added into each of **"background of the test compound"** (row C, between 2C to 9C and row D, between 2D to 9D).

5. Forty microliters of the solvent used to dissolve the test compound and 50 µL of Master Pre-mix were added into each well of **"solvent control"** (1A, 1B, 10A and 10B).

6. Forty microliters of the solvent used to dissolve the test compound and 50 µL of Vivid® Reaction Buffer were added into each well of **"background of the solvent control"** (1C, 1D, 10C and 10D).

The solution mixture in the plate was gently and *horizontally* shaken for 30 seconds. The plate was pre-incubated for 20 minutes at room temperature.

7. The reaction was initiated by an addition of 10 µl of the pre-mixture of reconstituted Vivid® 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 for CYP1A2, CYP2C9, CYP2E1 and CYP3A4 or 60 minutes for 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 Fluorescent Reader and the fluorescence intensities were detected by using the excitation wavelength of 405 ± 20 nm and emission wavelength of 460 ± 25 nm.

11. The experiment from step 1 to 10 was performed for at least 3 times for each CYP isoform.

Calculations

Percent inhibition was calculated for each concentration of the *P. emblica* aqueous extract solution by using following equation.

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

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 remarkable known inhibitor of each CYP of interested. The reaction was performed as mentioned above using α-naphthoflavone, sulfaphenazole, miconazole, imipramine and ketoconazole as inhibitor for CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4, respectively. 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 (for correcting the fluorescence intensity due to the known inhibitor), the solvent control (for correcting the inhibitory effects of the solvent using to dissolving the known inhibitor) and the background of the solvent control (for correcting the fluorescence intensity due to the solvent using to dissolving the known inhibitor). The experiment was performed 3 times for each known inhibitor.

2.2 Assessment of mechanism-based inhibition of *P. emblica* aqueous extract on human CYP

Evaluation of mechanism-based inhibition of *P. emblica* aqueous extract on human CYP used in this study was modified from the method of Obach et al. (2007).

As CYP3A4 was found to be inhibited by the extract from the screening test mentioned earlier and because CYP3A4 is the major CYP isoform in human which metabolized the largest number of currently used medicines, this study further focused on the mechanism of inhibition of only CYP3A4.

The test compound was evaluated for the ability to inhibit CYP3A4 using the IC_{50} ratio between IC_{50} of the test compound without NADPH pre-incubation and IC_{50} of the test compound with NADPH in pre-incubation. If the test compound with IC_{50} ratio of more than 1 was potentially a mechanism-based inhibitor. In contrast, the test compound with IC_{50} ratio of less than 1 was potentially a non mechanism-based inhibitor.

In this procedure, the Vivid® substrate used for determining the mechanism-based inhibition of the test compound on CYP3A4 was DBOMF.

Reagents

1. Preparation of *P. emblica* aqueous extract solution

P. emblica aqueous extract solution was freshly prepared on each day of the experiment. Ten milligram of *P. emblica* aqueous extract was dissolved in 1000 µL of ultra-pure water to make a concentration of 10 mg/mL of stock solution. Then a serial dilution was prepared to various concentrations with ultra-pure water depending upon each assay of CYP as following.

CYP isoform	Final concentrations of *P. emblica* aqueous extracts solution (µg/mL)
3A4	750, 1000, 1500, 2000, 2500, 3000

2. Preparation of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System 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 gentle inversion. The solutions were kept on ice until used.

CYP isoform	Vivid® CYP450 Reaction Buffer (µL)	CYP450 BACULOSOME® Reagent (µL)	Regeneration system (µL)
3A4	425 (Buffer I)	37.5	37.5

3. Preparation of substrate solution

Anhydrous acetonitrile was used to reconstitute Vivid® Substrate as following. The solution was kept at room temperature for the immediate use or stored at -20ºC.

CYP isoform	Vivid® Substrate (0.1 mg/tube)	Anhydrous acetonitrile added per tube (µL)
3A4	DBOMF	85

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

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

CYP isoform	$NADP^+$ solution (µL)	Vivid® CYP450 Reaction Buffer (µL)
3A4	22.5	477.5 (Buffer I)

5. Preparation of the mixture of the reconstituted Vivid® Substrate, $NADP^+$ solution and Regeneration system in Vivid® CYP450 Reaction Buffer for 100 wells.

The mixture of reconstitute Vivid® Substrate, $NADP^+$ solution and Regeneration system was prepared in Vivid® CYP450 Reaction Buffer as following. It was mixed well by vortex mixer and kept on ice.

CYP isoform	Vivid® Substrate	Reconstituted Vivid® Substrate (µL)	$NADP^+$ solution (µL)	Regeneration system (µL)	Vivid® CYP450 Reaction Buffer I (µL)
3A4	DBOMF	7.5	22.5	93.75	5876.25

6. 0.5 M Tris-HCl buffer pH 10.5

Trizma base of 6.06 g. was dissolved and adjusted the volume to 100 ml with ultra-pure water. The solution was adjusted to pH 10.5 with NaOH or HCl.

Procedure

1. Background fluorescent intensity of the 96-well black plate was measured before using in the experiment

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, the solvent control and the background of the solvent control with and without NADPH pre-incubation (**Figure 14**).

<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">Solvent control of the test compound with NADPH pre-incubation</td><td colspan="8">Test compound (various concentrations) with NADPH pre-incubation</td><td rowspan="2">Solvent control of the test compound with NADPH pre-incubation</td><td></td><td></td></tr>
<tr><td>B</td><td colspan="8">Test compound (various concentrations) with NADPH pre-incubation</td><td></td><td></td></tr>
<tr><td>C</td><td rowspan="2">Solvent control of the test compound without NADPH pre-incubation</td><td colspan="8">Test compound (various concentrations) without NADPH pre-incubation</td><td rowspan="2">Solvent control of the test compound without NADPH pre-incubation</td><td></td><td></td></tr>
<tr><td>D</td><td colspan="8">Test compound (various concentrations) without NADPH pre-incubation</td><td></td><td></td></tr>
<tr><td>E</td><td rowspan="2">Background of the solvent control</td><td colspan="8">Background of the test compound</td><td rowspan="2">Background of the solvent control</td><td></td><td></td></tr>
<tr><td>F</td><td colspan="8">Background of the test compound</td><td></td><td></td></tr>
<tr><td>G</td><td></td><td colspan="8"></td><td></td><td></td><td></td></tr>
<tr><td>H</td><td></td><td colspan="8"></td><td></td><td></td><td></td></tr>
</table>

Figure 14 The well assignment of 96-well black plate for determining the mechanism-based inhibition of *P. emblica* aqueous extract

3. From Figure 14, wells in rows 2A-9A and 2B-9B 2C-9C were assigned to be the wells of **"Test compound (various concentrations) with NADPH pre-incubation"** which were added with these following solutions:

- 5 µl of the test compound (*P. emblica* aqueous extract solution) at various concentrations

- 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. From Figure 14, wells in rows 2C-9C and 2D-9D 2F-9F were assigned to be the wells of **"Test compound (various conc.) without NADPH pre-incubation"** which were added with these following solutions:

- 5 µl of the test compound (*P. emblica* aqueous extract solution) at various concentrations
- 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. From Figure 14, wells in rows 2E-9E and 2F-9F were assigned to be the wells of **"Background of the test compound"** which were added with these following solutions:

- 5 µl of the test compound (*P. emblica* aqueous extract solution) at various concentrations
- 5 µl of Vivid® CYP450 Reaction Buffer
- 5 µl of Vivid® CYP450 Reaction Buffer

6. From Figure 14, wells in rows 1A, 1B, 10A and 10B were assigned to be the wells of **"Solvent control of the test compound with NADPH pre-incubation"** which were added with these following solutions:

- 5 µl of solvent used to dissolve the test compound
- 5 µl of the mixture of CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffers
- 5 µl of $NADP^+$ solution in Vivid® CYP450 Reaction Buffer

7. From Figure 14, wells in rows 1C, 1D, 10C and 10D were assigned to be the wells of **"Solvent control of the test compound without NADPH pre-incubation"** which were added with these following solutions:

- 5 µl of solvent used to dissolve 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

8. From Figure 14, wells in rows 1E, 1F, 10E and 10F were assigned to be the wells of **"Background of the solvent control"** which were added with these following solutions:

- 5 µl of solvent used to dissolve the test compound
- 5 µl of Vivid® CYP450 Reaction Buffer
- 5 µl of Vivid® CYP450 Reaction Buffer

The solution mixture in the plate was gently and *horizontally* shaken for 30 seconds. The plate was pre-incubated for 30 minutes at room temperature.

9. The reaction was initiated by an addition of 60 µl of the mixture of reconstituted Vivid® 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 as well as incubated at room temperature for 10 minutes.

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 485 ± 20 nm and emission wavelength of 535 ± 10 nm.

13. The experiment from step 1 to 12 was performed for at least 3 times.

Calculations

Percent inhibition for each concentration of the test compound and IC_{50} of the test compound were calculated as mentioned above.

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

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

The test compound with an IC_{50} ratio of more than 1 was potentially a mechanism-based inhibitor.

Verification of the mechanism-based inhibition screening protocol

The mechanism-based inhibition screening protocol was verified by using known mechanism-based and the non mechanism-based inhibitors of CYP3A4 which were erythromycin ethylsuccinate and ketoconazole, respectively. The reaction was performed as mentioned above. Each experiment plate was performed duplicates for each concentration of the known inhibitor either with or without NADPH pre-incubation as well as the background of each concentration of the known inhibitor, the solvent control and the background of the solvent control. The experiment was performed 3 times for each known inhibitor.

