EFFECTS OF *COSCINIUM FENESTRATUM* STEM EXTRACT ON FUNCTION AND EXPRESSION OF P-GLYCOPROTEIN

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ผลของสารสกัดเถาแห้มต่อการทำงานและการแสดงออกของพี-ไกลโคโปรตีน

นางสาวนารีรัตน์ ทองดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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การศึกษานี้ มีวัตถุประสงค์ เพื่อศึกษาผลของสารสกัด เถาแห้มต่อการทำงานของพี- ไกลโคโปรตีน และการแสดงออกของยีน *MDR1* ในเซลล์เยื่อบุไตหมู (แอลแอลซี-พีเควัน) และเซลล์เยื่อบุไตหมูที่มีการแสดงออกของพี-ไกลโคโปรตีนมากกว่าปกติ (แอลแอลซี-เอ็มดีอาร์วัน) และเซลล์เยื่อบุไตหมูที่มีการแสดงออกของพี-ไกลโคโปรตีนมากกว่าปกติที่ถูกชัก นำด้วยวินบลาสติน (แอลแอลซี-วีบีแอล) โดยหมักเถาแห้มด้วยเอทานอลแปดสิบเปอร์เซ็นต์ ผลการทดลองพบว่าที่ความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ของสารสกัดแห้ม (100 ไมโครกรัมต่อมิลลิลิตร) สามารถเพิ่มความเป็ นพิษของ วินบลาสตินซึ่งเป็นสับสเตรทของพี-ไกลโคโปรตีนในเซลล์แอลแอลซี-เอ็มดีอาร์วัน และเซลล์แอลแอลซี- วีบีแอลได้อย่างมีนัยสำคัญ ซึ่งสอดคล้องกับผลการเพิ่มการสะสมของสารเรืองแสงโรดามีนหนึ่งสองสามที่เป็นสับสเตรทของพี-ไกลโคโปรตีนภายในเซลล์ได้อย่างมีนัยสำคัญ อีกทั้งสารสกัดแห้มยังสามารถลดการทำงานของเอนไซม์เอทีพีเอส ในพี- ไกลโคโปรตีน ในทั้งสองเซลล์ด้วย ถึงแม้ว่าความเข้มข้นที่ไม่เป็นพิษต่อเซลล์ของสารสำคัญเบอร์บะรีน (1 ไมโครกรัมต่อมิลลิลิตร) ที่พบในสารสกัดแห้มสามารถเพิ่มความเป็นพิษของวินบลาสตินในเซลล์แอลแอลซี-วีบีแอลได้อย่างมีนัยสำคัญ แต่ไม่มีผลต่อการสะสมของโรดามีนหนึ่งสองสามภายในเซลล์ และไม่มีผลต่อการทำงานของเอนไซม์เอทีพีเอสในพี-ไกลโคโปรตีน อย่างไรก็ตามในการศึกษาผลของสารสกัดแห้มต่อการแสดงออกของ พี-ไกลโคโปรตีน ด้วยวิธี Western blot analysis พบว่าทั้งสารสกัดแห้ม และเบอร์บะรี น ไม่มีผลต่อการแสดงออกของพี-ไกลโคโปรตีน จากการศึกษานี้สรุปได้ว่าสารสกัดแห้มสามารถยับยั้งการทำงานของพี-ไกลโคโปรตีน โดยยับยั้งการทำงานของเอนไซม์เอทีพีเอส แต่ไม่มีผลต่อการสังเคราะห์พี- ไกลโคโปรตีน ซึ่งอาจส่งผลทำให้เกิดปฏิกิริยาไม่พึงประสงค์ร่วมกัน เมื่อรับประทานยาที่เป็นสับสเ ตรทของพี-ไกลโคโปรตีนร่วมกับแห้มได้

ภาควิชา ชีวเคมี และจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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This study aimed to investigate the effects of *Coscinium fenestratum* stem extract on P-glycoprotein (Pgp) function and *MDR1* expression in porcine renal epithelial (LLC-PK1) and its *MDR1* transfected (LLC-MDR1) and its vinblastine (VBL)-induced *MDR1* transfected (LLC-VBL) cell lines. *C. fenestratum* stems were extracted by maceration with 80% ethanol. The result showed that the non-toxic concentration of *C. fenestratum* extract (100 µg/ml) potentiated the effect of VBL (Pgp-substrate)-induced cytotoxicity in LLC-VBL and LLC-MDR1 cells. This effect was stronger in LLC-VBL cells than in LLC-MDR1 cells which it was well correlated with significantly increase accumulation of intracellular fluorescent rhodamine 123, a Pgp-substrate. Interestingly, the *C. fenestratum* extract decreased Pgp-ATPase activity in both Pgp-overexpressed cells. The major compound in *C. fenestratum* extract, berberine, at the non-toxic concentration of 1 µg/ml significantly potentiated the effect of VBL-induced cytotoxicity in LLC-VBL cells, but it did not affect LLC-MDR1 cells. It had no effect on rhodamine 123 accumulation and Pgp-ATPase activity in both Pgp-overexpression cells. However, both *C. fenestratum* extract and berberine had no effect on Pgp expression determined by Western blot analysis. Taken together, our findings indicated that *C. fenestratum* ethanolic stem extract is a P-glycoprotein inhibitor by inhibiting ATPase activity without affecting Pgp-expression. Therefore, co-administration of Pgp-substrate medicines with *C. fenestratum* extract may lead to undesirable drug-herb interaction.

Department : Biochemistry and Microbiology Student's Signature .....
Field of Study : Biomedicinal Chemistry Advisor's Signature .....
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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius (centigrade)
µg	Microgram (s)
µl	Microliter
µM	Micromolar
Ab	Antibody
ADP	Adenosine diphosphate
ATCC	American type culture collection, Maryland, USA
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine triphosphatase
AUC	Area under the plasma concentration-time curve
C. fenestratum	*Coscinium fenestratum*
C_{max}	Maximum plasma concentration
CO_2	Carbon dioxide
CYPs	Cytochrome P450s
CYP3A4	Cytochrome P450, family 3, subfamily A, polypeptide 4
DDW	Double distilled water
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
et al.	*et alii*, and others
g or RCF	Relative centrifugal force
g	Gram (s)
hr	Hour
HRP	Horseradish peroxidase
IC_{50}	50% inhibitory concentration
Ig	Immunoglobulin
KCl	Potassium chloride
kDa	Kilodalton

KH_2PO_4	Monopotassium phosphate
LLC-PK1	Porcine renal epithelial cell line
LLC-MDR1	*MDR_1* gene-transfected procine epithelial renal cell line
LLC-VBL	Vinblastine-induced Pgp-overexpressed LLC-MDR1 cell line
MAOIs	Monoamine oxidase inhibitors
MDR	Multidrug resistance
mg	Milligram (s)
min	Minute (s)
ml	Milliliter (s)
mM	Millimolar
mm	Millimeter (s)
M199	Medium 199
MRP	Multridrug resistance-associated protein
MTT	Methyl thiazolyl tetrazolium
NaCl	Sodium chloride
Na_2HPO_4	Disodium phosphate
NaF	Sodium fluoride
$NH_4 \cdot 6Mo_7O_{24}$	Ammonium molybdate
NBDs	Nucleotide binding domains
$(NH_4)_3PO_4 \cdot 12MoO_3$	phosphomolybdate complex
ng	Nanogram (s)
nm	Nanometer (s)
nmol	Nanomole (s)
OD	Optical density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline, 0.1 % Tween 20
Pgp	P-glycoprotein
pH	The negative logarithm of hydrogen ion concentration
Pi	phosphate

PMSF	Phenyl-methyl-sulfonyl fluoride
qs.	Make to volume
R_f	Retention factor
Rh 123	Rhodamine 123
RIPA	Radio immune precipitation assay buffer
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SPSS	Statistical package for social sciences
$t_{½}$	The elimination half-life
TLC	Thin layer chromatography
TMDs	Transmembrane domains
UGTs	Uridine 5'-diphospho-glucuronosyltransferase
UV	Ultraviolet
V	Volt
VBL	Vinblastine
% (w/w)	Weight-weight percentage

CHAPTER I
INTRODUCTION

Herb supplements are widely used currently in daily life and are commonly co-administered with prescribed medicines, which may result in drug-herb interaction. The mechanism of P-glycoprotein (Pgp) on drug interaction was previously reported (Zhou *et al.*, 2007). Pgp is a transmembrane protein encoded by human multidrug resistance 1 (*MDR1*) gene, a member of ATP-binding cassette (ABC) transporters. Pgp functions as an efflux of xenobiotics and other Pgp-substrates such as drugs, organic cations, carbohydrates and amino acids (Zhou, 2008). Under a normal condition, Pgp is found at apical membrane of epithelial barriers of kidney, liver and intestinal lumen. The major role of Pgp is involved in drug absorption, distribution and elimination leading to drug–herb interaction (Lin and Yamazaki, 2003). Moreover, several types of herbs could affect Pgp functions, for examples, curcumin could increase VBL cytotoxicity in the multidrug-resistant human cervical carcinoma cells, KB-V1, which overexpressed Pgp (Anuchapreeda *et al.*, 2002). Piperine could inhibit the efflux of Pgp-substrates, digoxin and cyclosporine A (Balayssac *et al.*, 2005), and grapefruit juice could increase cyclosporine A concentration in blood circulation (Romiti *et al.*, 2004).

Coscinium fenestratum is widely used in traditional medicine for treatment of many diseases, such as high blood cholesterol, hyperglycemia and hypertension (Rojsanga and Gritsanapan, 2005; Wongcome *et al.*, 2007). Chemical constituents of *C. fenestratum* are isoquinoline alkaloids such as berberine, palmatine, tetrahydropalmatine, crebanine and jatrorrhizine. Berberine is a major compound found in highest content of *C. fenestratum* (Rojsanga and Gritsanapan, 2005). Interestingly, the common structure of berberine and palmatine is planar aromatic ring which is found in most Pgp inhibitors (Shitan *et al.*, 2007). Consistently, both berberine and palmatine were Pgp-substrates (Pan *et al.*, 2002; Suzuki *et al.*, 2010). Therefore, *C. fenestratum* may has an effect on Pgp and cause drug-herb ineraction when co-administration with other Pgp-substrate drugs.

Nowadays, *C. fenestratum* is manufactured in many commercial products in the market claiming to reduce blood glucose. Therefore, diabetic patients who may have various complications, *e.g.*, neuropathy, nephropathy, cardiovascular and cerebrovascular diseases (Yibchok-anun *et al.*, 2009) commonly used *C. fenestratum* containing product together with other prescribed medicines. There is a concern about lacking information about drug and herb interaction, particularly interaction with *C. fenestratum*, that may cause undesirable effects. Therefore, the aim of this study is to investigate the effect of *C. fenestratum* extract on Pgp function and *MDR1* expression in porcine renal epithelial cells (LLC-PK1) and its *MDR1*-transfected counterparts cells (LLC-MDR1 and LLC-VBL).

1.1 Objectives

1. To study the effect of *C. fenestratum* extract on Pgp function.
2. To study the effect of *C. fenestratum* extract on *MDR1* gene expression.

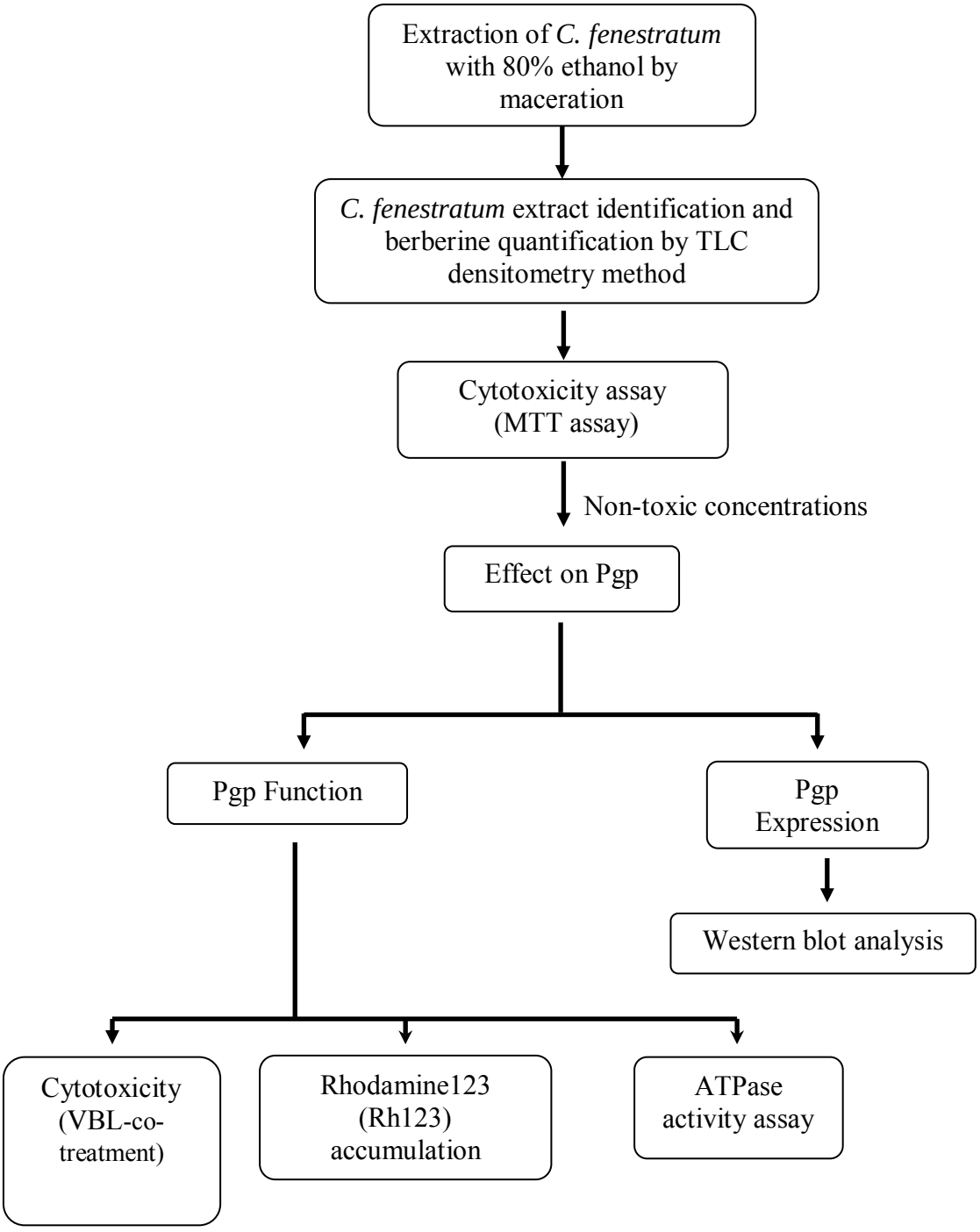


Figure 1. The conceptual framework of this study

1.2. Scope of study

The first step of this study is the extraction of *C. fenestratum* stems by maceration with 80% ethanol. Subsequently, the berberine contents in the *C. fenestratum* extract was quantified by TLC-densitometry method. The next step is cytotoxicity determination which was divided into two parts. In the first part, non-toxic concentrations of *C. fenestratum* extract and berberine were evaluated in wild type cell, LLC-PK1, and the Pgp-overexpression cells, LLC-MDR1 and LLC-VBL. In the second part, the cytotoxicity of vinblastine (VBL) was evaluated for the 50 % inhibitory concentrations (IC_{50}) in LLC-PK1, LLC-MDR1, and LLC-VBL cells in order to be used in VBL-cotreated cytotoxicity. MTT cytotoxicity assay, Rhodamine 123 accumulation assay and ATPase activity assay were used to evaluate the effect of *C. fenestratum* extract and berberine on the Pgp function. Firstly, MTT cytotoxicity assay was used to evaluate the effect of *C. fenestratum* extract and berberine on the toxicity of VBL, a Pgp-substrate, in all three cells. Secondly, rhodamine 123 accumulation assay was used to evaluate the effect of *C. fenestratum* extract and berberine on the accumulation of the fluorescence dye Rh 123, a Pgp-substrate, in all three cells. Thirdly, the effects of these samples on ATPase activity assay were performed, since ATPase enzyme activity in Pgp is important for Pgp function. Finally, Western blot analysis was used to examine the effect of *C. fenestratum* extract and berberine on the *MDR1* gene expression.