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

To determine the type of reversible inhibition and the apparent K_i of *P. emblica*, the selective substrate of the approximate concentrations of $K_m/4$, $K_m/2$, K_m, and $2K_m$ were performed the reaction with various concentrations of the test compound as described earlier in the IC_{50} determination assay. The kinetic parameters of the reaction (V_{max} and K_m) and the type of inhibition were estimated from Lineweaver–

Burk plot. K_i were determined via the second plot of the slopes from Lineweaver–Burk plots *versus* the inhibitor concentrations.

Reagents

1. Preparation of *P. emblica* aqueous extract solution

P. emblica aqueous extract solution was freshly prepared on each day of the experiment. Ten milligram of *P. emblica* aqueous extract was dissolved in 1000 µL of ultra-pure water to make a concentration of 10 mg/mL of stock solution. Then a serial dilution was prepared to various concentrations with ultra-pure water depending upon each assay of CYP as following.

CYP isoform	Final concentrations of *P. emblica* aqueous extracts solution (µg/mL)
3A4	100, 200, 300, 400 and 500

2. Preparation of Master Pre-mix solution for 100 wells

According to the Vivid® CYP450 Screening Kits Protocols, Master Pre-Mix was prepared by diluting CYP450 BACULOSOME® Reagent and Regeneration System in Vivid® CYP450 Reaction Buffer as following.

CYP isoform	Vivid® CYP450 Reaction Buffer (µL)	CYP450 BACULOSOME® Reagent (µL)	Regeneration system (µL)
3A4	4850 (Buffer I)	50	100

Throughout the dilution processes, the solution was strictly prepared on ice and mixed well by gentle inversion. Master Pre-mix solution was kept on ice during the preparation and the experimentation.

3. Preparation of substrate solution

Substrates solution was prepared according to the Vivid® CYP450 Screening Kit Protocols as following.

CYP isoform	Vivid® Substrate (0.1 mg/tube)	Anhydrous acetonitrile added per tube (µL)
3A4	BOMCC	160

Anhydrous acetonitrile was used to reconstitute Vivid® Substrate. The solution was kept at room temperature for the immediate use or stored at -20ºC.

4. Preparation of Pre-mixture of reconstituted Vivid® Substrate and $NADP^+$ solution for 100 wells

Pre-mixture of reconstitute Vivid® Substrate and $NADP^+$ solution was prepared in Vivid® CYP450 Reaction Buffer as following.

CYP isoform	Vivid® Substrate	Concentration of substrate (µM)	Reconstitute Vivid® Substrate (µL)	$NADP^+$ solution (µL)	Vivid® CYP450 Reaction Buffer I (µL)
3A4	BOMCC (K_m =10µM)	2.5	12.5	100	887.5
		5.0	25.0	100	875.0
		10.0	50.0	100	850.0
		20.0	100.0	100	800.0

Then the mixture was mixed well by vortex mixer and kept on ice.

5. Preparation of the fluorescence standard solution

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

CYP isoform	Assay Standard	µmol per tube [X]	DMSO added per tube (µL) [X x 10000]
3A4	Blue standard	0.1	1000

Procedures

1. Preparation of the standard curve

Standard curve preparation was performed according to the Vivid® CYP450 Screening Kits Protocols.

1.1 The 1X Reaction Buffer prepared from 2X Reaction Buffer by diluting with ultrapure water was used as the solvent for serial dilution of standard curve.

1.2 Using a 96-well black plate, one standard curve containing 8 different concentrations of the standard can be done with 8 wells using totally of the 1 ml of reaction buffer. Each concentration of the standard curve was performed in duplicate.

1.3 Beginning at the first well of the first row, 195 µl of 1X reaction buffer was added.

1.4 Reaction buffer (1X) of 100 µl was added to each of the remaining wells in the first row.

1.5 Five microliters of the reconstituted fluorescent standard solution was added to the first well of the first row 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.

1.6 One hundred microliters of the solution in 1.5 was transferred to the next well containing 100 µl of 1X reaction buffer and the solution was mixed by pipetting.

1.7 The dilution was continued in the same way as 1.6 except the last well of the row which was a standard blank containing only 1X reaction buffer. Thus the resulting fluorescent standard concentrations were: 2.5 µM, 1.25 µM, 625 nM, 312.5 nM, 156.25 nM, 78.125 nM, 39.063 nM and 0 nM.

2. Determination of the type of reversible inhibition of *P. emblica* aqueous extract

2.1 Fluorescence intensity of the 96-well black plate was measured before use so as to correct the background fluorescent intensity. In each plate, the experiment was performed in duplicate for each concentration of the test compound and the solvent control (for correcting the reaction without the test compound) (**Figure 15**).

<table>
<tr><td></td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td><td>11</td><td>12</td></tr>
<tr><td>A</td><td rowspan="2">Solvent control</td><td colspan="6">Test compound (various concentrations)</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>B</td><td colspan="6">Test compound (various concentrations)</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>C</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>
<tr><td>D</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>F</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>
<tr><td>G</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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</table>

Figure 15 The well assignment of 96-well black plate for determining the type of reversible inhibition of *P. emblica* aqueous extract

2.2 Forty microliters of the test compound (*P. emblica* aqueous extract solution of various concentrations) and 50 µL of Master Pre-mix were added into each well of **"test compound"** (row A, between 2A to 9A and row B, between 2B to 9B).

2.3 Forty microliters of the solvent used to dissolve the test compound and 50 µL of Master Pre-mix were added into each well of **"solvent control"** (1A and 1B).

The solution mixture in the plate was gently and *horizontally* shaken for 30 seconds. The plate was pre-incubated for 20 minutes at room temperature.

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

2.5 The plate was put into the Fluorescent Reader and the fluorescence intensity was continually detected for 20 minutes by using the excitation wavelength of 405 ± 10 nm and emission wavelength of 460 ± 25 nm.

2.6 The experiment from step 2.1-2.5 was repeated using the various concentrations of reconstituted substrate in pre-mixture solution.

The procedures (preparation of the standard curve and determination of the type of reversible inhibition of *P. emblica* aqueous extract) were performed for at least 3 times.

Calculations

The amount of fluorescent product (in nmol) in the reaction could be calculated by using the standard curve. Initial velocity values could be determined from the slope of the graph plotted between the amounts of fluorescent product *versus* time (**Figure 16**).

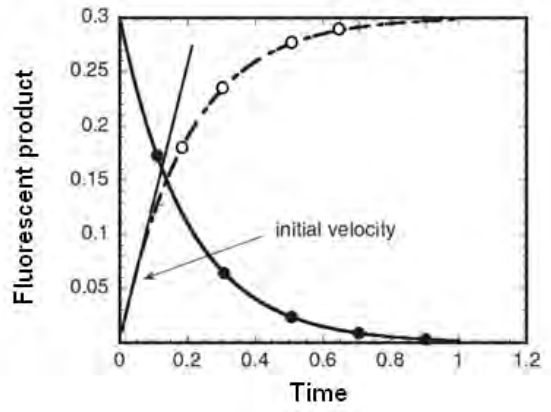


Figure 16 Typical enzyme reaction progress curve showed the initial velocity (modified from: Lee and Berdis, 2005).

The graphical plot that was used in analyzing enzyme kinetic data was the double-reciprocal plot of velocity (v) *versus* substrate concentration ([S]) or Lineweaver–Burk plot. The kinetic parameters and the mechanism of inhibition were estimated graphically from these plots. K_i were calculated via second plot of the slope from Lineweaver–Burk plots *versus* inhibitor concentrations.

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

The enzyme kinetic study protocol was verified by using the known competitive and non competitive inhibitor of CYP3A4 which were erythromycin and ketoconazole, respectively. Each experiment plate was performed duplicates for each concentration of the known inhibitor as well as the background of each concentration of the known inhibitor, the solvent control and the background of the solvent control. The experiment was performed 3 times for each known inhibitor.

CHAPTER IV

RESULTS

1. Verification of the Vivid® CYP450 Screening Kits Protocol

IC_{50} and the corresponding 95% confidence interval (95% CI) of the known inhibitor of each CYP isoform were shown in **Table 3**. The corresponding concentration - inhibition curves of the known inhibitors of each CYP was shown in **Figure 17**. α-Naphthoflavone, sulfaphenazole, miconazole, imipramine and ketoconazole were used as known inhibitor of CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4, respectively.