1.3. Contribution of the study

The information about the effect of *C. fenestratum* extract on Pgp function and *MDR1* gene expression may be useful information in order to avoid the undesirable drug-herb interaction when co-administration of prescribed medicines with *C. fenestratum*.

CHAPTER II
LITERATURE REVIEWS

2.1. Drug-herb interaction

Recently, herbs are becoming popular used as alternative medicine worldwide. The various commercial herbal products are produced which are easy for selection to use in healthcare or therapy. Many people believe that the herbal products from natural sources are safe and healthy (Kuhn, 2002). Moreover, the herbal medicines usages currently increase in developed countries, such as the United State. Many patients, especially patients with chronic illnesses, such as heart disease, stroke, cancer, chronic respiratory disease, depression and diabetes, usually take herbal products together with the prescribed drugs. These patients trend to take more prescribed drugs, particularly drugs with a narrow therapeutic index (Gardiner *et al.*, 2008). Therefore, there are risk of clinically significant drug-herb interactions (Gouws *et al.*, 2012; Marchetti *et al.*, 2007). The popular herbs such as ginseng which has hypoglycemic activity can show additive effect in diabetic patients taking oral hypoglycemic agents or insulin (Gardiner *et al.*, 2008). St John's wort, one of the most popular herbs in the United States for the management of depression, can decrease or increase plasma drug concentrations of prescribed drugs such as amitriptyline, atorvastatin, bupropion, buspirone, cyclosporine and erythromycin. Kava kava which is cultivated throughout the South Pacific area, has been used as a ceremonial drink and can relieve anxiety, nervousness and tension. It acts as a dopamine antagonist, therefore, it may increase tremor and made prescribed drugs less effective in patients with Parkinson disease (Kuhn, 2002). The other examples of herbs that can interact with drugs were shown in Table 1.

Table 1. Examples of drug-herb interaction. They were modified from Kuhn in 2002, Shi and Klotz in 2012 and Zhou's group in 2007 (Kuhn, 2002; Shi and Klotz, 2012; Zhou *et al.*, 2007).

Herbal products	Drugs	Results from herb-drug interaction
Internal aloe vera, saparilla, marshmallow	All drugs	Binds with drugs
Evening primrose oil	Phenothiazines	Increase likelihood of seizures
Bittermelon, Garlic, Ginseng	Antidiabetic drugs	May increase likelihood of hypoglycemia
Ginseng, guarana	Phenelzine sulfate (Nardil) and other MAOIs*	Increase likelihood of headache tremulousness and manic episodes and blood pressure
Ginkgo	Aspirin	Spontaneous hyphema (Hemorrhage in the anterior cavity of eyes)
Kava kava	Alprazolam	Coma (lethargy, disorientation)
	Chlorzoxazone	Decreased 6-hydroxychlorzoxazone /chlorzoxazone serum ratios
	Levodopa	Reduced efficacy
St John's wort	Amitriptyline	Decreased amitriptyline AUC[a]
	Atorvastatin	Reduced efficacy of atorvastatin
	Bupropion	Decreased bupropion AUC
	Buspirone	Serotonin syndrome
	Cyclosporine	Decreased cyclosporine AUC and C_{max}[b]
	Erythromycin	Increased erythromycin metabolism
Milk thistle	Losartan	Increased losartan AUC; decreased metabolic ratio of losartan

* MAOIs = Monoamine oxidase inhibitors which are class of antidepressant drugs.

[a] AUC = area under the plasma concentration-time curve

[b] C_{max} = maximum plasma concentration

2.1.1. Mechanisms of drug-herb interaction

The mechanisms of drug-herb interaction can be divided into two major classes: pharmacodynamic interaction and pharmacokinetic interaction (Kuhn, 2002). First class, pharmacodynamic interaction occurs when the pharmacological action of a herbal medicine synergizes or increases or antagonizes the biological activity of a prescribed drug by coincident effects on the same drug targets such as enzymes or drug receptors (Shi and Klotz, 2012; Zhou *et al.*, 2007). Because a herbal medicine may contain many active compounds, all of which may have various unknown biological activities (Zhou *et al.*, 2007). Therefore, if the biological active compounds in herb had synergistic or additive properties, the drug efficacy may increase. On the other hand, the antagonistic properties of herbal compounds may reduce drug efficacy as shown in Figure 2 (Gouws *et al.*, 2012; Zhou *et al.*, 2007). For example, the hypnotic activity of benzodiazepines is increased by valerian, and the anticoagulant action of warfarin is enhanced by ginko (Zhou *et al.*, 2007).

Second class, pharmacokinetic interaction is potentially cause of drug-herb interaction (Shi and Klotz, 2012). This interaction intervenes when a herb can change the concentration of co-administered drug at the site of action by altering its absorption, distribution, metabolism and excretion (Gouws *et al.*, 2012). Pharmacokinetic interactions come to be clinically significant when considerable changes occur in pharmacokinetic parameters, such as the area under the plasma concentration-time curve (AUC), the maximum plasma concentration (C_{max}) or the elimination half-life ($t_{½}$), of prescription drug, particularly a narrow therapeutic index drug, *e.g.*, warfarin and digoxin (Shi and Klotz, 2012).

Cytochrome P450s (CYPs) enzymes are one of the important mechanism of drug metabolism in liver and intestine. Many herb remedies are substrates of CYPs. The induction or inhibition of these hepatic and intestinal metabolic enzymes is considered to be a main mechanism of pharmacokinetic herb-drug interaction. Moreover, drug transporter, P-glycoprotein (Pgp) which is responsible for drug efflux out of cells in the intestine, liver and kidney, plays significant role in drug absorption, distribution or excretion. Therefore, the herb medicine interacts with co-administered drug through Pgp may significantly affect to the bioavailability of drug, cause drug-herb interaction. In addition, many active components of drugs and herbs are

substrates for both Pgp and CYP3A4, are one of CYPs member (Gouws *et al.*, 2012; Pal and Mitra, 2006; Zhou *et al.*, 2007). The activity of CYPs and Pgp influence oral bioavailability, thus, the modulating activity of co-administered herbal products has been shown to significantly result in decrease or increase the drug levels in blood, known as pharmacokinetic drug-herb interaction (Fasinu *et al.*, 2012). However, changed of protein binding of drug by herbal medicines has slightly effect on pharmacokinetic parameters. Because many herbal compounds can highly bound to plasma proteins which may displace the drugs from the binding sites (Zhou *et al.*, 2007) as shown in Figure 2.

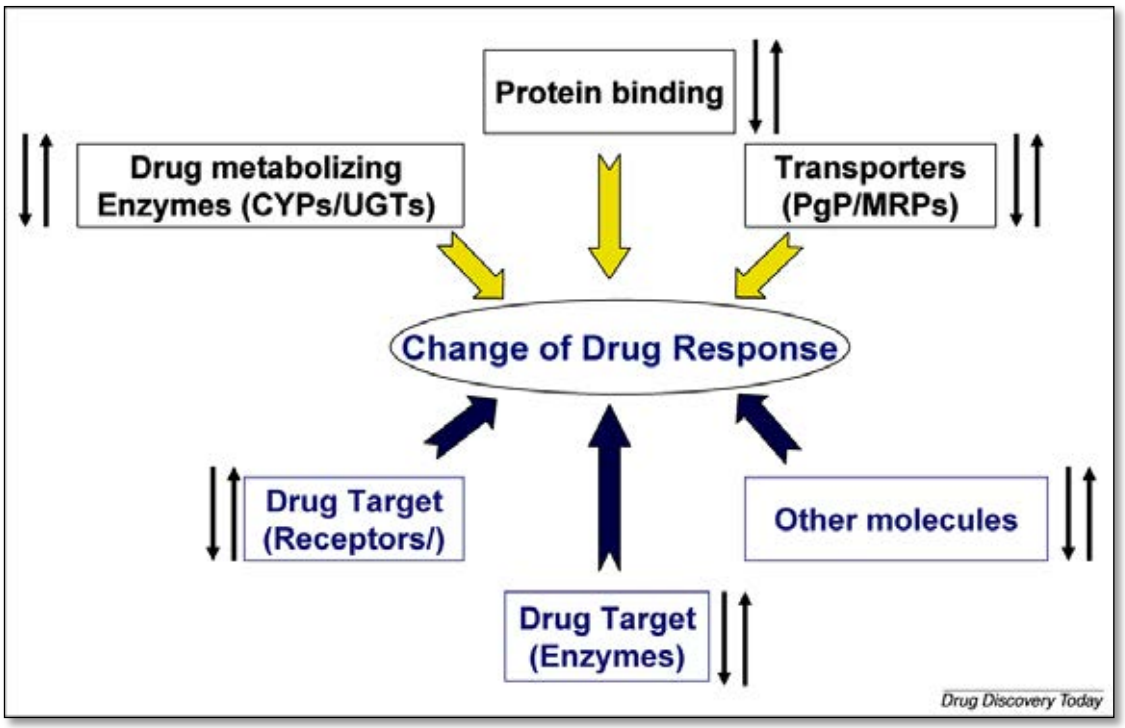


Figure 2. The possible mechanisms of drug-herb interaction. Both pharmacokinetic and pharmacodynamic interactions may play important roles in drug-herb interaction (Zhou *et al.*, 2007).

2.1.2. Clinical significance of the identification of drug-herb interaction

The possible clinically important effects of drug-herb interaction may occur as shown in Figure 3. Herbs can modulate intestinal and hepatic CYP enzymes and Pgp which lead to modify the bioavailability and clearance of co-administered drugs (Zhou *et al.*, 2007). The clinically effects of drug-herb interaction can occur in two different ways, *i.e.*, synergism or addition and antagonism. Synergism is the effect of drug-herb interaction which herb can increase the drug concentrations in blood result in increasing the efficacy of drug or causing toxicity. On the other hand, antagonism is the effect of drug-herb interaction which herb can decrease the drug concentrations in blood result in lowering the efficacy of drug and therapeutic failure. For example, St John's wort decreased the plasma concentration of cyclosporine, amitriptyline, digoxin, indinavir, nevirapine, oral contraceptives, warfarin, phenprocoumon, theophylline or simvastatin (Shi and Klotz, 2012), garlic decreased the plasma concentrations of saquinavir, but not ritonavir (Fugh-Berman, 2000).

The clinical importance of drug-herb interactions depend on factors that are related to co-administered drugs, *i.e.*, dose, dosing regimen, administration route, pharmacokinetic and therapeutic range; factors related to herbs which consist of species, dose, dosing regimen, and administration route; and factors related to patients which consist of genetic polymorphism, age, gender and pathological conditions (Gurley, 2012).

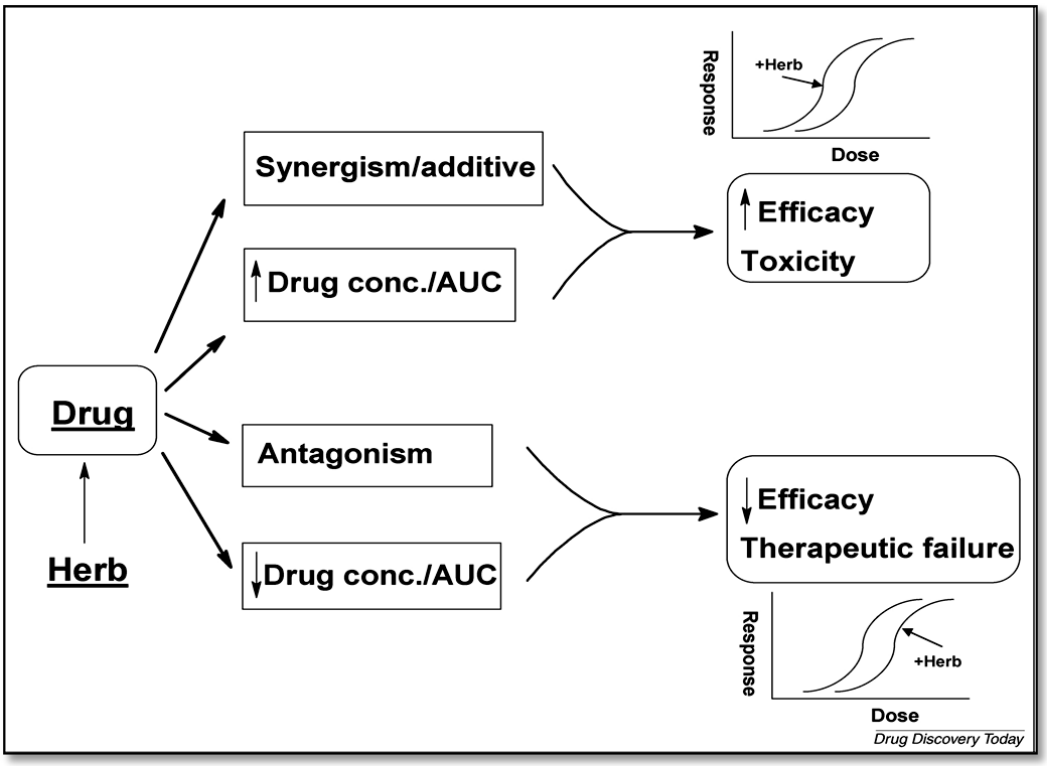
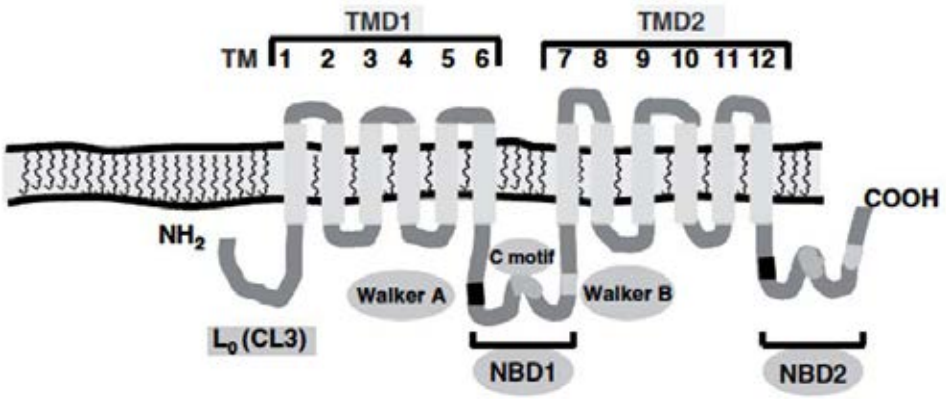


Figure 3. The possible clinical effects of drug-herb interaction (Zhou *et al.*, 2007).

A



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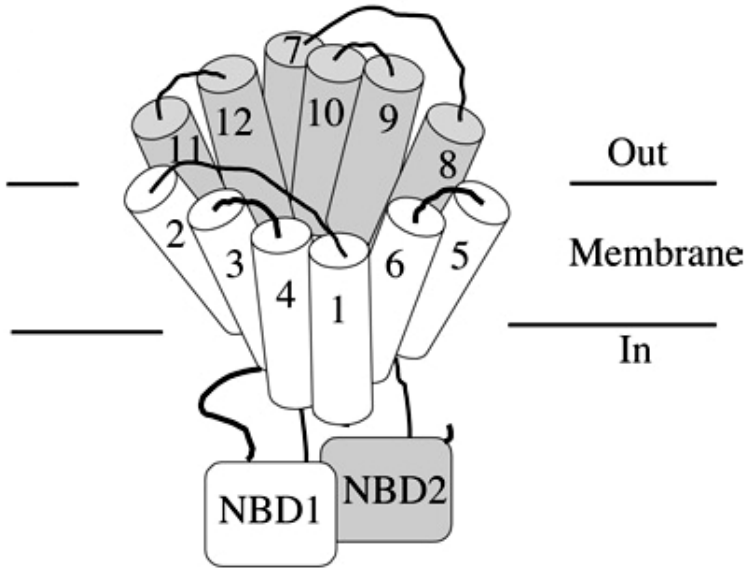


Figure 4. The schematic picture of Pgp structure. **(A)** The secondary structure of Pgp as embedded in the plasma membrane, consists of 12 transmembrane domains (TMD1 and TMD2), with each half of the molecule containing a nucleotide binding domain (NBD) and reveals six predicted and highly hydrophobic transmembrane domains (TMDs) (Zhou, 2008). **(B)** The 2 dimensional structure of Pgp. The two homologous domains (grey and white color) of Pgp which consist of transmembrane domains (TMDs) and nucleotide binding domains (NBDs) (Loo *et al.*, 2006).

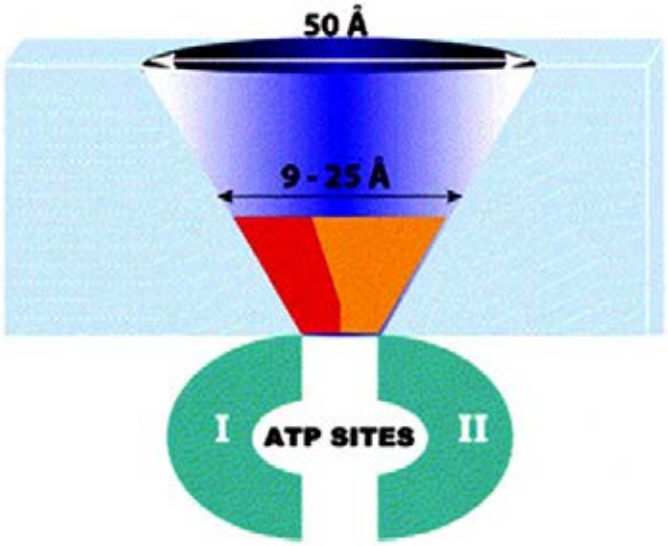


Figure 5. The schematic picture showing drug-substrate binding site of Pgp. The cross-section schematic picture shows the approximate dimensions and drug-substrate binding pocket and central pore of Pgp. The drug-substrate binding pocket consists of two size of funnel shapes, the narrow (9-25A°) funnel shape and the wide (approximately 50 A°) funnel shape. The narrow size is a high affinity site for substrate binding which may have two sites for simultaneously binding of substrates as shown in red and orange color. On the other hand, the wide size is a low affinity site to release substrate out of cell (Ambudkar *et al.*, 2006; Hennessy and Spiers, 2007).

2.2. Permeability-glycoprotein, Pgp

Permeability-glycoprotein or P-glycoprotein (Pgp) is an apical transmembrane protein, encoded from human multidrug resistance 1 or *MDR1* gene in human or *Mdr1a* and *Mdr1b* in mouse, as a transporting Pgp isoform. The Pgp is found at apical or luminal membrane of excretory function tissues, such as liver, kidney and adrenal gland, and barrier function tissues, such as intestine, blood-brain barrier, placenta, blood-testis and blood-ovarian barrier (Balayssac *et al.*, 2005; Marchetti *et al.*, 2007). Pgp is a member of ATP binding cassette (ABC) transporter proteins, subclass ABCB1, it can efflux xenobiotics out of cell by using ATP energy from ATP hydrolysis (Ambudkar *et al.*, 2006). From the cellular locations and functions of Pgp suggested that Pgp has significant effect on pharmacokinetic parameters of drugs.

2.2.1. Structure of Pgp

The Pgp is a single chain, N-glycosylated protein and consists of 1,280 amino acid residues with 12 transmembrane domains (170 kDa). The structure of Pgp consists of 2 homologous halves, each containing 6 transmembrane domins (TMDs) and nucleotide binding domains (NBDs), separated by a highly charged flexible linker region which is phosphorylated at several sites by protein kinase C as shown in Figure 4 (Ambudkar *et al.*, 2006; Zhou, 2008).

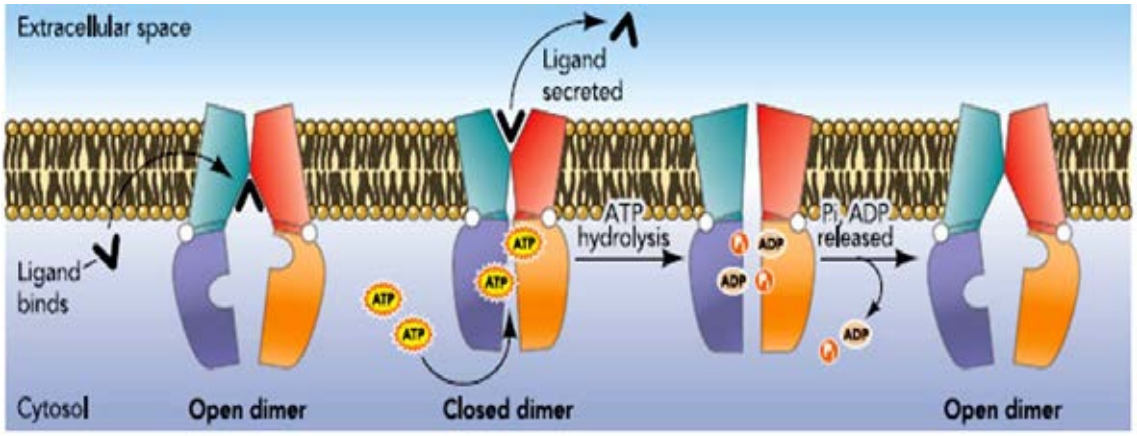
The NBDs are located in intracellular site and composed of two core consensus motifs which are the Walker A and B motifs and the S signature of ABC transporter as shown in Figure 4A. All of these motifs are usually contained a wide range of ATPase, and are directly involved in the binding and hydrolysis of nucleotide (Ambudkar *et al.*, 2006; Zhou, 2008).

Both NBDs are important for suitable Pgp function which can change the conformation of substrate binding site of Pgp to efflux substrate out of cell. The Pgp activity is depended on the presence of ATP, thus, the NBDs acts as ATPase enzyme which changes the ATP to ADP and provides the energy for Pgp to pump substrates out of cells across membrane. ATP has to bind to both sites to allow activity of the entired Pgp protein, but the hydrolysis of both bound ATP molecules is uncertain necessary to produce this activity (Zhou, 2008). The ATP binding, rather than ATP hydrolysis, induces a conformational change in the tertiary Pgp structure. Although ATP hydrolysis drives the transport process, the substrates binding to Pgp are also due to ATP binding rather than hydrolysis. Therefore, ATP binding seems to drive the conformational change which can reduce substrate binding affinity and expose the substrate binding site to the extracellular milieu to efflux substrate out of cell; ATP hydrolysis might, therefore, simply ‘reset’ the transporter molecule (Hennessy and Spiers, 2007; Zhou, 2008).

The TMDs are highly hydrophobic part in Pgp structure, they are the substrate binding site which drug or substrate molecules bind and cross the membrane (Zhou, 2008). The substrate binding sites within the TMDs of Pgp are anywhere from two to at least four sites (van der Heide and Poolman, 2002). In addition, several research groups have shown that two different substrates can bind to Pgp at the same time. Since, the substrate binding sites may overlap or be allosterically coupled,

leading to the possibility that there is only a single common site. Moreover, the binding site can be classified as transport and modulating site which can switch between high and low affinity state to adjust substrates or inhibitors. The substrate binding site is closely associated with transmembrane domains 1, 4, 5, 6, 9, 10, 11 and 12 by thiol cross-linking which is the key feature of the Pgp binding site for substrate or modulator. Mapping of the drug-binding pocket using thiol specific cross-linker with spacer arms, in conjunction with Pgp mutants (cysteine residues introduced), points towards the central pore being funnel shaped narrow at the cytosolic side, at least 0.9-2.5 nm wide in the middle and wider again at its extracellular surface, and is accessible to water as shown in Figure 5. Pgp-substrates get in binding pocket after partitioning into plasma membrane through gate which formed by transmembrane domine 5 and 8 on one site of the transporter and domains 2 and 11 on the opposite side. Furthermore, the binding of the substrate causes a conformational change in the substrate binding pocket and alters the cross-linking pattern between transmembrane domains 5/8 on one side of the transporter and domain 2/11 on the opposite side, which may represent closing of the gate (Hennessy and Spiers, 2007).

A



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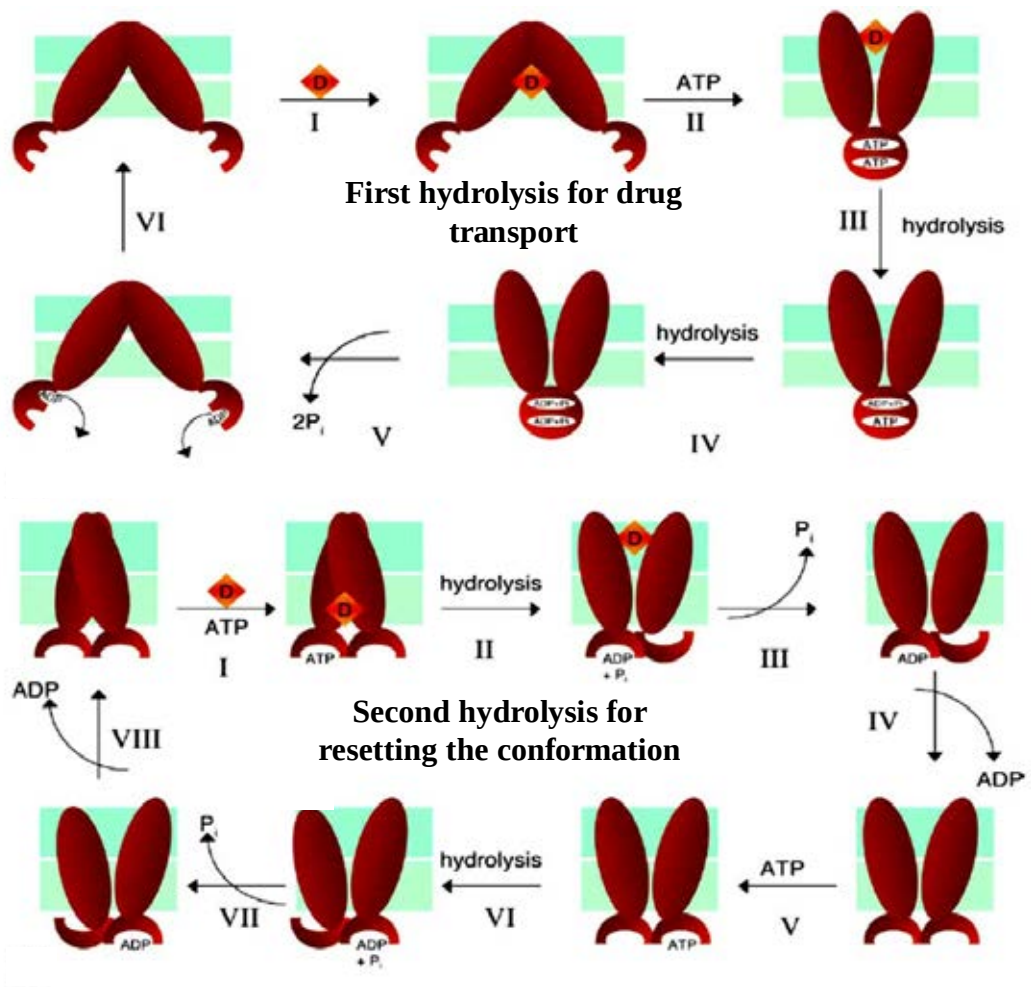


Figure 6. The Pgp mechanism models. (A) The ATP-switch model (Linton, 2007). (B) ATP hydrolysis driven efflux pump model (Ambudkar *et al.*, 2006).