Table 3 IC_{50} of the known inhibitor of each CYP isoform

CYP isoform	**Known Inhibitor**	**IC_{50} ± SE (μM)**	**95% confidence interval (μM)**
1A2	α-naphthoflavone	$0.06 \pm 0.00_{47}$	0.04 – 0.07
2C9	sulfaphenazole	0.24 ± 0.02	0.19 – 0.29
2D6	miconazole	0.98 ± 0.06	0.72 – 1.29
2E1	imipramine	125.33 ± 2.91	97.78 – 151.91
3A4	ketoconazole	0.09 ± 0.01	0.07 – 0.11

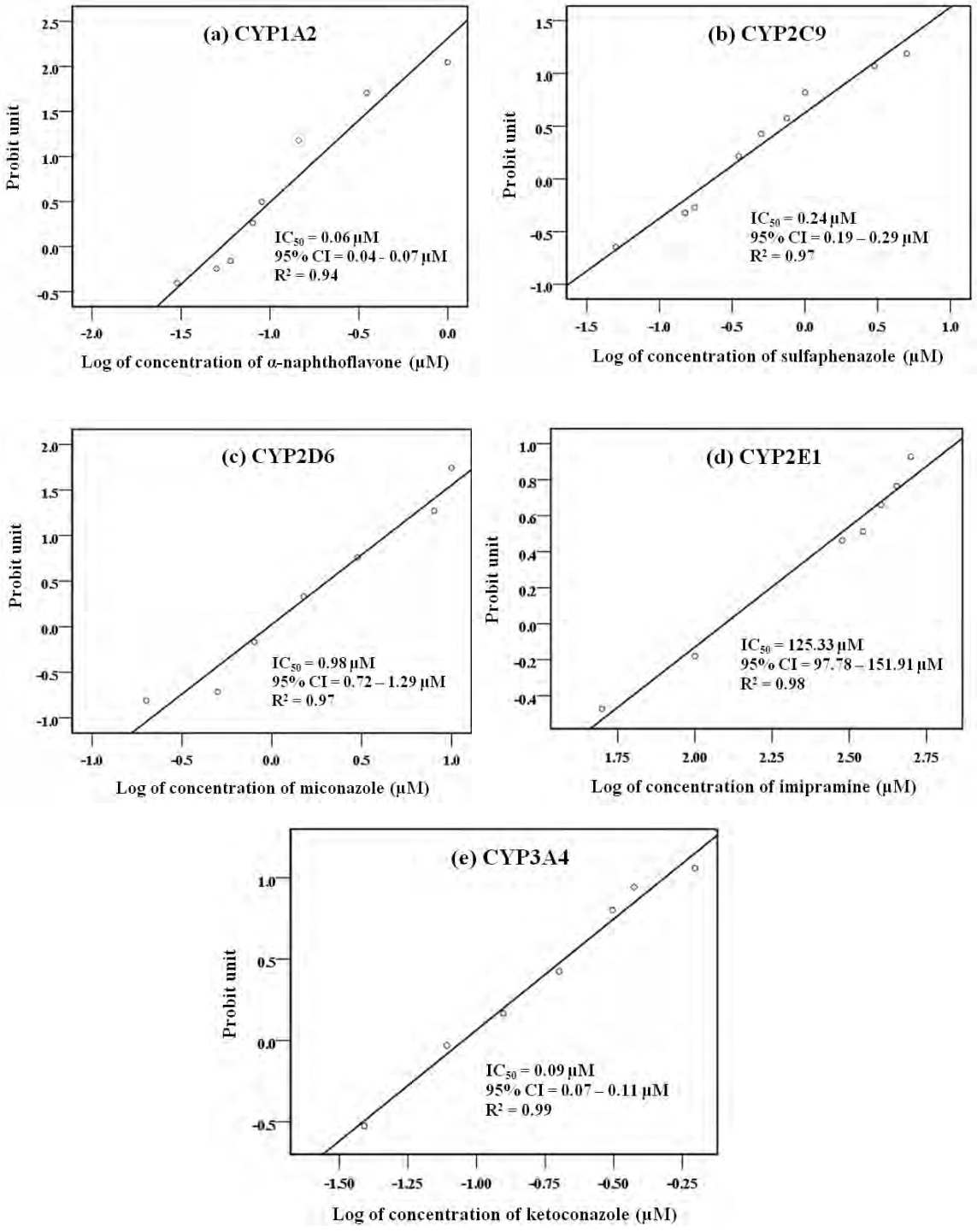


Figure 17 The concentration - inhibition curves of the known inhibitors of CYP1A2 (a), CYP2C9 (b), CYP2D6 (c), CYP2E1 (d) and CYP3A4 (e)

2. Determination of the inhibitory effect of *P. emblica* aqueous extract on recombinant human CYP

2.1 IC_{50} determining of *P. emblica* aqueous extract on CYP1A2 activity

The inhibitory effect of *P. emblica* aqueous extract on CYP1A2 activity was in the range between 100 to 500 µg/mL. Inhibition curve plotting between probit unit of percent inhibition of the extract on CYP1A2 versus logarithm of concentrations of *P. emblica* aqueous extract was shown in **Figure 18**. IC_{50} of *P. emblica* aqueous extract on CYP1A2 was 310.28 µg/mL with the 95% confidence interval of 254.57 to 398.17 µg/mL.

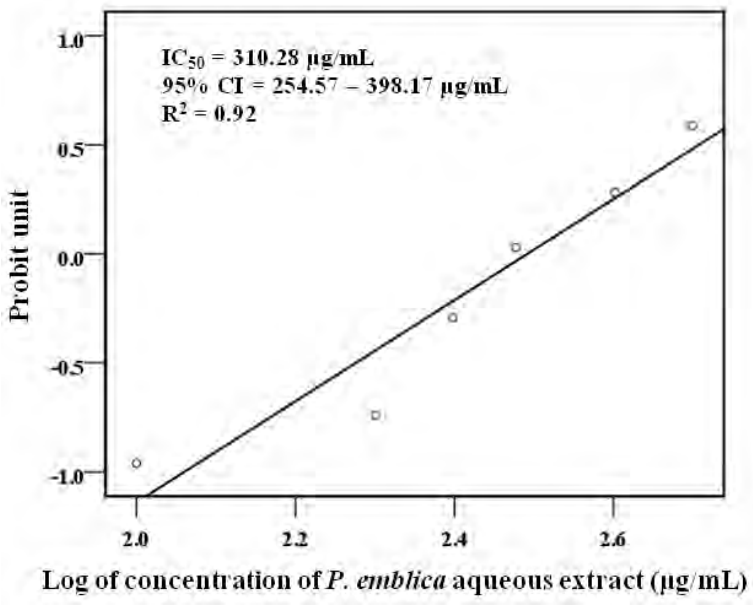


Figure 18 The concentration - inhibition curves of *P. emblica* aqueous extract on CYP1A2 activity. Each point represented the mean of triplicate experiments.

2.2 IC_{50} determining of *P. emblica* aqueous extract on CYP2C9 activity

The inhibitory effect of *P. emblica* aqueous extract on CYP2C9 activity was in the range between 80 to 400 µg/mL. Inhibition curve plotting between probit unit of percent inhibition of the extract on CYP2C9 versus logarithm of concentrations of *P. emblica* aqueous extract was shown in **Figure 19.** IC_{50} of *P. emblica* aqueous extract on CYP2C9 was 194.72 µg/mL with the 95% confidence interval of 177.75 to 214.29 µg/mL.

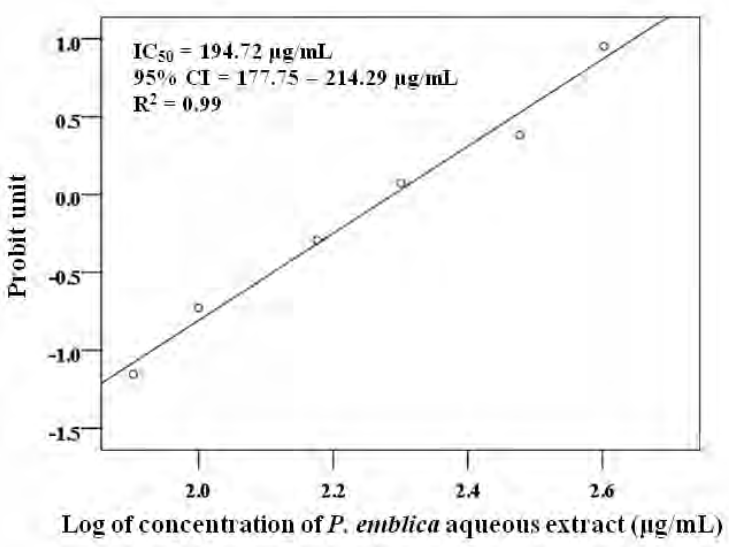


Figure 19 The concentration - inhibition curves of *P. emblica* aqueous extract on CYP2C9 activity. Each point represented the mean of triplicate experiments.

2.3 IC_{50} determining of *P. emblica* aqueous extract on CYP2D6 activity

The inhibitory effect of *P. emblica* aqueous extract on CYP2D6 activity was in the range between 400 to 2,000 µg/mL. Inhibition curve plotting between probit unit of percent inhibition of the extract on CYP2D6 versus logarithm of concentrations of *P. emblica* aqueous extract was shown in **Figure 20.** IC_{50} of *P. emblica* aqueous extract on CYP2D6 was 589.52 µg/mL with the 95% confidence interval of 540.03 to 638.58 µg/mL.

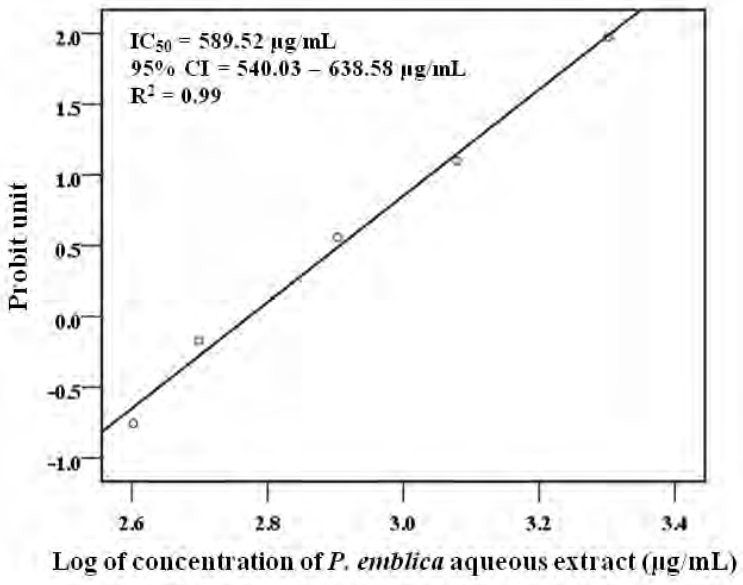


Figure 20 The concentration - inhibition curves of *P. emblica* aqueous extract on CYP2D6 activity. Each point represented the mean of triplicate experiments.