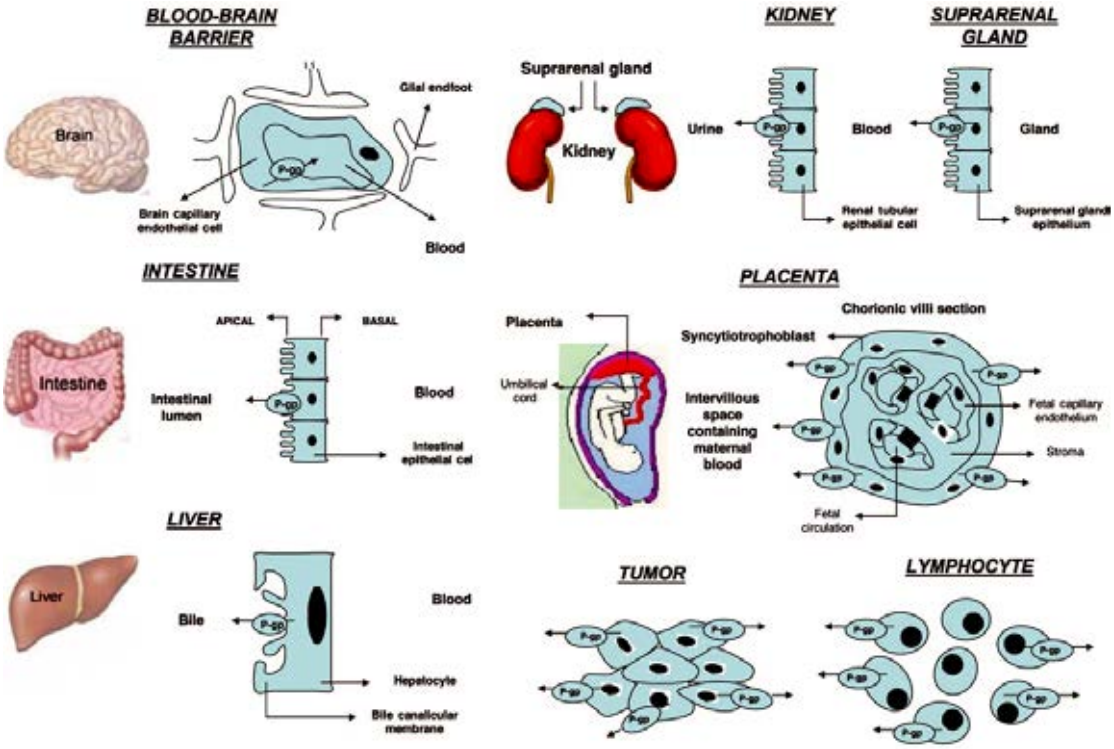


Figure 7. Schematic picture of the major Pgp localizations in the body (Marchetti *et al.*, 2007).

2.2.2. Mechanisms and functions of Pgp

Pgp action acts an active transporter by using energy (ATP) from ATP hydrolysis. The Pgp transport mechanisms could be explained by two models: the ATP-switch model and ATP hydrolysis driven efflux pump model (Ambudkar *et al.*, 2006; Linton, 2007). In the first model, ATP-switch model starts when substrate binds to substrate binding site in TMDs and induces the nucleotide binding site in NBDs to have high affinity to ATP. Then, the binding of two molecules of ATP to NBDs lead to change the TMDs conformation to low affinity for substrate which can pump substrate out of the cell. After that, the ATP hydrolysis occurs in NBDs in order to change the conformation of TMDs to the resting state. After hydrolysis, ADP and Pi are released from NBDs lead to the conformation of NBDs and TMDs change back to be ready to function again as shown in Figure 6A (Linton, 2007).

The second model is the ATP hydrolysis driven efflux pump model as shown in Figure 6B. First, substrate and ATP bind to Pgp without energetic requirement. The prior binding of ATP is not essential for substrate interaction with Pgp. Therefore, ATP binding could go forward, follow or together with the binding of substrate. The hydrolysis of ATP is accompanied by large conformational change that extremely reduces the affinity of both substrate and nucleotide. Following hydrolysis ATP to ADP, the Pgp becomes conformational change to allow the binding of nucleotide, but not substrate. A second ATP hydrolysis is initiated which is kinetically indistinguishable from the first. The subsequent ADP-release completes one catalytic cycle, bringing the Pgp molecule back to the original state where it can bind both substrate and nucleotide to initiate the next cycle (Ambudkar *et al.*, 2006).

The main physiological functions of Pgp are detoxification and protection of the body and the specific cell types from toxic xenobiotics and metabolites by excreting them out of cells or body into bile, urine and intestinal lumen. Therefore, Pgp is found mostly in intestine, blood-brain barrier, kidney, liver, adrenal gland, placenta, blood-testis and blood-ovarian barriers as shown in Figure 7 (Marchetti *et al.*, 2007; Zhou, 2008).

2.2.3. Substrates and modulators of Pgp

Owing to the nature of Pgp as an efflux pump for cell protection, a variety of substances or xenobiotics, which are Pgp substrates, are vary greatly in sizes and structures, ranging from small molecules, such as organic cations, carbohydrates, amino acids, and some antibiotics to macromolecules such as polysaccharides and proteins (Zhou, 2008) as shown in Table 2.

The main structure of substrates is consisted of hydrogen bonding potential and the presence of an amine. Molecular weight, size, surface area and the presence of aromatic ring structure are also important determinants of substrate binding and functionality. Most of Pgp-substrates hold two or three electron-donor groups with a fixed spatial separation of 2.5 and 4.6 A°, respectively, since an increased number of these elements increase the affinity for substrate binding. Accordingly, there are a high percentage of amino acids with hydrogen bonding donor side-chains in the transmembrane sequences of Pgp which are responsible for substrate recognition.

Some physicochemical characteristic features such as lipophilicity, hydrogen-bonding ability, molecular weight, and surface area are contributed to the ability of drug binding of Pgp. Such chemical characteristics are lipophilicity with a log-*P* value of 2.92 or higher, an 18-atom-long or longer molecular axis, a high energy of the highest occupied orbital value and the presence of at least one tertiary basic nitrogen atom. However, a small number of Pgp-substrates are large molecules greater than 3,000 Dalton in size and contain organic cationic property at physiological pH (Hennessy and Spiers, 2007; Zhou, 2008).

The modulators of Pgp have two types which are inhibitors and inducers, examples are shown in Table 2. Some Pgp-substrates are able to inhibit Pgp-mediated transport of other substrates. Most of Pgp inhibitors participate some common chemical features, such as aromatic ring structures, a tertiary or secondary amino groups and high lipophilicity, therefore, Pgp substrates as a whole have varying classes of inhibitory action. The main ways in which an Pgp inhibitor can exert its activity are either by being a very high-affinity substrate for Pgp and binding non-competitively, thus, it does not allow other substrate to bind, or by being efficient inhibitors of ATP hydrolysis either at the ATP binding site or by inhibiting protein kinase C which is involved with ATP coupling to Pgp. For example, verapamil is the first Pgp inhibitor which can block Pgp activity by competing for substrate binding sites, as a competitive inhibitor (Hennessy and Spiers, 2007; Marchetti *et al.*, 2007; Zhou, 2008).

Inducers of Pgp are agents that can induce the Pgp expression. The Pgp expression is regulated by nuclear receptor, like the pregnane X receptor (PXR), constitutive androstane receptor, and vitamin D binding receptor. The Pgp inducers consist of many drugs including phenytonin, ritonavir, nelfinavir, rifampicin and the herbal antidepressant St John's wort. However, Pgp is induced not only by a chemical compounds, but also by physical stress, such as X-irradiation, ultraviolet light irradiation, and heat shock (Marchetti *et al.*, 2007; Zhou, 2008).

Table 2. Examples of the Pgp-substrates, Pgp-inhibitors and Pgp-inducers. They were modified from Hennessy and Spiers in 2007, Sharom in 1997 and Zhou in 2008 (Hennessy and Spiers, 2007; Sharom, 1997; Tornio *et al.*, 2012; Zhou, 2008)

Substrates of Pgp	Inducers of Pgp	Inhibitors of Pgp
Doxorubicin	Amiodarone	Cyclosporin A
Daunorubisin	Cisplatine	Carvedilol
Glibenclamide	Daunorubicin	Diltiazem
Glyburide	Nelfinavir	Erythromycin
Metformin	Phenytonin	Verapamil
Rhodamine 123	Ritonavir	Tamoxifen
Rosiglitazone	Rifampicin	Midazole
Hoechst 33342	St John's wort	
Vinblastine		
Vincristine		

2.2.4. How to study of Pgp function *in vitro*.

Cell culture is used as a model to study various biological activities *in vitro*. Interestingly, the cell model used to study Pgp function selected from epithelial tissues which found high Pgp levels such as kidney (*i.e.*, porcine renal epithelial cell; LLC-PK1), intestine (*i.e.*, epithelial colorectal adenocarcinoma cell; CaCo-2) and blood-brain barrier (*i.e.*, rat brain microvessel endothelial cell; RBE4) (Hockmann, 2001; Marchetti *et al.*, 2007). Moreover, the Pgp-overexpression or drug-resistant cells were used to compare with the wild type cell in the *in vitro* study (Zhang *et al.*, 2003).

Since Pgp can efflux substrate out of cell by using energy from ATP hydrolysis (Ambudkar *et al.*, 2006), the *in vitro* methods for determining Pgp function can be divided into four groups; Chemosensitization assay or Cytotoxicity assay, Accumulation and efflux assay, Transport assay and ATPase assay (Gottesman and Pastan, 1993; Zhang *et al.*, 2003). In the first assay, Chemosensitization assay or cytoxicity assay is evaluated by comparing the ability to sensitize cell to cytotoxic

effect of cytotoxic Pgp-substrates, such as vinblastine, doxorubicin and daunorubicin (Gottesman and Pastan, 1993). For the second assay, Accumulation and efflux assay are used to determine the level of fluorescent or radiolabeled Pgp-substrate, *e.g.*, Rh 123, calcein-AM, intracellular or extracellular. Third assay, Transport assay is determination of transporting the compound of interest as Pgp-substrate, from the apical to basolateral side of a confluent monolayer of polarized epithelial cells. The last assay, ATPase assay is determination of Pgp-ATPase activity for ATP hydrolysis in nucleotide binding site of Pgp (Zhang *et al.*, 2003).

In this study, three assays were chosen to evaluate the Pgp function, which are Cytotoxicity (VBL co-treatment) assay, Rh 123 accumulation assay and ATPase assay. Since *C. fenestratum* extract contained several biological active compounds, the transport assay is not suitable.

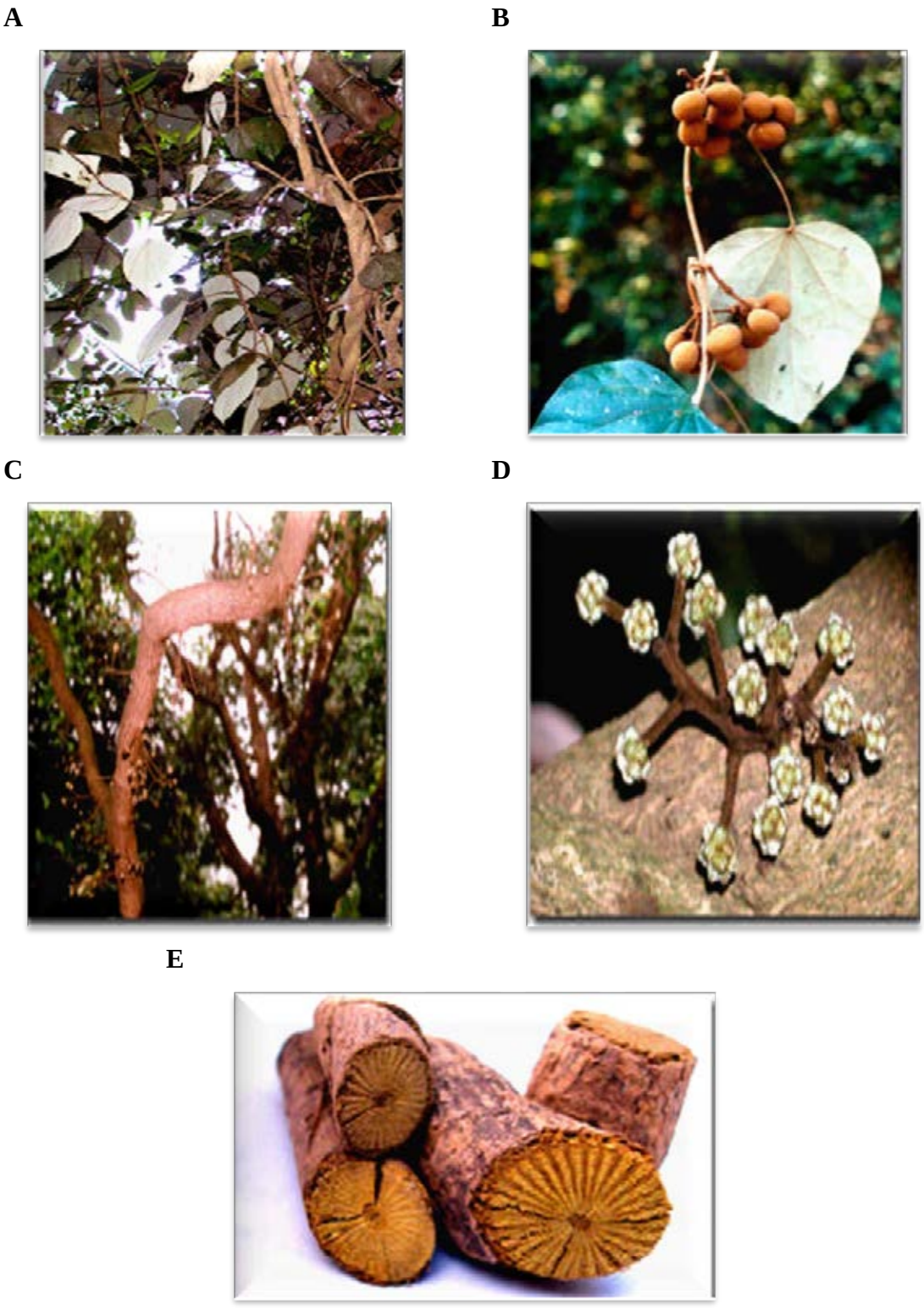


Figure 8. The photographs of *C. fenestratum* morphology. The pictures are habit (A), fruiting twig (B), male inflorescences flower arising from stem (C), female inflorescences flower arising from stem (D) and raw dried stem pieces (E) (Tushar *et al.*, 2008).

2.3. *Coscinium fenestratum* (Gaertn.) Colebr.

Coscimium fenestratum (Gaertn.) Colebr. (*C. fenestratum*) is a medicinal plant belonging to family Menispermaceae. It is called "Ham" or "Khamin khruea" in Thai name (Rojsanga and Gritsanapan, 2005; Rungsimakan, 2001). The Khamin khruea were used in Thai name in 4 species which consist of *Arcangelisia flava* (L.) Merr, *Coscimium fenestratum* (Gaertn.) Colebr., *Fibraurea tinctoria* Lour. and *Combretum latifolium* Blume. All of these species could characterized by the detail pharmacognostic properties and they contained significant differences of TLC patterns (Rungsimakan, 2001). Therefore, TLC patterns can be used to identify the species of interest, *C. fenestratum*, among these 4 species.

C. fenestratum extract is a traditional medicine of the North and Northeastern parts of Thailand, India, Sri Lanka, Cambodia and Vietnam. It is a dioeciously, large and woody climber. It grows well in humus rich soil having food drainage and areas having more than 2,000 mm. rainfalls with an annual mean temperature of 27 °C. The slow growing climber takes 15 years to reach its reproductive stage (Tushar *et al.*, 2008).

The Figure 8 showed the morphology of *C. fenestratum*, a large dioeciously climber up to 10 m. long which is yellow wood and sap. The stem and root slices are hard and woody. The external wood is yellowish-brown color and the internal one is yellow color. Leaves are alternate and exstipulate. Leaves shape are usually broadly ovate or ovate, truncate or shallowly cordate at base, acuminate at apex approximately size about 10-13 x 8-22 cm. Leaves have glabrescent above and hoary yellowish-white tomentellous beneath. Inflorescence globose heads on 1-3 cm. long peduncles, of 5-11 cm long racemes, 6-7 mm across, supra-axillary or on old leafless stems; bracts subulate, villous, closely pressed on the calyx, those of peduncles small, 4-5 mm long. Flowers are unisexual, small, yellowish or whitish; sepals 9, in 3 whorls, imbricate, densely sericeous-pilose; petals absent. Male flowers are sessile or shortly pedicellate, 1-mm long. Their sepals are broadly elliptic to obovate, the inner 3-6 spreading, yellow, and 1.5-2 mm long and the outermost is smaller 1-1.5 mm long, inserted lower. The stamens are 6, 1-mm long. Female flowers are 3-6 free, subglobose, carpels with slender subulate recurve or filiform styles, staminodes 6. Drupes are subglobose, tomentellous, brown to orange or yellowish, 2.8-3 cm

diameter, pericap drying woody, 1-mm thick, endocarp bony, 2.2-2.5 cm diameter, wall 3-mm thick covered with anastomosing fibrous ridges; condyle deeply intrusive, thickly clavate. Seeds are whitish, subglobose and enveloping the condyle (Dechwisissakul *et al.*, 2002; Tushar *et al.*, 2008).

C. fenestratum is widely used in traditional medicine for the treatment of many diseases, such as high blood cholesterol, hyperglycemia and hypertension (Rojsanga and Gritsanapan, 2005; Wongcome *et al.*, 2007). Nowadays, *C. fenestratum* is manufactured as many commercial products in the market, and widely used for co-admistration with other prescribed medicines. There is a concern about lacking information about drug and *C. fenestratum* interaction that may cause undesirable effects.

The chemical compounds found in the stem and root of *C. fenestratum* are berberine, oxy-berberine, palmatine, tetrahydropalmatine, crebanine and jatrorrhizine which are isoquinoline alkaloids. Berberine is a major compound found in the highest quantity in *C. fenestratum* (Malhotra *et al.*, 1989; Rojsanga and Gritsanapan, 2005; Tushar *et al.*, 2008). Interestingly, the common structure of berberine and palmatine are planar aromatic ring (Figure 9) which are found in most Pgp inhibitors (Shitan *et al.*, 2007). Consistently, it was reported that both berberine and palmatine were Pgp-substrates (Pan *et al.*, 2002; Suzuki *et al.*, 2010). Therefore, *C. fenestratum* may has an effect on Pgp and cause drug-herb interaction when co-administration with other Pgp-substrate drugs.

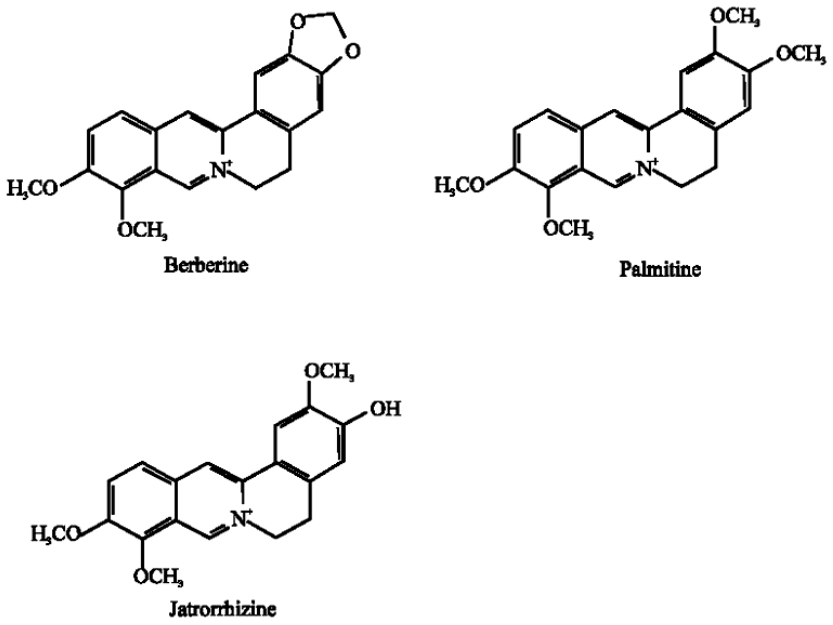


Figure 9. The structure of berberine, plamitine and jatrorrhizine. All of these are compounds found in *C. fenestratum* (Tushar *et al.*, 2008).

2.4. Berberine

Berberine (5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxole[5,6-d-a] quinolizinium) is an isoquinoline alkaloid which is a major compound found in *C. fenestratum* (Rojsanga and Gritsanapan, 2005). It has various pharmacological activities, such as antimicrobial activity against bacteria, fungi and viruses; antimalarials; anti-inflammatory activity; antihypertension; lower blood glucose level; reduce blood lipid level; and antiproliferative activity (Rudeewan *et al.*, 2011).

However, there are limited reports of berberine effect on Pgp function. Whether it is an inducer or inhibitor of Pgp were debatable. According to previous studies, berberine decreased Rh 123 accumulation in oral cancer cell lines (OC2 and KB cells) and increased Paclitaxel-resistant cancer cell lines, such as oral cancer (OC2), human gastric carcinoma (SC-M1) and human colon cancer cell line (COLO 205) (Lin *et al.*, 1999a; Lin *et al.*, 1999b). However, berberine was reported to inhibit rat intestinal Pgp which lead to increase bioavailability of digoxin and cyclosporine A (Qiu *et al.*, 2009). Besides, berberine could increase Rh 123 accumulation in bovine brain capillary endothelia cells (BCEC) (He and Liu, 2002). In addition, berberine and

palmatine, the compounds found in *C. fenestratum* extract, slightly up-regulated the mRNA of *Mdr1a* and *Mdr1b*, but they had no effect on protein expression (Suzuki *et al.*, 2010).

CHAPTER III
MATERIALS AND METHODS

3.1. Materials

C. fenestratum stems were purchased from Thai traditional drug store Ran-Khay-Ya-Chao-Krom-Poe in January 2007, Bangkok, Thailand. Porcine renal epithelial (LLC-PK1) cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *MDR_1* gene-transfected epithelial cells (LLC-MDR1) were a gift from Dr. A. H. Schinkel (Netherlands Cancer Institute, Amsterdam, the Netherlands). Medium 199 (M199) and penicillin-streptomycin were purchased from Gibco (Carlsbad, CA, USA). Fetal bovine serum was purchased from Hyclone (Cramlington, UK). Berberine standard, dimethyl sulfoxide (DMSO), DL-dithiothreitol (DTT), ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), methyl thiazolyl tetrazolium (MTT), phenyl-methyl-sulfonyl fluoride (PMSF), rhodamine 123 (Rh 123), sodium orthovanadate, trypsin, verapamil hydrochloride, vinblastine sulfate (VBL) and ouabain octahydrate (ouabine) were purchased from Sigma Chemical Inc. (St. Louis, MO, USA). Butanol, sodium hydroxide and titriplex III were purchased from Ajax Finechem (Taren Point, Australia). Adenosine 5'-triphosphate disodium salt (ATP), glacial acetic acid and silica gel 60 F_{254} plates (20 x 10 cm, 0.2 mm thickness) were purchased from Merck (Darmstadt, Germany). Ammonium persulfate (APS), nitrocellulose membrane, precision plus protein^{TM} standards, sodium dodecyl sulfate (SDS), and skimmed milk were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Rabbit polyclonal P-glycoprotein antibody, rabbit polyclonal β-Actin antibody and HRP-conjugated rabbit IgG secondary antibody were purchased from Abcam (Cambridge, MA, USA). The enhanced chemiluminescence Western blotting detection reagent was purchased from Thermo Scientific Pierce Protein Research Products (Rockford, IL, USA). All other chemicals used were commercially available reagents or analytical reagent quality.

3.2. Cell culture

Porcine renal epithelial (LLC-PK1) cells, *MDR$_1$* gene-transfected epithelial cells (LLC-MDR1) and VBL-induced Pgp overexpressed LLC-MDR1 (LLC-VBL) cells were cultured in M199 containing 2.2 g/L sodium bicarbonate plus 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 µg/ml) in a humidified incubator with the atmosphere of 95% air and 5% CO_2 at 37 °C. VBL at the concentration of 1.1 pM which is non-toxic VBL concentration at 90 % cell viability was added only for LLC-VBL cells. Cells that reached about 70 – 80 % confluency were harvested and plated for either subsequent passages or experiments (Batrakova *et al.*, 2001).

3.3. Preparation of *C. fenestratum* extract

C. fenestratum stems were dried overnight at 60 °C, ground and passed through a sieve No. 40 to obtain *C. fenestratum* powder. Then, 30 g of *C. fenestratum* powder were macerated with 80% ethanol everyday for 5 days. Each crude extract was filtered through Whatman filter paper No. 1 and combined together. Finally, the combined extract was dried in rotary evaporator (Rojsanga *et al.*, 2006).

3.4. Identification of *C. fenestratum* extract and quantification of berberine content by TLC-densitometer

C. fenestratum was identified by One Stop Service Center from Department of Medical Sciences, Ministry of Public Health and extract with 80% ethanol to yield authentic *C. fenestratum* extract.

C. fenestratum stems purchased from the Thai traditional drug store were confirmed for their identity by comparison of TLC fingerprint with the authentic *C. fenestratum* extract. Berberine, the major compound in *C. fenestratum* extract, was quantified by TLC-densitometer.

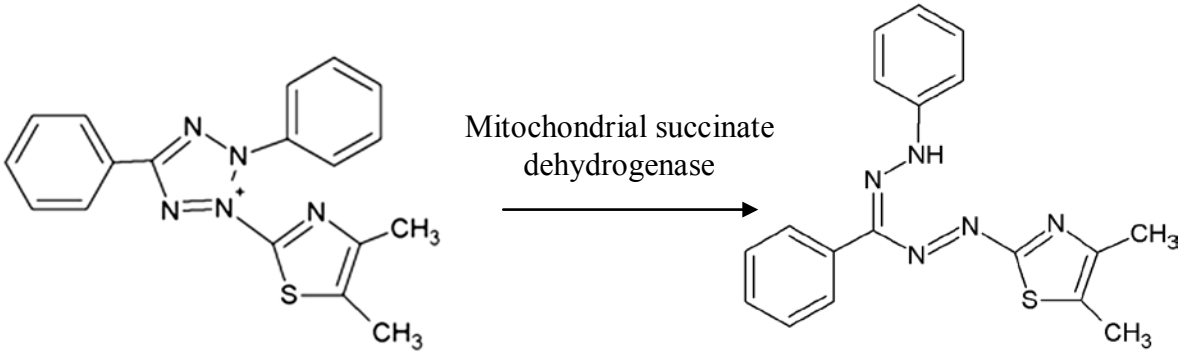
Chromatography was performed on silica gel 60 F_{254} plates with a 100 µl Camag syringe by application device Linomat IV. Development of the plates was carried out in saturated solvent system, butanol: glacial acetic acid: water (14:3:4, v/v/v). Total volume of solvent mixture was 30 ml and the migration distance was 80 mm. Chromatograms

obtained from Camag TLC Scanner III were evaluated *via* peak area with winCATS 1.3.5 software. The absorbance mode at 350 nm with a scanning speed of 20 mm/s using a slit diamension of 6 x 0.30 mm was used in this software. TLC fingerprint was examined under ultraviolet light 365 nm (Rojsanga *et al.*, 2006).

The stock solution of berberine and extract were prepared in 80% ethanol. The concentrations of berberine were 10, 20, 40, 60 and 80 ng/spot (N=3) and spot volumes were 2 µl. This series of concentrations of solution were used for constructing the calibration curve of berberine. The 400 ng/spot of extract was used for quantifying the content of berberine. The 5 µg/spot of extract and the authentic *C. fenestratum* extract were used for identification.

3.5. Cytotoxic effects of *C. fenestratum* extract and berberine

The non-toxic concentrations of *C. fenestratum* extract and berberine were evaluated prior to be used in this study by MTT assay. This assay measured the reduction of MTT by mitochondrial succinate dehydrogenase only in metabolically active cells, thus, the level of activity is the determination of cell viability. Briefly, the yellow tetrazolium salt, MTT, entered the cells and passed into mitochondria where it was reduced to insoluble purple formazan product. The formazan crystals were solubilized with dimethyl sulfoxide (DMSO) and the concentration was measured using a microplate reader at 570 nm (Carmichael *et al.*, 1987).



Yellow tetrazolium salt, MTT Purple formazan product

Figure 10. The reaction of MTT in cell mitochondria (Stockert *et al.*, 2012).