2.4 IC_{50} determining of *P. emblica* aqueous extract on CYP2E1 activity

The inhibitory effect of *P. emblica* aqueous extract on CYP2E1 activity was in the range between 120 to 1,000 µg/mL. The inhibition curve plotting between probit unit of percent inhibition of the extract on CYP2E1 versus logarithm of concentrations of *P. emblica* aqueous extract was shown in **Figure 21.** IC_{50} of *P. emblica* aqueous extract on CYP2E1 was 310.27 µg/mL with 95% confidence interval of 275.56 to 350.43 µg/mL.

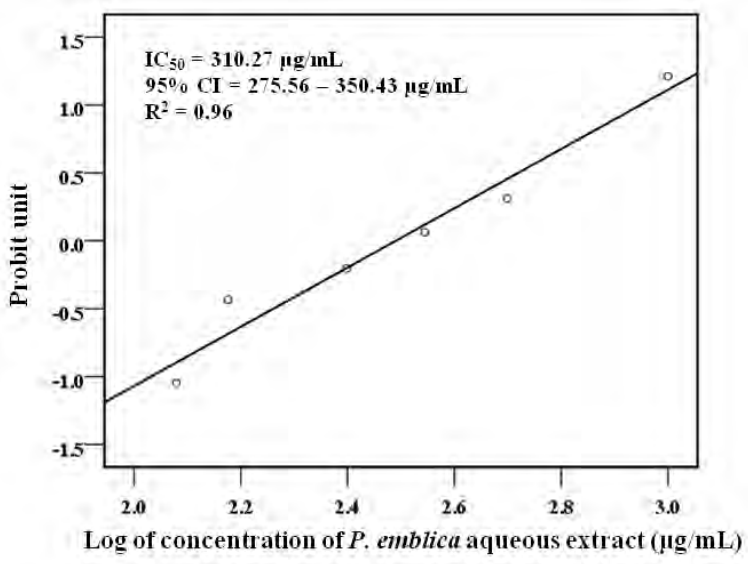


Figure 21 The concentration - inhibition curves of *P. emblica* aqueous extract on CYP2E1 activity. Each point represented the mean of triplicate experiments.

2.5 IC_{50} determining of *P. emblica* aqueous extract on CYP3A4 activity

The inhibitory effect of *P. emblica* aqueous extract on CYP3A4 activity was in the range between 200 to 1200 µg/mL. Inhibition curve plotting between probit unit of percent inhibition of the extract on CYP3A4 versus logarithm of concentrations of *P. emblica* aqueous extract was shown in **Figure 22.** IC_{50} of *P. emblica* aqueous extract on CYP3A4 was 325.54 µg/mL with 95% confidence interval of 298.64 to 351.97 µg/mL.

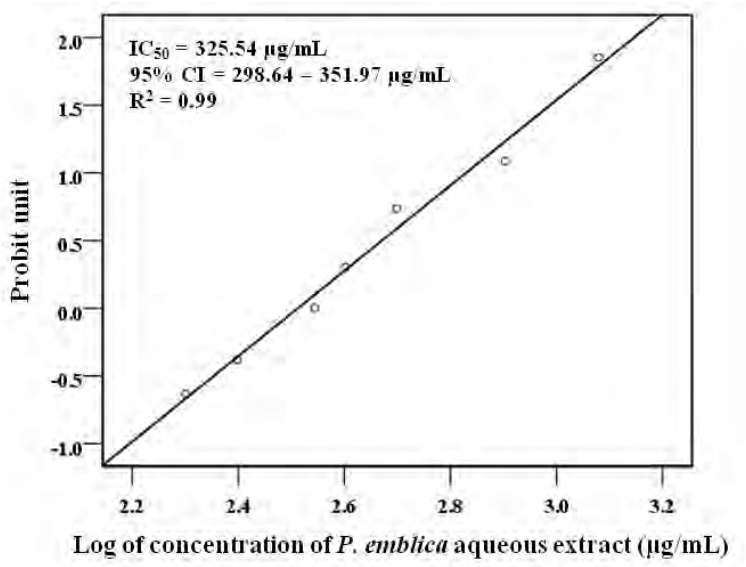


Figure 22 The concentration - inhibition curves of *P. emblica* aqueous extract on CYP3A4 activity. Each point represented the mean of triplicate experiments.

3. Verification of the mechanism-based inhibition screening protocol

The protocol was performed by determining the IC_{50} ratio of the known mechanism-based inhibitor of CYP3A4 in the absence of NADPH pre-incubation and in the presence of NADPH pre-incubation. IC_{50} ratio of non mechanism-based inhibitors of CYP3A4 was also determined in the same manner. Erythromycin and ketoconazole were used as mechanism-based inhibitor and non mechanism-based inhibitor of CYP3A4 isoform, respectively.

Inhibition curves of probit unit of percent inhibition of CYP3A4 activity versus logarithm of concentration of erythromycin and ketoconazole were shown in **Figure 23 and 24**, respectively. IC_{50} ratio of erythromycin and ketoconazole on CYP3A4 activity were shown in **Table 4.** It was shown that IC_{50} ratio of erythromycin which is the mechanism-based inhibitor of CYP3A4 was 1.06 (more than 1) while the IC_{50} ratio of ketoconazole which is the non mechanism-based inhibitor of CYP3A4, was 0.85 (less than 1).

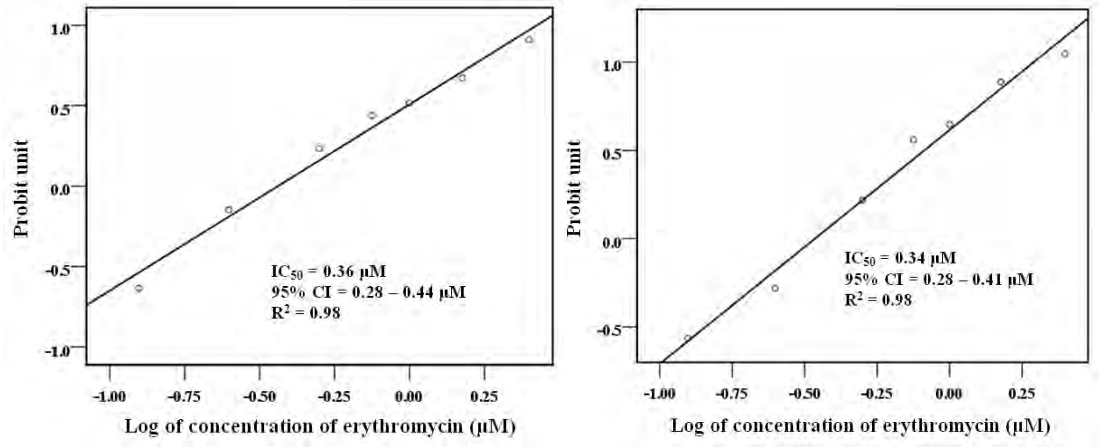


Figure 23 The inhibition curves of erythromycin on CYP3A4. Erythromycin was used in the reaction without NADPH pre-incubation (left) and in the reaction with NADPH pre-incubation (right).

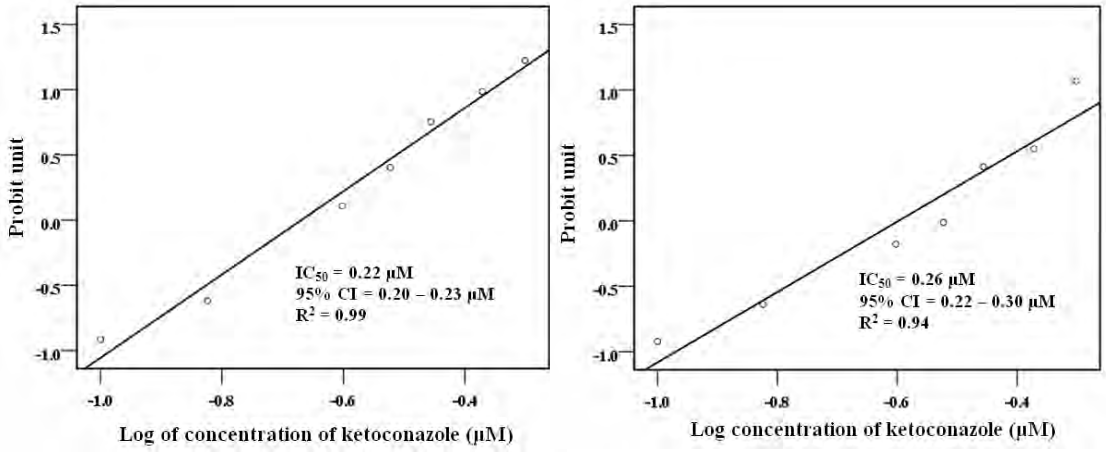


Figure 24 The inhibition curves of ketoconazole on CYP3A4. Ketoconazole was used in the reaction without NADPH pre-incubation (left) and in the reaction with NADPH pre-incubation (right).

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

CYP isoform	**Known inhibitors**	**IC_{50} (µM) without NADPH pre-incubation**	**IC_{50} (µM) with NADPH pre-incubation**	**IC_{50} ratio ± SE**
3A4	erythromycin **(mechanism-based inhibitor)**	0.36	0.34	1.06 ± 0.04
	ketoconazole **(non mechanism-based inhibitor)**	0.22	0.26	0.85 ± 0.02

4. Evaluation of the mechanism-based inhibition of *P. emblica* aqueous extract on CYP3A4

IC_{50} of *P. emblica* aqueous extract on CYP3A4 in the reaction without NADPH pre-incubation was 943.15 µg/mL whereas the IC_{50} of *P. emblica* aqueous extract on CYP3A4 in the reaction with NADPH pre-incubation was 916.92 µg/mL. Then, IC_{50} ratio ± SE of the extract on CYP3A4 was 1.03 ± 0.10 (more than 1) indicating that *P. emblica* aqueous extract is potentially a mechanism-based inhibitor of CYP3A4.