The cytotoxic effect of *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1 and LLC-VBL cells were determined by MTT assay. All cell lines were incubated at concentration of 1 x 10^5 cells/ml in 96-well plates contained 100 µl of medium in humidified CO_2 incubator at 37 °C (5% CO_2 atmosphere) for 24 hr. After 24 hr of incubation, the cells were treated in 200 µl of medium which consisted of berberine at various concentrations from 0.0001 to 100 µg/ml or *C. fenestratum* extract at concentrations from 50 to 500 µg/ml and incubated for 48 hr. The viable cells were determined by the MTT assay and compared with that of untreated cells. Briefly, after totally removal of tests – agent medium, 100 µl of MTT dye solution (0.4 mg/ml in medium, freshly prepared) was added to each well, and the plates were incubated in CO_2 incubator for 4 hr. Then, 100 µl of DMSO were added to each well and the plates were shaken for 15 min in the dark thoroughly to dissolve the dye crystals. Absorbance was measured at 570 nm by using a microplate reader. The percentage of cell viability was calculated. Dose-response curve was plotted between percentages of cell viability and the concentrations used in a log scale. The 50% inhibitory concentration (IC_{50}) was calculated using CurveExpert 1.4. The non-toxic concentrations were the maximum concentrations which all cells were still alive.

% Cell viability is calculated as following:

$$\% \text{ Cell viability} = \frac{A_{570} \text{ (Treatment – Blank)}}{A_{570} \text{ (Without treatment – Blank)}} \times 100$$

Where A_{570} = Absorbance at 570 nm

3.6. Evaluation of the effects of *C. fenestratum* stem extract and berberine on Pgp functions.

After determining the non-toxic concentrations of *C. fenestratum* extract and berberine in all cells: LLC-PK1, LLC-MDR1 and LLC-VBL cells, the effect of *C. fenestratum* extract and berberine on Pgp functions in these cells were investigated. VBL-cotreated cytotoxicity assay, Rh 123 accumulation assay and ATPase activity assay were used.

3.6.1. Effects of *C. fenestratum* stem extract and berberine on the cytotoxicity of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells.

VBL is a cytotoxic drug and also known as Pgp substrate. Therefore, Pgp function could be evaluated by comparing the ability to sensitize cells to the cytotoxic effects of VBL. The IC_{50} values of VBL-cotreated with verapamil (a known Pgp inhibitor, positive control), or *C. fenestratum* extract or berberine at the non-toxic concentrations in each cell type were evaluated.

LLC-PK1, LLC-MDR1 and LLC-VBL cells (1 x 10^5cells/ml) were seeded in 100 µl of media in 96-well plates and incubated in humidified CO_2 incubator at 37 °C for 24 hr. All cells were treated with 200 µl of media in presence or absence of 20 µM of verapamil, 1µg/ml of berberine or 100 µg/ml of *C. fenestratum* stem extract in combination with various concentrations of VBL ranging from 0.00128 to 200 ng/ml after 24-hr of incubation. Then, cells were continued to incubate in humidified CO_2 incubator at 37 °C for 48 hr. The cell viability was determined by MTT assay, as described previously in 3.5.

3.6.2. Effects of *C. fenestratum* stem extract and berberine on Rh 123 accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells.

Rh 123 is a fluorescence dye and a known Pgp substrate. The amount of intracellular accumulation of Rh 123 can be used to determine Pgp function of cells. The effect on the activity of Pgp was evaluated by comparing quantity of the intracellular accumulation of Rh 123 in co-treatment with verapamil (a known Pgp inhibitor, positive control), or berberine, or *C. fenestratum* stem extract.

All cells were incubated in 24-well plates ($1x10^5$ cells/ml) with 1 ml of medium in humidified CO_2 incubator at 37 °C (in 5% CO_2) for 7 days. Then, cells were washed 3 times with PBS. The cells were co-treated with 10 µM of Rh 123 and PBS (vehicle control), or 20 µM of verapamil (positive control), or 1 µg/ml of berberine, or 100 µg/ml of *C. fenestratum* extract and incubated in the dark at 37 °C for 2 hr. Then, cells were rinsed 3 times with ice-cold PBS to stop the accumulation and centrifuged at 1,500 rpm for 4 min at 4 °C. After that, cells were solubilized with 0.2 M NaOH for 1 hr. The concentration of Rh 123 was determined fluorometrically (excitation, 485 nm; emission, 535 nm) using microplate reader and calculated using Rh 123 standard curve. The protein content of the cells was measured by Bradford assay using bovine serum albumin as a standard. The concentration of Rh 123 per mg protein (mmole/mg protein) was used as the amount of Rh 123 accumulation (Quesada *et al.*, 1996; Yoshida *et al.*, 2005).

3.6.3. Eeffects of *C. fenestratum* stem extract and berberine on ATPase activity of Pgp in LLC-PK1, LLC-MDR1 and LLC-VBL cells.

Pgp is an ATP-dependent efflux transporter; it pumps substrates out of cell by using energy from ATP hydrolysis which can be described by following reaction:

$$ATP + H_2O \longrightarrow ADP + Pi + Energy$$

The amount of phosphate (Pi) released is proportional to the activity of Pgp and can be detected by colorimetric reaction as following reaction.

$$Pi + NH_4 \cdot 6Mo_7O_{24} + H^+ \longrightarrow (NH_4)_3PO_4 \cdot 12MoO_3 + 21\ NH_4^+ + 12\ H_2O$$

Ammonium molybdate was reacted with Pi in an acidic medium to form a phosphomolybdate complex. This complex is reduced to an intensely blue-colored complex by ascorbic acid which can be measured by using microplate reader at 820 nm. (Debruyne, 1982; Li-Blatter *et al.*, 2009; Litman *et al.*, 1997).

All cells were incubated in 100 mm-diameter culture dish (2.2×10^5 cells/ml) contained medium 13 ml in humidified CO_2 incubator at 37 °C (in 5% CO_2 atmosphere) for 7 days. Cells were rinsed with PBS and harvested by scraping in ice-cold PBS, pH 7, contained 1 mM of PMSF, a protease inhibitor, and centrifugation at 1,000 g for 10 min at 4 °C. Cells were rinsed with ice-cold buffer A (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM $MgCl_2$ and 1 mM PMSF) and centrifuged at 1,000 rpm for 10 min at 4 °C. The cell pellet was resuspended with 5 ml ice-cold buffer and sonicated in a steel beaker set in ice for three 30-s bursts with 30-s cooling periods between. Then, the cell extract was diluted 1:1 with ice-cold buffer B (10 mM Tris-HCl pH 7.4, 120 mM sucrose and 1 mM PMSF) followed by centrifugation at 800 g for 10 min at 4 °C to remove the unbroken cells and nuclei. Accordingly, mitochondria were removed by centrifugation at 6,000 g for 10 min at 4 °C. Finally, the crude membranes were pellet at 100,000 g for 1 hr at 4 °C. Then, the pellet was dissolved in buffer B and homogenized by repeatedly passing pellet through a 23-gauge syringe. Aliquots of the plasma membrane vesicles were stored at – 80 °C until use.

The detection of ATPase activity was started by incubating 10 µg of plasma membrane vesicle with ATP 3 mM and 20 µM of verapamil (positive control), or 1 µg/ml

of berberine, or 100 µg/ml of *C. fenestratum* extract in ice-cold phosphate release assay buffer (25 mM Tris-HCl pH 7, 50 mM KCl, 2.5 mM $MgSO_4$, 3 mM DTT, 0.5 mM EGTA; to inhibit Ca-ATPase, 2 mM ouabain; to inhibit the N/K-ATPase, and 3 mM sodium azide; to inhibit mitochondrial ATPase) at 37 °C for 1 hr in water bath. Then, the reaction was terminated by rapid cooling on ice. Adding freshly prepared the ice-cold solution contained 0.2% (w/v) ammonium molybdate, 1.43% (v/v) sulfuric acid, 1% (w/v) ascorbic acid and 0.9% (w/v) SDS to determine the inorganic phosphate (Pi) released and incubated for 30 min at room temperature. After incubation, the concentration of Pi was determined calorimetrically using microplate reader at 820 nm and calculated using Pi standard curve. In the assay, samples were incubated with 1 mM vanadate (Pgp ATPase inhibitor) in parallel.

Pgp ATPase activity (nmol Pi/mg protein/min) was calculated from the amount of Pi in the absence of vanadate minus that in the presence of vanadate (Aanismaa and Seelig, 2007).

3.7. Evaluation of the effects of *C. fenestratum* stem extract and berberine on Pgp expression.

After evaluating the effects of *C. fenestratum* stem extract and berberine on Pgp function, their effects on the expression of Pgp were investigated by Western blot analysis.

The Western blot analysis is an analytical technique for detecting specific proteins in a given sample of tissue homogenate or cell extract. Gel electrophoresis was separated the denatured proteins by molecular weight of the protein. After selecting protein by molecular weight, proteins were transferred to membrane; the target protein was detected using specific antibody.

All cells were incubated in 100-mm diameter culture dish (2.2×10^5 cells/ml) containing cultured medium 13 ml in humidified CO_2 incubator at 37 °C (in 5% CO_2 atmosphere) for 7 days. Then, cells were treated in the absence or presence of 100 µg/ml of *C. fenestratum* extract, or 1 µg/ml of berberine, and further incubated at 37 °C for 48 hr. After 48-hr incubation, cells rinsed with PBS and harvested by trypsinization, rinsed

with PBS twice and centrifuged at 1,500 rpm for 4 min at 4 °C. The cells were lysed with ice-cold RIPA lysis buffer (50 mM Tris pH 8, 100 mM NaCl, 10mM NaF, 1% sodium deoxycholate, 0.1% SDS, 2% Triton X-100, 1mM Na_3VO_4 and 1mM PMSF, freshly prepared RIPA for use) for 45 min on ice followed by centrifugation at 4,000 g for 10 min at 4^{o}C. The supernatants were ultracentrifuged at 41,000 rpm for 1-hr at 4 °C and the pellet was collected in Laemmle buffer (50 mM Tris pH 6.8, 2% SDS and 10% glycerol), freshly prepared before use. Protein concentration was determined using Lowry assay. Samples containing 20 μg of proteins were loaded and separated on 8% sodium dodecyl sulphate polyacrylaminde gel electrophoresis (SDS-PAGE) with a constant 100 V for about 1.5-hr. Protein bands were then transferred to nitrocellulose membranes with a constant 45 V overnight in ice box and blocked for 1-hr at room temperature with 5% skimmed milk in PBST buffer (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 and 0.1% tween 20), then, washed membranes for 5 min, three times with PBST. The membranes were immunoblotted with rabbit polyclonal Pgp antibody (1:200, 1^{o}Ab) in 5% skimmed milk PBST overnight at 4 ^{o}C. After that, membranes were washed and incubated with HRP-conjugated rabbit IgG secondary antibody (1:3,000) and rabbit polyclonal β-actin antibody (1:3,000) for 1-hr at room temperature and washed before detection. Protein bands were visualized using an enhanced chemiluminescence Western blot analysis system and exposed to film (Khantamat *et al.*, 2004; Kitada *et al.*, 2007).

The protein band intensities were determined using ImageJ 1.43 software (NIH, USA).

3.8. Data summation and statistical analysis

All results were expressed as means ± SEM from triplicate samples of at least three independent sets of experiments. Data shown in figures were from a representative set of experiment. Differences between the mean were analyzed by one-way analysis of variance and student t-test. Result were considered to be statistically significance when $P < 0.05$.

CHAPTER IV
RESULTS

4.1. Preparation of *C. fenestratum* extract

Thirty grams of *C. fenestratum* powder from dry stems were macerated with 80% ethanol. The totally macerated supernatants were collected everyday for 5 days and dried in a rotary evaporator. The dark-yellow viscous extract was obtained. The dried weight and percent yield of crude extract were 5.91 g and 19.7% (w/w), respectively. Our yield is comparable to that of previous studies. By using the same extraction method and TLC densitometry, Rojsanga's group reported the percent yield of crude extract as 18.41 ± 0.16 % (Rojsanga *et al.*, 2006). In addition, the study of berberine content variation in *C. fenestratum* stems in Thailand market showed that the percent yield of crude extract from 80% ethanol maceration were varied from 9.87 to 16.38 % (Rojsanga and Gritsanapan, 2005).

4.2. Identification of *C. fenestratum* extract and quantification of berberine content by TLC-densitometer

TLC analysis is a valuable method for identification and quality guarantee of herb. The *C. fenestratum* extract from this study was identified and confirmed by comparing the TLC fingerprint with that of the authentic *C. fenestratum* extract of the *C. fenestratum* stems identified by the Department of Medical Science, Ministry of Public Health. The content of berberine, a major compound in *C. fenestratum* extract, was determined by TLC-densitometer.

The pattern of *C. fenestratum* extract was observed under UV light at the 365 nm wavelength. This pattern contained 6 bands which were similar to those of the previous study (Rojsanga and Gritsanapan, 2005). Figure 11 showed the TLC fingerprints of *C. fenestratum* extract from this study compared with the authentic *C. fenestratum* extract. Our *C. fenestratum* extract had similar chromatographic profile as that of the authentic *C. fenestratum* extract. Therefore, our extract was identified as *Coscinium fenestratum* extract.

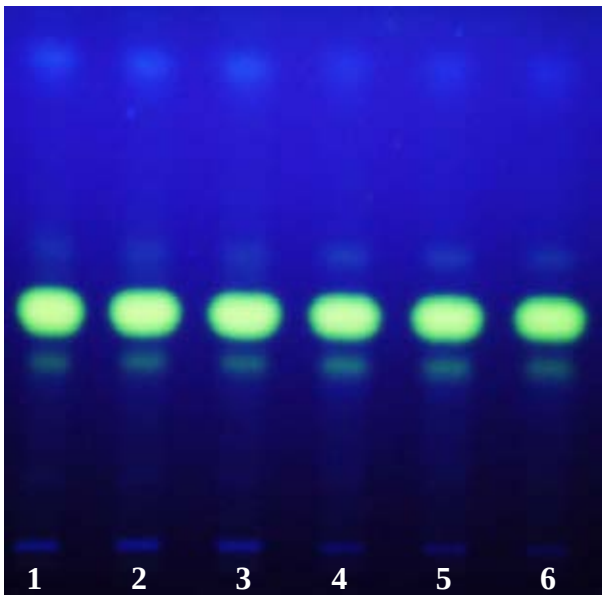
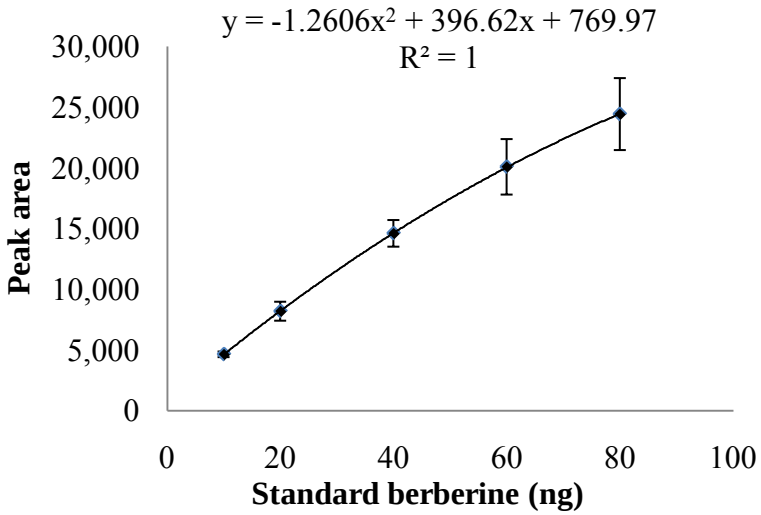


Figure 11. The TLC fingerprints of our *C. fenestratum* extract compared with that of the authentic *C. fenestratum* extract. They were visualized under UV light at 365 nm. Lanes 1, 2 and 3 were authentic *C. fenestratum* extract samples and 4, 5 and 6 were *C. fenestratum* extract samples from this study.