Inhibition curves of probit unit of percent inhibition versus logarithm of concentration of *P. emblica* on CYP3A4 in the reaction without and with NADPH pre-incubation were shown in **Figure 25.**

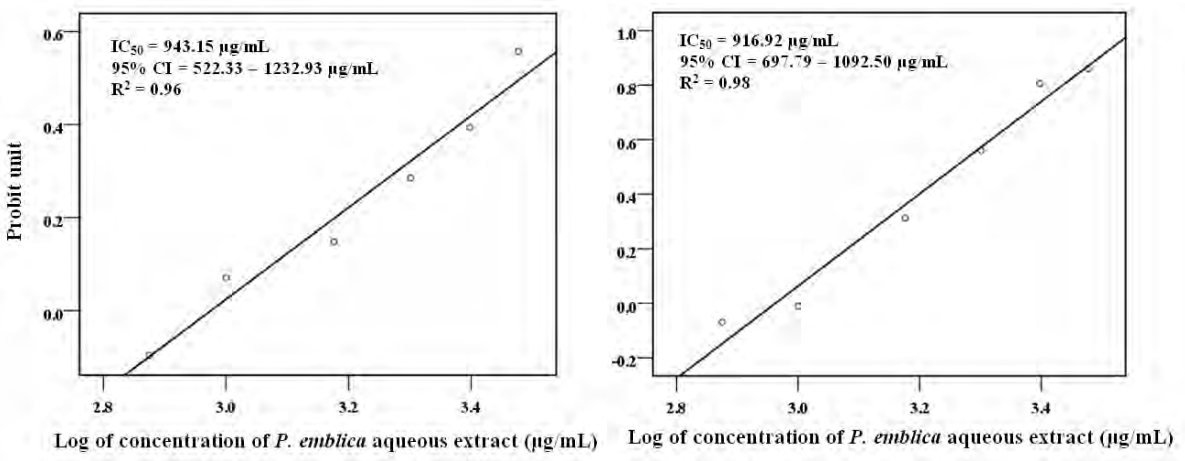


Figure 25 The inhibition curves of *P. emblica* aqueous extract on CYP3A4. *P. emblica* aqueous extract was used in the reaction without NADPH pre-incubation (left) and in the reaction with NADPH pre-incubation (right).

5. Standard curve for analysis of the type of inhibition of *P. emblica* on CYP3A4

The standard curve was constructed from the serial dilutions of the reconstituted Fluorescence Blue Standard in Vivid® CYP450 Reaction Buffer of which RFU were measured. The standard curve plotted between RFU and the fluorescence standard concentrations as well as the linear equation of the standard curve was shown in **Figure 26.**

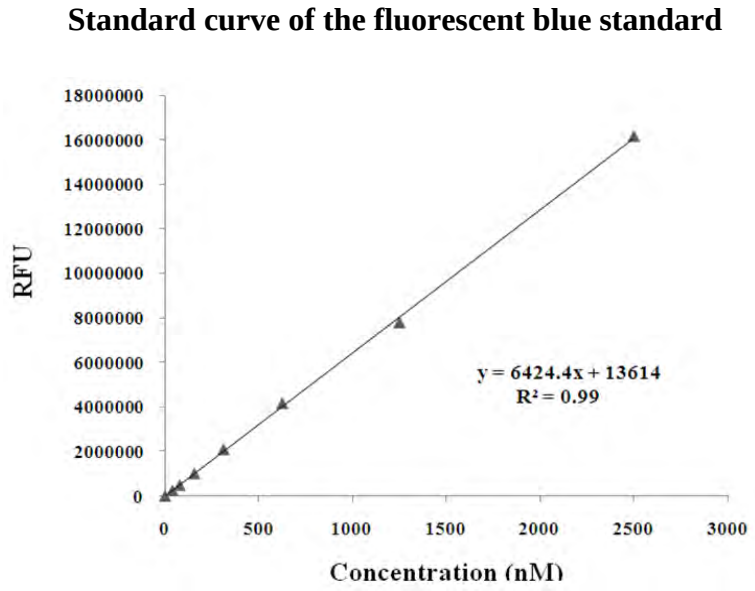


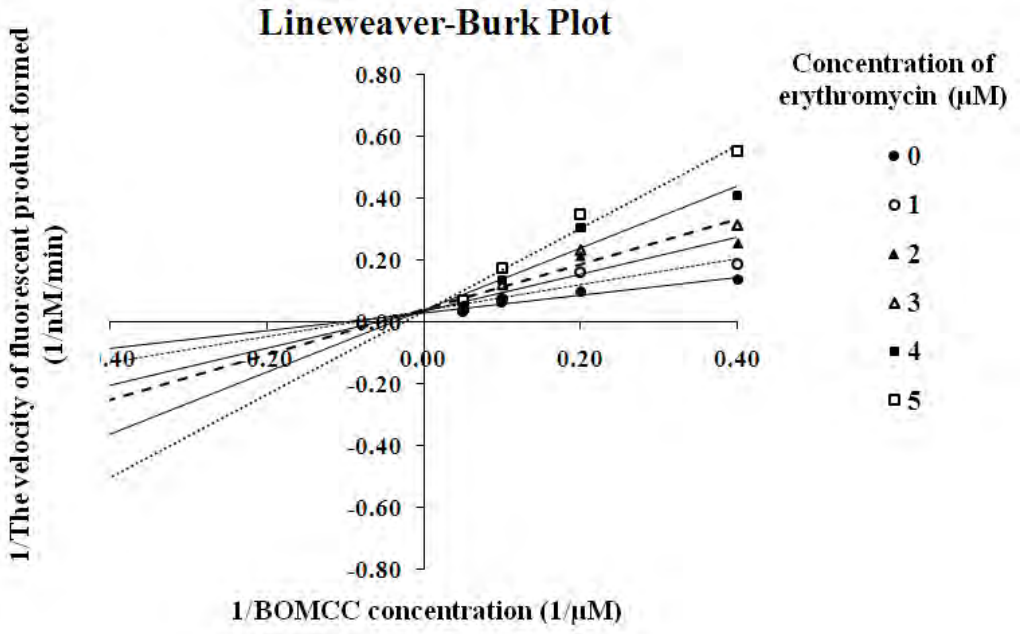
Figure 26 Standard curve of the fluorescent blue standard using for studying the type of inhibition on CYP3A4

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

6.1 Determination of the type of reversible inhibition of erythromycin on CYP3A4

The protocol using for determining the competitive type of inhibition of a compound on CYP3A4 was verified using the corresponding known inhibitor of this type of inhibition, erythromycin. The results showed that characteristic of the Lineweaver - Burk plot showed the pattern of competitive inhibition. The second plot was shown with the mean ± SE of K_i value of 1.12 ± 0.30 µM. The Lineweaver-Burk plot and the second plot of erythromycin on CYP3A4 were shown in **Figure 27.**

(a)



(b)

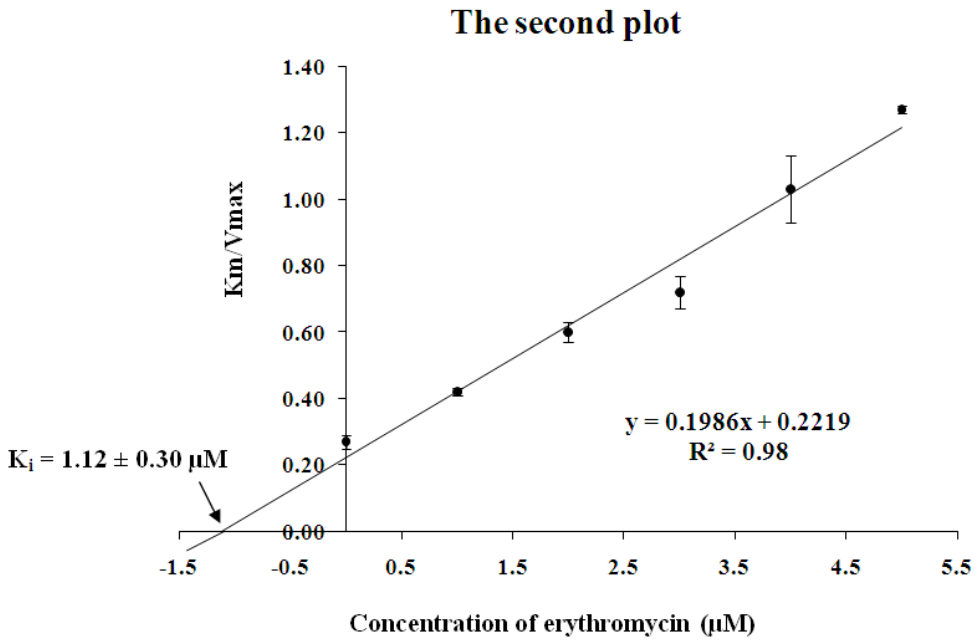
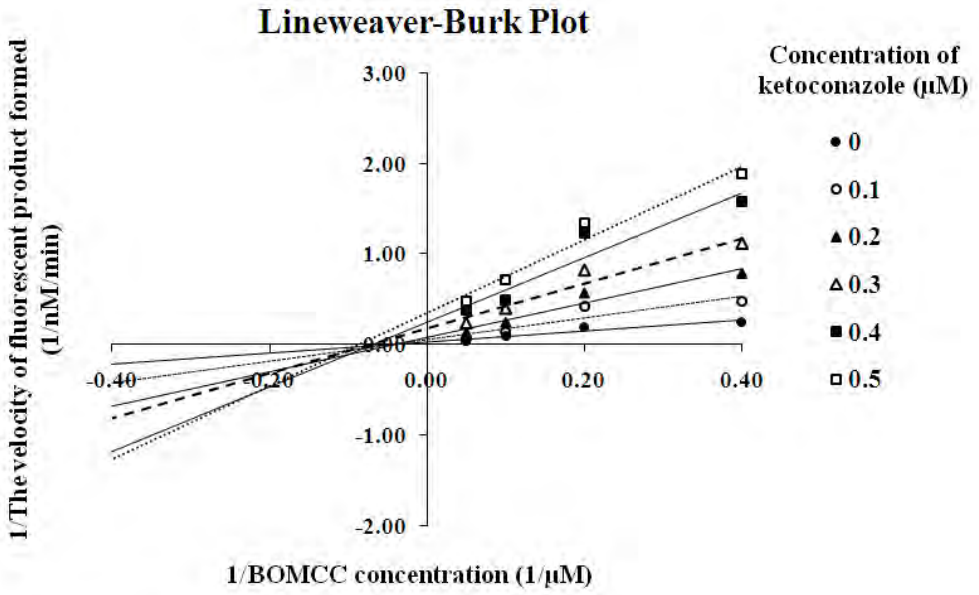


Figure 27 Lineweaver - Burk plots for the inhibition of erythromycin, the known competitive inhibitor of CYP3A4 (a) and the second plot of erythromycin on CYP3A4 (b). Each point represented the mean ± SE of triplicate experiments.

6.2 Determination of the type of reversible inhibition of ketoconazole on CYP3A4

The protocol using for determining the non competitive type of inhibition of a compound on CYP3A4 was verified using the corresponding known inhibitor of this type of inhibition, ketoconazole. The result showed that characteristic of the Lineweaver - Burk plot showed the pattern of non-competitive inhibition. The second plot was shown with the mean ± SE of K_i value of 0.07 ± 0.03 µM. The Lineweaver-Burk plot and the second plot of ketoconazole on CYP3A4 were shown in **Figure 28.**

(a)



(b)

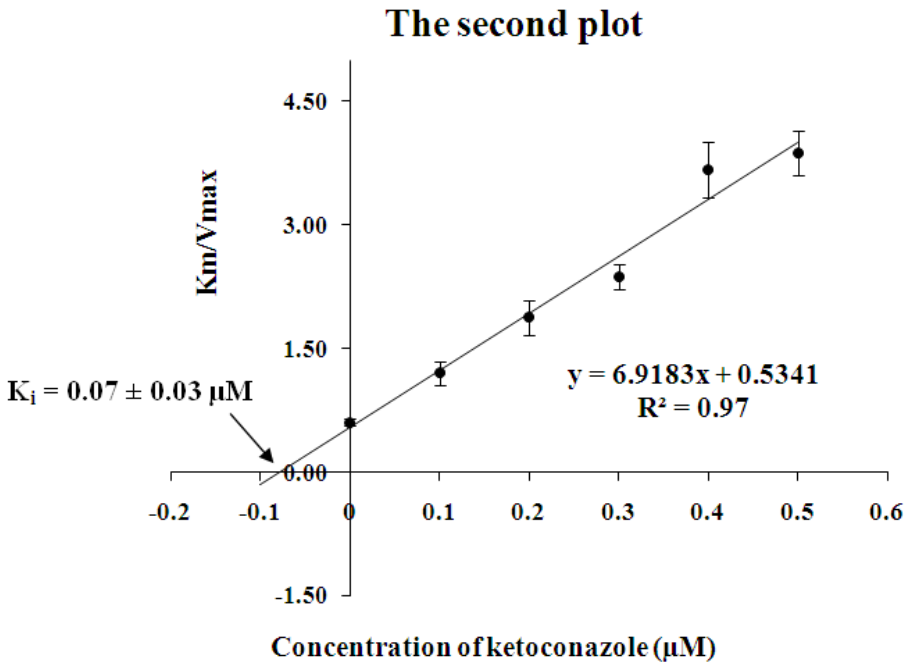


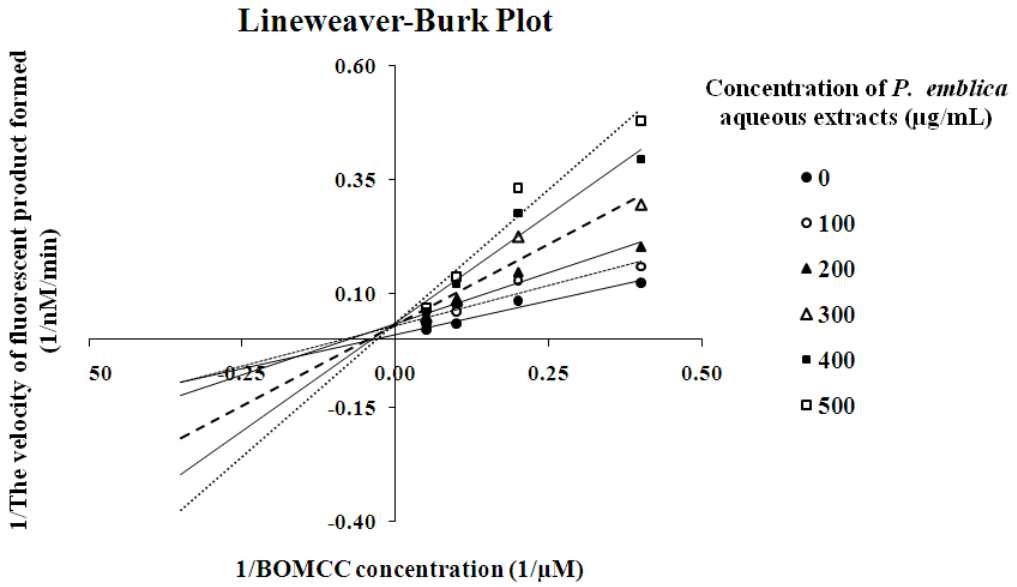
Figure 28 Lineweaver - Burk plot for the inhibition of ketoconazole, the known non competitive inhibitor on CYP3A4 (a) and the second plot of ketoconazole on CYP3A4 (b). Each point represented the mean ± SE of triplicate experiments.

7. Determination of the type of reversible inhibition of *P. emblica* aqueous extract on CYP3A4

The protocol was used to determining the reversible type of inhibition of *P. emblica* on CYP3A4. The results showed that the characteristic of the Lineweaver - Burk plot showed the pattern of non-competitive inhibition. The second plot was shown with the mean ± SE of the K_i value of 117.11 ± 1.55 µg/mL. The Lineweaver-Burk plot and the second plot of *P. emblica* were shown in **Figure 29.**

Summary of the K_i values of erythromycin, ketoconazole and *P. emblica* aqueous extract is shown in **Table 5**.

(a)



(b)

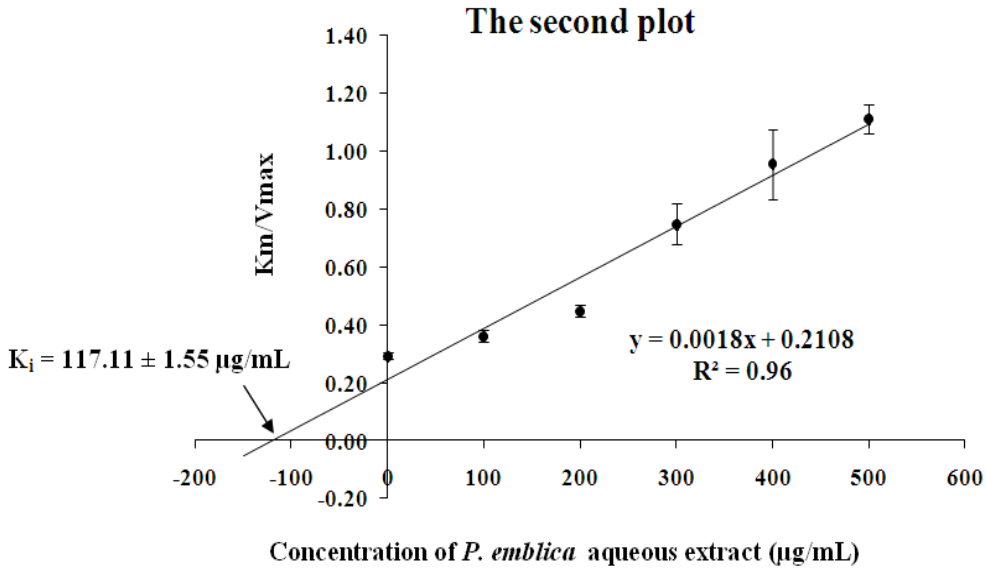


Figure 29 Lineweaver - Burk plot for the inhibition of *P. emblica* aqueous extract of CYP3A4 (a) and the second plot of *P. emblica* aqueous extract on CYP3A4 (b). Each point represented the mean ± SE of triplicate experiments.

Table 5 The K_i values ± SE (µg/mL) of known competitive inhibitor, known non-competitive inhibitor and *P. emblica* aqueous extract of CYP3A4 from enzyme kinetic study.