The berberine bands were well resolved from other components with R_f values of 0.45 ± 0.02 in a solvent system of butanol: glacial acetic acid: water (14:3:4, v/v/v). The polynomial regression data from the calibration curve of berberine shown in Figure 12A was used to quantify the berberine content in *C. fenestratum* extract. The calibration curve was plotted between the peak area and the berberine contents (ng/lane). The TLC fingerprints of berberine standard were compared with berberine found in 400 ng/lane of *C. fenestratum* extract as shown in Figure 12B. The berberine contents were calculated from the polynomial equation: $y = -1.2606x^2 + 396.62x + 769.97$; $R^2 = 1$. The quantity of berberine in 1 µg of *C. fenestratum* extract is 0.10 ± 0.002 µg or 10 ± 0.002 %w/w.

A



B

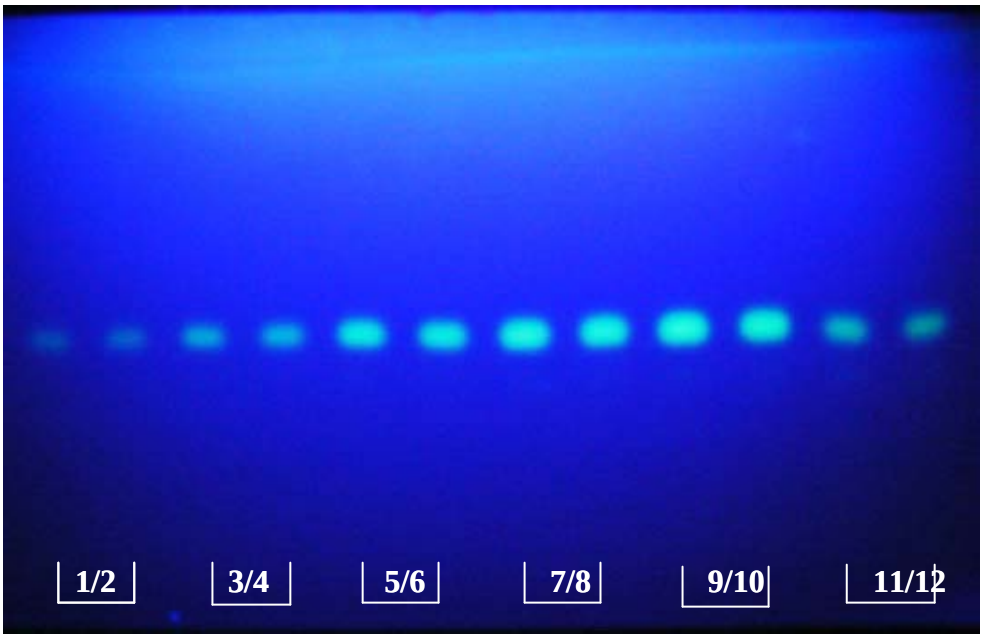


Figure 12. Berberine calibration curve (A) and TLC fingerprints of berberine standard and *C. fenestratum* extract (B) determined by TLC densitometry. TLC fingerprints were visualized under UV light at 365 nm. Lanes 1 to 10 were berberine standard at concentrations ranging from 10 to 80 ng/lane which each concentration consisted of 2 lanes. Lanes 11 and 12 were *C. fenestratum* extract samples from this study. Data presented as mean ± SEM with N = 3.

The berberine contents in *C. fenestratum* extract and R_f values were consistent with the previous studies using similar system (Rojsanga and Gritsanapan, 2005; Rojsanga *et al.*, 2006).

4.3. Baseline data of Pgp expression and Pgp function in LLC-PK1, LLC-MDR1 and LLC-VBL cells

The levels of Pgp in wild type cell (LLC-PK1), Pgp-overexpression cell (LLC-MDR1), and vinblastine (VBL)-induced Pgp-overexpression cell (LLC-VBL), were evaluated by Western blot analysis. In addition, the VBL cytotoxicity and Rh 123 accumulation were also compared among all these three cell types in order to measure the baseline of Pgp function.

Western blot analysis indicated that the LLC-MDR1 and LLC-VBL cells expressed Pgp at high levels, but there was undetectable low Pgp level in LLC-PK1 cells. Figure 13A and 13B showed the levels of Pgp in overexpression cells, LLC-MDR1 and LLC-VBL which were significantly higher than in wild type cell, LLC-PK1 for 4.11- and 4.60-fold when compared with wild type, respectively. Furthermore, the level of Pgp in LLC-VBL cells was slightly higher than in LLC-MDR1 cells but they were not significantly difference when compared using independent-samples t-test (Figure 13B).

In addition, accumulation of the Rh 123, a fluorescence dye substrate for Pgp, in LLC-PK1 cells was significantly higher than those of the other cells as shown in Figure 13C. However, the Rh 123 accumulation in LLC-MDR1 and LLC-VBL cells were not much different. They were significantly lower than in LLC-PK1 cells by 2.26- and 1.79-fold, respectively. Rh 123 can be pumped out of the cells by Pgp, therefore, the Pgp-overexpression cells cannot accumulate high Rh 123 levels (Quesada *et al.*, 1996).

Moreover, the toxicity of vinblastine (VBL) in all three cells are evaluated, the results were shown in Figure 14. VBL is a cytotoxic drug and known as a Pgp-substrate. Therefore, Pgp function was evaluated by comparing the ability of the sample to sensitize cells to the cytotoxic effect of VBL (Bruggemann *et al.*, 1992). To determine the cytotoxicity of VBL, IC_{50} values of VBL in all three cells were investigated. The viability of the cells was observed after exposure to VBL at various

concentrations ranging from 0.00128 to 200 ng/ml for 48 hr. Cytotoxicity was determined by MTT assay. Upon exposure to VBL, the cytotoxic effect of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells were shown in concentration-dependent manners as shown in Figure 14A with the IC_{50} values of 2.04 ± 0.15, 7.67 ± 1.18, and 13.71 ± 2.96 ng/ml, respectively (Figure 14B). This result is consistent with the previous study which the IC_{50} values of VBL in LLC-PK1 cells were 3.71 ng/ml (Booth-Genthe *et al.*, 2006).

Therefore, Pgp-overexpression cells, LLC-MDR1 and LLC-VBL; and wild type cell, LLC-PK1, were used to study the effect of *C. fenestratum* extract and berberine on Pgp functions and expression. The baseline data of Pgp functions and expression of these cells were summarized in Table 3.

Table 3. The baseline data of Pgp function and expression of LLC-PK1, LLC-MDR1 and LLC-VBL cells.

	Relative ratio to wild type cell; LLC-PK1		
	LLC-PK1	**LLC-MDR**	**LLC-VBL**
Pgp expression levels	1	4.11	4.60
Rh 123 accumulation	1	0.44	0.56
VBL toxicity (IC_{50} value ratio)	1	3.76	6.72

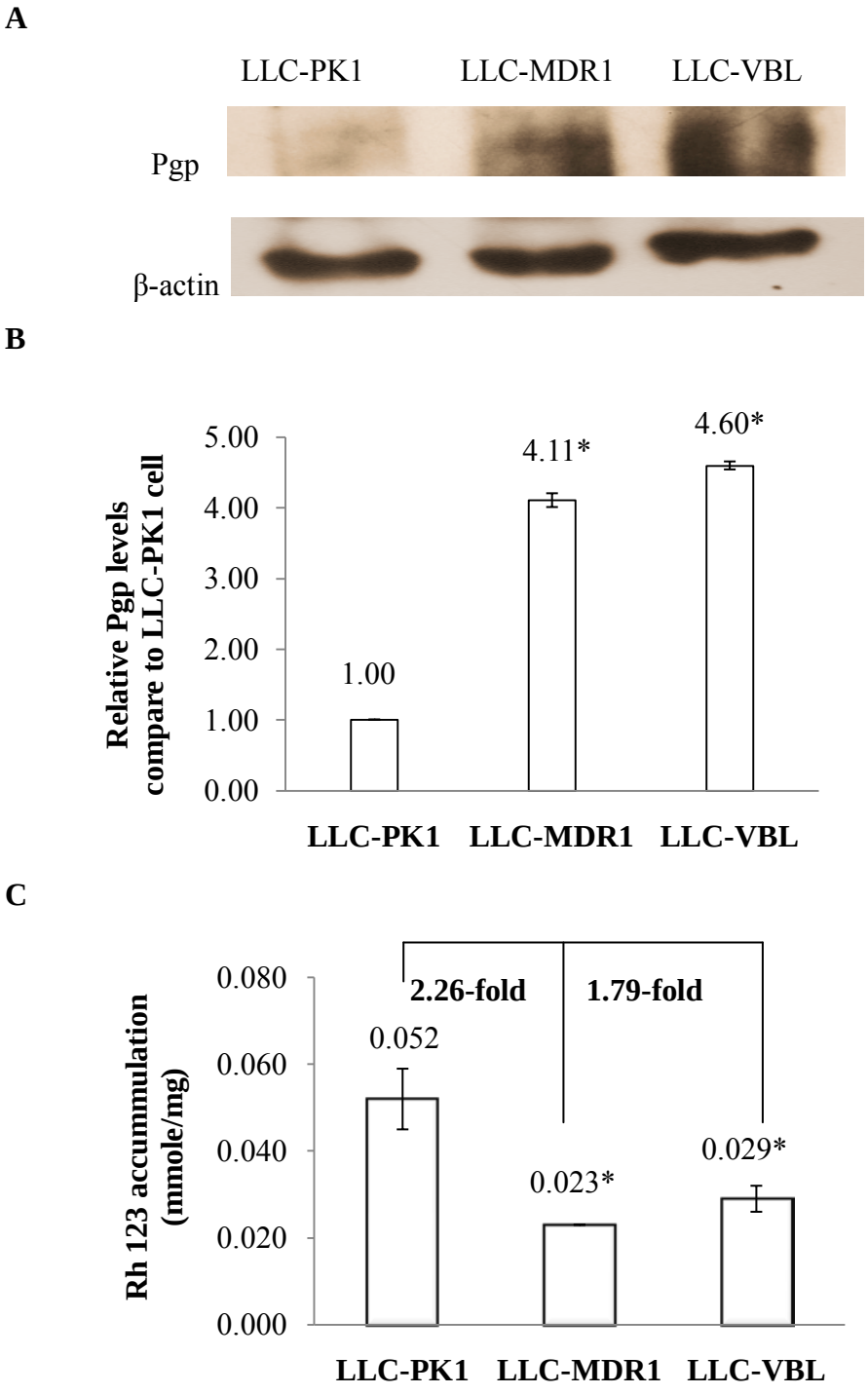


Figure 13. The correlation between Pgp expression levels and Rh 123 accumulations in LLC-PK1, LLC-MDR1 and LLC-VBL cells. **(A)** The Pgp expression levels and **(B)** the relative Pgp levels in LLC-PK1, LLC-MDR1 and LLC-VBL cells compared to LLC-PK1 cell. All three cells cultured in M199 media for 7 days. The Pgp levels were evaluated by Western blot analysis. **(C)** The Rh 123

accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells. (* = significantly different from Pgp levels or Rh 123 accumulation in wild type cell; LLC-PK1, $P<0.05$).

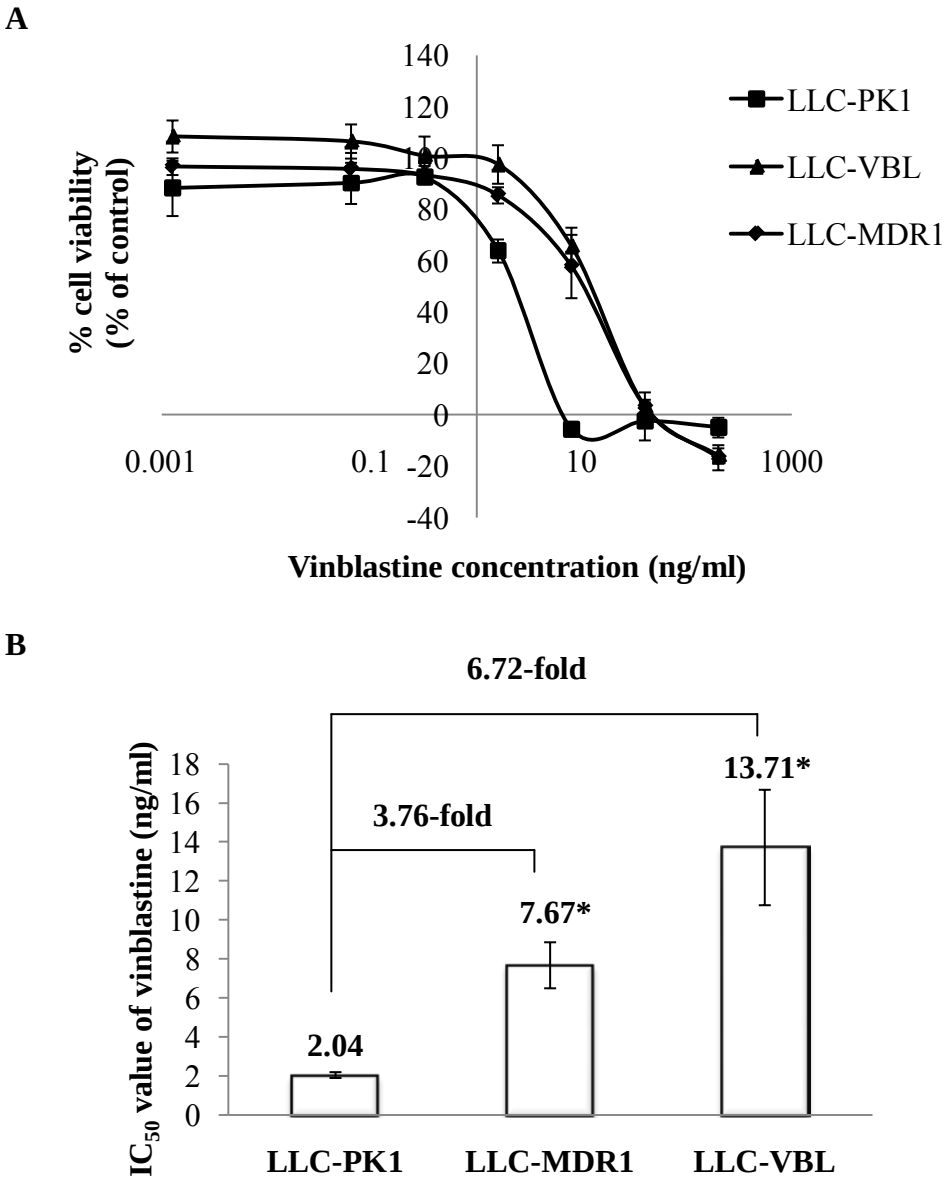


Figure 14. Cytotoxic effect of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells. **(A)** Concentration-response curve of VBL in cells incubated for 48 hr. Data were shown as percent cell viability compared to the vehicle control cells (0.5% DMSO) measured by MTT assay. **(B)** The IC_{50} values in all three cells. Each point presented as the mean ± SEM values for three independent experiments

performed in triplicate. (* = significantly different from VBL cytotoxicity in wild type; LLC-PK1 cells, $P<0.05$).