Compound	Type of reversible inhibition	K_i values (µM) (SE)	K_i values (µg/mL) (SE)	Other studies
Erythromycin	Competitive inhibition	1.12 (0.30)	0.97 (0.26)	-
Ketoconazole	Non competitive inhibition	0.07 (0.03)	0.04 (0.02)	0.04 µM (Thidarat, 2009)
P. emblica aqueous extract	Non competitive inhibition	-	117.11 (1.55)	-

CHAPTER V

DISCUSSION AND CONCLUSION

I. The inhibitory effect of *P. emblica* aqueous extract on recombinant human cytochrome P450

P. emblica has been used as traditional medicine and studied for several interesting pharmacological effects. Despite the safety data regarding toxicity testing in animal, drug interaction potential data of this plant has not been reported. Drug interaction study as recommended in the process of drug development focus on effect of a new compound on CYP enzyme either inhibition or induction effect by screening study *in vitro* before confirmatory study *in vivo* in human. Thus, to provide a drug interaction data for *P. emblica*, the aim of the study is to investigate the inhibitory effect of *P. emblica* aqueous extract on human drug metabolizing enzyme, CYP including CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 by an *in vitro* study using recombinant human CYP.

In This study, the Vivid® CYP450 Screening Kit consisted of CYP450 BACULOSOME® reagent which are microsomes prepared from insect cells expressing the particular CYP isozyme and rabbit NADPH CYP reductase. The Vivid® CYP450 substrate such as BOMCC (for CYP2C9 and CYP3A4) and EOMCC (for CYP1A2, CYP2D69 and CYP2E1) which were used as substrates, are metabolized by the particular CYP isoforms into products that are highly fluorescent and measured by Fluorescence Reader. The method was verified using the known inhibitor of each particular CYP isoform prior to assaying inhibitory effect of the extract on each CYP isoform. The IC_{50} values of the known inhibitor of each CYP isoform are summarized in **Table 6** and compared with those reported by other groups of researchers that used the same procedure. The results showed that IC_{50} values of this study were either closed to or somewhat different from the corresponding IC_{50} of other studies. Despite using the same procedure, the IC_{50} values can normally be different due to several factors such as different lot number of the reagents, different environment conditions (such as temperature) during experimentation, instrument factors and analyst factor, etc.

Table 6 IC_{50} values of the known inhibitor of each CYP isoform comparing to those reported in other studies

CYP isoform	**Known inhibitor**	**IC_{50} (µM) (95% Confidence interval)**	**IC_{50} (µM) (References)**
1A2	α-naphthoflavone	0.056 (0.042 – 0.070)	0.060 (Pichayapa, 2008) 0.130 (Duangjai, 2008) 0.110 (Patthanan, 2009) 0.080 (Thidarat, 2009) 0.029 (Mark and Larson, 2011a)
2C9	sulfaphenazole	0.238 (0.188 – 0.293)	0.110 (Duangjai, 2008) 1.076 (Pichayapa, 2008) 0.260 (Patthanan, 2009) 0.310 (Mark and Larson, 2011a)
2D6	miconazole	0.981 (0.724 – 1.293)	1.991 (Pichayapa, 2008) 1.14 (Thidarat, 2009) 0.430 (Mark and Larson, 2011a)
2E1	imipramine	125.325 (97.777 – 151.908)	345.930 (Pichayapa, 2008) 163.770 (Thidarat, 2009) 380.000 (Mark and Larson, 2011b)
3A4	ketoconazole	0.090 (0.071 – 0.108)	0.107 (Pichayapa, 2008) 0.270 (Patthanan, 2009) 0.080 (Thidarat, 2009) 0.130 (Mark and Larson, 2011a)

Summary of the IC_{50} values of *P. emblica* aqueous extract on CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 are shown in **Table 7**. As compared to the known inhibitor, it was shown that the inhibitory effect of *P. emblica* aqueous extract on all CYP isoforms were much weaker. These weak inhibitory effects *in vitro* may not be found or significant *in vivo*, however, the further confirmatory study *in vivo* was recommended (FDA, 2006).

Table 7 The IC_{50} values (µg/mL) of *P. emblica* aqueous extract on each CYP isoform comparing to those of the known inhibitors in this studies

CYP isoforms	IC_{50} (µg/mL) of *P. emblica* aqueous extract (SE, 95%CI)	IC_{50} (µg/mL) of known inhibitor (SE, 95%CI)
1A2	310.28 (5.07, 254.57 - 398.17)	0.02 (1.28×10^{-3}, 0.01 - 0.02)
2C9	194.72 (2.94, 177.75 - 214.29)	0.07 (6.29×10^{-3}, 0.06 - 0.09)
2D6	589.52 (14.32, 540.03 - 638.58)	0.47 (0.03, 0.34 - 0.62)
2E1	310.27 (15.06, 275.59 - 350.43)	35.09 (0.81, 27.38 - 42.53)
3A4	325.54 (7.44, 298.64 - 351.97)	0.05 (5.31×10^{-3}, 0.04 - 0.06)

II. Mechanism-based inhibition of *P. emblica* aqueous extract on CYP3A4

Mechanism of inhibition of *P. emblica* aqueous extract either mechanism-based inhibition or type of reversible inhibition was investigated on only CYP3A4 due to it is the major CYP isoform in human and responsible for metabolism of a large number of currently used medicines.

For the mechanism-based inhibition study, verification of the procedure was performed using a known mechanism-based inhibitor of CYP3A4, erythromycin, and known non mechanism-based inhibitor of CYP3A4, ketoconazole. It was shown that IC_{50} ratio of erythromycin was 1.06 ± 0.04 µM (more than 1) while the IC_{50} ratio of ketoconazole was 0.85 ± 0.02 (less than 1). Even though the IC_{50} ratio of those compounds was shown as expected, the values of the IC_{50} ratio between these two types of inhibitions were not obviously different from 1. This indicated that this method was not an appropriate procedure for assessing the mechanism-based inhibition of unknown compounds on CYP. Unlike this study, when the reactions were performed using human liver microsomes. The IC_{50} ratio for mechanism-based

inhibition of human CYPs were far different from 1 in the absence and presence of NADPH (Obach et al., 2007)

III. Determination of the type of reversible inhibition of *P. emblica* aqueous extract on CYP3A4

Enzyme kinetic study was performed to determine the type of reversible inhibition of *P. emblica* aqueous extract on CYP3A4. Before performing the experiment, the procedure was verified using a known competitive inhibitor and a known non competitive inhibitor of CYP3A4, which were erythromycin and ketoconazole, respectively. Then, *P. emblica* aqueous extract was assessed in the same manner and found that inhibitory effect of this extract on CYP3A4 was non competitive type with a K_i value of 117.11 ± 1.55 µg/mL.

K_i value obtained from the *in vitro* study is proposed to predict the *in vivo* drug-drug interaction by using $[I]/K_i$ ratio. A ratio of $[I]/K_i > 1$ suggests that an interaction is highly likely. While ratio of $0.1 < [I]/K_i < 1$ or $[I]/K_i < 0.1$ indicate that an interaction is likely or not likely, respectively. When [I] is the inhibitor concentration in serum and K_i is the inhibition constant obtained from the *in vitro* study (Zhang et al., 2008). For example the K_i of ketoconazole in the kinetic assay in this study was 0.07 µM. Generally, therapeutic serum concentration of ketoconazole is approximately 50 µg/mL or 94 µM (Brass et al., 1982). Then, $[I]/ K_i$ ratio of ketoconazole was 94/0.07 or 1342.86 which was much more than one. Therefore, ketoconazole is highly likely to cause drug-drug interaction *in vivo* which is truely reported in clinical practice (Salphati and Benet, 1998; Dutreix et al., 2004). *P. emblica* fruit extract at the concentration of 100 µg/mL was shown to inhibit both cytotoxicity- and apoptosis-induced by chromium (Ram et al., 2002). K_i of *P. emblica* aqueous extract in the kinetic assay in this study was 117.11 µg/mL. If the concentration of 100 µg/mL of the extract was approximately the concentration of the extract in the serum, the possibility of drug interaction caused by the extract using $[I]/ K_i$ ratio was 100/117.11 which was 0.85 (less than one). As a result, inhibitory effect of *P. emblica* extract on CYP3A4 was likely to cause drug-drug interaction *in vivo.* Thus, K_i value of *P. emblica* aqueous extract obtained from this study is useful to predict the possibility of drug-drug interaction *in vivo* if the concentration of this extract in serum is known.

From this study, *P. emblica* aqueous extract exhibited both mechanism-based inhibition and reversible inhibition of non-competitive type. This characteristic can be found that some parent drugs themselves exhibit some degree of reversible inhibition. The pre-incubation of the inhibitors with liver homogenates result in a substantial increase in the inhibitory effect of these drugs (Gibson and Skett, 2001).

In conclusion, *P. emblica* aqueous extract exhibited inhibitory effect on CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4. The inhibition of the extract on CYP3A4 was shown to be mechanism-based as well as reversible of non competitive type. Even though the inhibitory effect of *P. emblica* aqueous extract on these CYP isoforms were small, further study is recommended so as to assure the safety regarding drug-drug interaction if this extract is co-administered with other medicines that are metabolized by these CYP isoforms.

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APPENDICES

Appendix A

- Certificate of analysis of *P. emblica* extract

Certificate of Analysis

EMD

Date Printed : April 16, 2009

ITEM : **Emblica ® Natural Extract**

Phyllanthus emblica fruit extract

✓ ITEM NUMBER : 130165
BATCH NUMBER: CA0509059A

Data Order no. 179052

CHARACTERISTIC	REQUIREMENT Min.	Max.	RESULTS
Appearance			Passes test
Arsenic (As)		0.0002	0.0000 %
Date of Manufacturing			01-SEP-05
Heavy metals (as Pb)		0.002	<0.002 %
Identity (IR)			Infrared spectrum conforms
Microbial limits-Total aerobic bacteria		100	<100 CFU/g
Microbiological test-E coli			Absent in 1g
Microbiological test-Pseudomonas aeruginosa			Absent in 1g
Microbiological test-Salmonella			Absent in 10g
Microbiological test-Staphylococcus aureus			Absent in 1g
Microbiology test-Candida albican			Absent in 0.5 g
Minimum shelf life			31-OCT-2011
Sulfated ash		6.0	5.10 %
Water (K.F.)		6.0	4.90 %
Water soluble extractives	80.0		98.60 %
Assay-HPLC/HPTLC-Total low mol.wt hydrolyzable tannis	50.0		72.58 %
Microbial limits-Total combined mold and yeast		100	<100 CFU/g

Andrea Van Gilder

Quality Control Manager
Release Date: 04/16/2009

EMD Chemicals Inc.
480 S. Democrat Road
Gibbstown, NJ 08027

MERCK

Specification

http://certificates.merck.de

Date of print: 06.08.2009

1.30165.0500 Emblica™

	Spec. Values
Identity	IR spectrum conforms
Total small hydrolyzable tannoids (HPTLC)	≥ 50 %
As (Arsenic)	≤ 0.0002 %
Heavy metals (as Pb)	≤ 0.002 %
Sulfated ash	≤ 6 %
Water (according to Karl Fischer)	≤ 6 %
Microbiological purity (total viable aerobic count)	≤ 100 CFU/g
Candida albicans	absent in 1 g
E.coli	absent in 1 g
Pseudomonas aeruginosa	absent in 1 g
Staphylococcus aureus	absent in 1 g
Salmonella species	absent in 10 g

Dr. Sebastian Haertner

responsible laboratory manager quality control

This document has been produced electronically and is valid without a signature

MERCK

Safety Data Sheet

According to Regulation (EC) No.1907/2006 (REACH)

Date of issue: 26.07.2007
Supersedes edition of 10.05.2001

1. Identification of the substance/preparation and of the company/undertaking

Identification of the product

Catalogue No.: 130165
Product name: Emblica™

Use of the substance/preparation

Cosmetic raw material

Company/undertaking identification

Company: Merck KGaA * 64271 Darmstadt * Germany * Phone: +49 6151 72-0
Emergency telephone No.: Please contact the regional Merck representation in your country.
Contact for information: EHSQ/EHS-PI * e-mail:prodsafe@merck.de

2. Hazards identification

No hazardous product as specified in Directive 67/548/EEC.

3. Composition/information on ingredients

CAS-No.: 90028-28-7
EC-No.: 289-817-3

INCI-name:

Phyllanthus Emblica Fruit Extract

4. First aid measures

After inhalation: fresh air.
After skin contact: wash off with plenty of water. Remove contaminated clothing.
After eye contact: rinse out with plenty of water with the eyelid held wide open. Call in ophtalmologist if necessary.
After swallowing: make victim drink plenty of water. Consult doctor if feeling unwell.

5. Fire-fighting measures

Suitable extinguishing media:
Water, CO_2, foam, powder.

Special risks:
Combustible. Development of hazardous combustion gases or vapours possible in the event of fire.

Special protective equipment for fire fighting:
Do not stay in dangerous zone without self-contained breathing apparatus.

Other information:
Prevent fire-fighting water from entering surface water or groundwater.

6. Accidental release measures

Person-related precautionary measures:
Avoid generation of dusts; do not inhale dusts.

Environmental-protection measures:
Do not allow to enter sewerage system.

Procedures for cleaning / absorption:
Take up dry. Forward for disposal. Clean up affected area. Avoid generation of dusts.

7. Handling and storage

Handling:

No further requirements.

Storage:

Tightly closed. Dry. At +15°C to +25°C.

Protected from light.

8. Exposure controls/personal protection

Personal protective equipment:

Protective clothing should be selected specifically for the working place, depending on concentration and quantity of the hazardous substances handled. The resistance of the protective clothing to chemicals should be ascertained with the respective supplier.

Respiratory protection: required when dusts are generated.

Eye protection: required

Hand protection:

In full contact:

Glove material:	nitrile rubber
Layer thickness:	0.11 mm
Breakthrough time:	> 480 Min.

In splash contact:

Glove material:	nitrile rubber
Layer thickness:	0.11 mm
Breakthrough time:	> 480 Min.

The protective gloves to be used must comply with the specifications of EC directive 89/686/EEC and the resultant standard EN374, for example KCL 741 Dermatril® L (full contact), 741 Dermatril® L (splash contact). The breakthrough times stated above were determined by KCL in laboratory tests acc. to EN374 with samples of the recommended glove types.
This recommendation applies only to the product stated in the safety data sheet and supplied by us as well as to the purpose specified by us. When dissolving in or mixing with other substances and under conditions deviating from those stated in EN374 please contact the supplier of CE-approved gloves (e.g. KCL GmbH, D-36124 Eichenzell, Internet: www.kcl.de).

Industrial hygiene:
Change contaminated clothing. Wash hands after working with substance.

9. Physical and chemical properties

Form:		powder, fine grained	
Colour:		yellowish to brown	
Odour:		musty	
pH value			not available
Melting point			not available
Boiling point			not available
Ignition temperature			not available
Flash point			not available
Explosion limits	lower		not available
	upper		not available
Density			not available
Solubility in water		(20 °C)	freely soluble

10. Stability and reactivity

Conditions to be avoided
no information available

Substances to be avoided

no information available

Hazardous decomposition products
no information available

Further information

The following applies in general to flammable organic substances and preparations: in correspondingly fine distribution, when whirled up a dust explosion potential may generally be assumed.

11. Toxicological information

Acute toxicity

LD_{50} (oral, rat): >5000 mg/kg (own results).

Specific symptoms in animal studies:
Eye irritation test (rabbit): Slight irritations (own results).
Skin irritation test (human): No irritation (own results).

Subacute to chronic toxicity

Sensitization:
Patch test (humans): negative. (own results)

Bacterial mutagenicity: Ames test: negative. (OECD 471)

Further toxicological information

After eye contact: Slight irritations.

Further data

The product should be handled with the care usual when dealing with chemicals.

12. Ecological information

Ecotoxic effects:
Quantitative data on the ecological effect of this product are not available.

Further ecologic data:
Do not allow to enter waters, waste water, or soil!

13. Disposal considerations

Product:

Chemicals must be disposed of in compliance with the respective national regulations. Under www.retrologistik.de you will find country- and substance-specific information as well as contact partners.

Packaging:

Merck product packaging must be disposed of in compliance with the country-specific regulations or must be passed to a packaging return system. Under www.retrologistik.de you will find special information on the respective national conditions as well as contact partners.

14. Transport information

Not subject to transport regulations.

15. Regulatory information

Labelling according to EC Directives

Symbol:	---
R-phrases:	---
S-phrases:	---

16. Other information

Reason for alteration

Chapter 11: toxicological information.

General update.

Regional representation:

This information is given on the authorised Safety Data Sheet for your country.

The information contained herein is based on the present state of our knowledge. It characterizes the product with regard to the appropriate safety precautions. It does not represent a guarantee of the properties of the product.

MERCK Merck Chemicals | Thailand

130165 Emblica™

Add to Favorites

Ordering number	Packaging	Size	Quantity	Price
1301650500	Plastic bottle	500 g		**price on request**
1301655000	Plastic drum	5 kg		**price on request**
1301659015	Plastic drum	15 kg		**price on request**

Add to request

Product information

HS Code	1302 19 80	**EC number**	289-817-3
CAS number	90028-28-7		

Chemical and physical data

Solubility in water	(20 °C) soluble

Safety information

Storage class (VCI)	10 - 13 Other liquids and solids
WGK	WGK 1 slightly water endangering

Toxicological data

LD 50 oral	LD50 rat > 5000 mg/k

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

O-13873-r1 US 0405

TABLE OF CONTENTS

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	P2370	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.

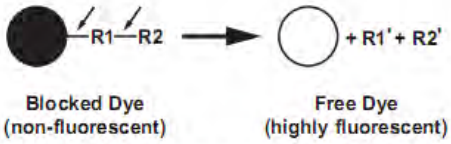

Figure 1. Schematic of the metabolism of the "blocked" dye substrate into a fluorescent metabolite

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).

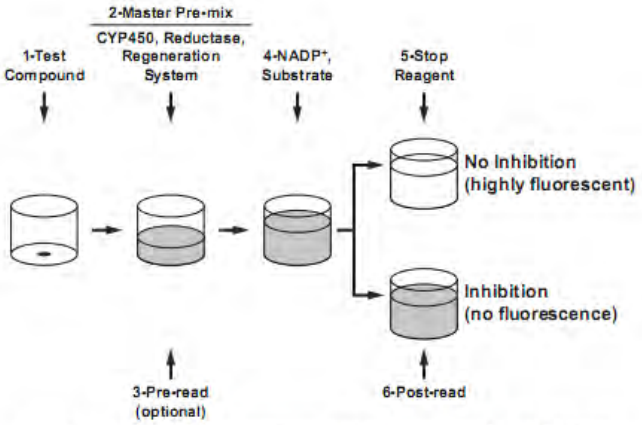

Figure 2. A schematic representation of an endpoint Vivid® CYP450 Assay

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†	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

† 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	58	9	46	61
	0.1	25	**	7	11
	0.01	**	--	**	**
2C9 Green	1	**	**	30	38
	0.1	**	--	**	**
	0.01	**	--	**	**
2C9 Red	1	21	3	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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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.

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VITAE

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