4.4. Cytotoxic effects of *C. fenestratum* stem extract and berberine

Prior to the study of the effect of *C. fenestratum* extract and berberine on Pgp functions and expression, the non-toxic concentrations were determined in LLC-PK1, LLC-MDR1 and LLC-VBL cells. The cells were exposed to various concentrations of *C. fenestratum* extract ranging from 50 to 500 µg/ml and of berberine ranging from 0.0001 to 100 µg/ml for 48 hr, and cytotoxicity was determined by MTT assay. The non-toxic concentrations were determined by using maximum concentrations that allowed cells to still being alive.

The effect of *C. fenestratum* extract (Figure 15A) and berberine (Figure 15B) on the viability of all three cells were decreased in concentration-dependent manners. The non-toxic concentrations of *C. fenestratum* extract and berberine determined from this study were 100 and 1 µg/ml, respectively. The 50% inhibitory concentration (IC_{50}) values of *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1 and LLC-VBL cells were shown in Table 4.

From Table 4, *C. fenestratum* extract had toxic effect with IC_{50} values higer in Pgp-overexpressed cells, LLC-MDR1 and LLC-VBL, than in wild type, LLC-PK1 cell. On the other hand, berberine had similar cytoxicity to all three cells.

There were no information on the cytotoxicity of *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1 and LLC-VBL cells reported elsewhere. From literature reviews, there were several articles reported about *C. fenestratum* extract and berberine toxicity in other cell lines. For example, the IC_{50} values of *C. fenestratum* extract in HL-60 (Acute promyelocytic leukemia cells) and L929 (mouse fibroblast cells) were reported as 120 ± 10 µg/ml and 100 µg/ml, respectively, from 24-hr incubation (Narasimhan and Nair, 2005; Tungpradit *et al.*, 2010). The IC_{50} values of berberine in NCI-H838 (Non-small cell lung adenocarcinoma cells), HL-60 (Acute promyelocytic leukemia cells), PBMC (Peripheral Blood Mononuclear Cells) and L929 (mouse fibroblast cells) were 92.4 ± 1.2 µM (31.86 µg/ml), 1.41 ± 0.7 µg/ml, 9.93 ± 5.4 µg/ml, and 130 µg/ml, respectively, from 24- to 72-hr incubation (Narasimhan and Nair, 2005; Rudeewan *et al.*, 2011; Tungpradit *et al.*, 2010).

According to those previous studies, the difference in the IC_{50} values of *C. fenestratum* extract and berberine depended on the cell types and incubation time.

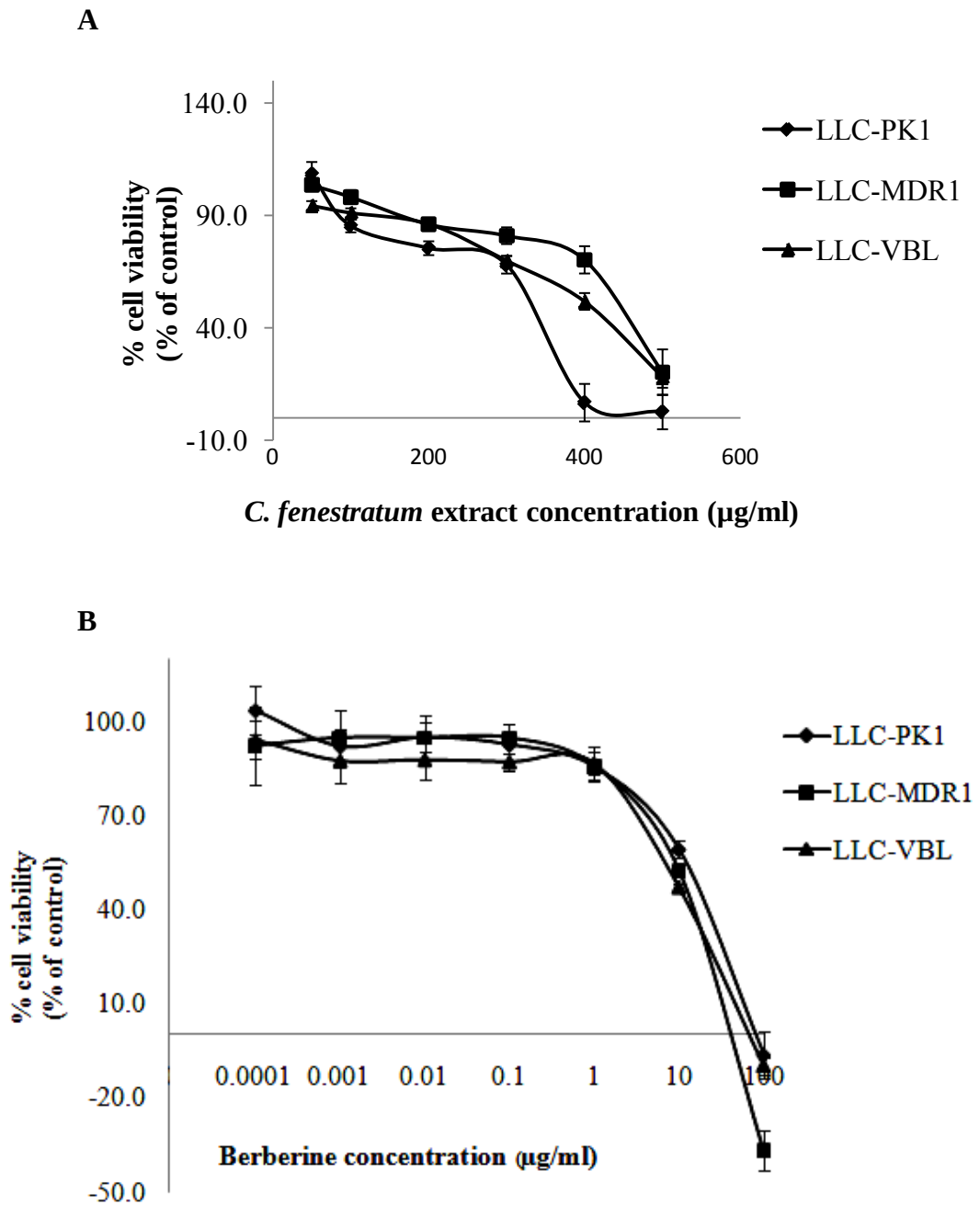


Figure 15. Cytotoxic effects of *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1, and LLC-VBL cells. All three cells were treated with *C. fenestratum* extract **(A)** or berberine **(B)** at various concentrations**.** The concentration-response curve of both samples in LLC-PK1, LLC-MDR1 and LLC-VBL cells

incubated for 48 hr were plotted. Data was shown as % cell viability comparing with vehicle control (0.5% DMSO) measured by MTT assay. Each point represented the mean value from three independent experiments performed in triplicate.

Table 4. The IC_{50} values for cytotoxic effect of *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1 and LLC-VBL cells. Data presented as mean ± SEM with N=3.

Treatment	IC_{50} values (µg/ml)		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
C. fenestratum extract	332.6 ± 5.1	453.5 ± 6.5	406.2 ± 25.3
Berberine	13 ± 1.7	9.3 ± 0.2	10.5 ± 0.3

4.5. Effects of *C. fenestratum* stem extract and berberine on Pgp functions

After the non-toxic concentrations of *C. fenestratum* extract and berberine were already obtained in the LLC-PK1, LLC-MDR1 and LLC-VBL cells, the effects of *C. fenestratum* extract and berberine on Pgp functions were investigated. Viblastine co-treated cytotoxicity assay, Rhodamine 123 accumulation assay and ATPase activity assay were used to measure these functions.

4.5.1. Effects of *C. fenestratum* stem extract and berberine on the cytotoxicity of vinblastine in LLC-PK1, LLC-MDR1 and LLC-VBL cells

In order to determine whether *C. fenestratum* extract and berberine can modify the Pgp function or not, non-toxic concentrations of *C. fenestratum* extract (100 µg/ml) and berberine (1 µg/ml) were used when co-treated with various VBL concentrations in LLC-PK1, LLC-MDR1 and LLC-VBL cells. The cytotoxic effects of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells were increased in concentration-dependent manners. Figure 16 showed the IC_{50} values of VBL in the presence or absence of verapramil, *C. fenestratum* extract and berberine in LLC-PK1, LLC-MDR1, and LLC-VBL cell lines. *C. fenestratum* extract increased the cytotoxic effect of VBL, similar to verapamil (a known Pgp inhibitor, positive control), in both Pgp-overexpression cells; LLC-MDR1 and LLC-VBL. However, *C. fenestratum* extract, berberine, and verapamil could significantly decrease the IC_{50} values of VBL only in LLC-VBL cells, by 0.50-, 0.74- and 0.51-fold, respectively. There was no

significant difference of the cytotoxic effect of VBL in presence or absence of 10 µg/ml of verapamil (positive control) or 1 µg/ml of berberine or 100 µg/ml of *C. fenestratum* extract in LLC-PK1, a wild type cells lacking Pgp.

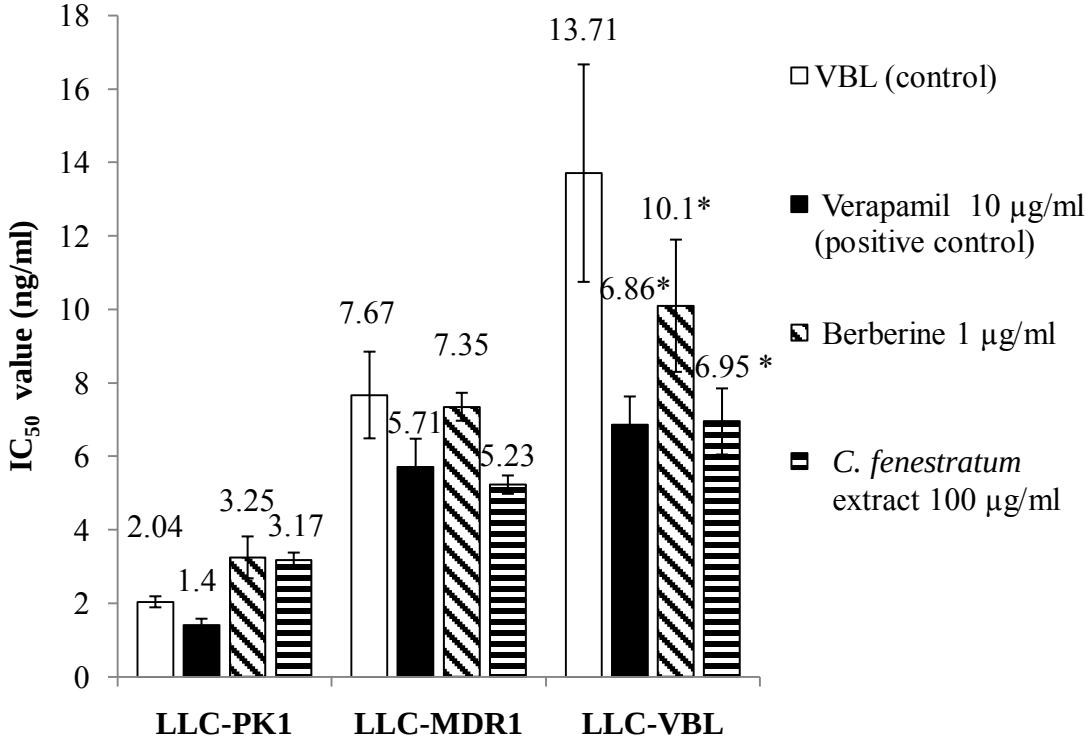


Figure 16. Cytotoxic effects reported as IC_{50} values of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml. Each point represented the mean ± SEM values from more than three independent experiments performed in triplicate. (* = significantly different from VBL control in the same cell type, $P<0.05$).

These results suggested that the *C. fenestratum* extract blocked the VBL-pumping out of cells and increased VBL toxicity in Pgp-overexpressed cells. Similarly, verapamil, an inhibitor of Pgp, increased the VBL toxicity in these cells. Interestingly, berberine, a major compound in *C. fenestratum* extract, slightly

increased the VBL toxicity in Pgp-overexpression cells, particularly in LLC-VBL cells.

In order to confirm the inhibitory effect of *C. fenestratum* extract and berberine on the Pgp function, the Rh 123 accumulation assay was performed.

4.5.2. Effects of *C. fenestratum* stem extract and berberine on Rhodamine 123 (Rh 123) accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells

Rh 123 accumulation assay was used to measure the accumulation of Pgp fluorescence substrate (Rh 123) to confirm the effect of *C. fenestratum* extract and berberine on Pgp function (Quesada *et al.*, 1996). The relative Rh 123 accummulation compared to the control was evaluated.

Figure 17 showed the effect of *C. fenestratum* extract and berberine on the Rh 123 accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells. The *C. fenestratum* extract significantly increased the accumulation of Rh 123 in LLC-PK1, LLC-MDR1 and LLC-VBL cells by approximately 1.76-, 2.49- and 1.91-fold when compared to each vehicle control, respectively. The positive Pgp inhibitor control, verapamil significantly increased the accumulation of Rh 123 in both Pgp-overexpression cells; LLC-MDR1 and LLC-VBL, by approximately 1.30- and 1.41-fold when compared to each vehicle control, respectively, but not in wild type LLC-PK1 cells. However, berberine, a major compound of the *C. fenestratum* extract at the concentration of 1 μg/ml, had no effect on the accumulation of Rh 123 in all three cells.

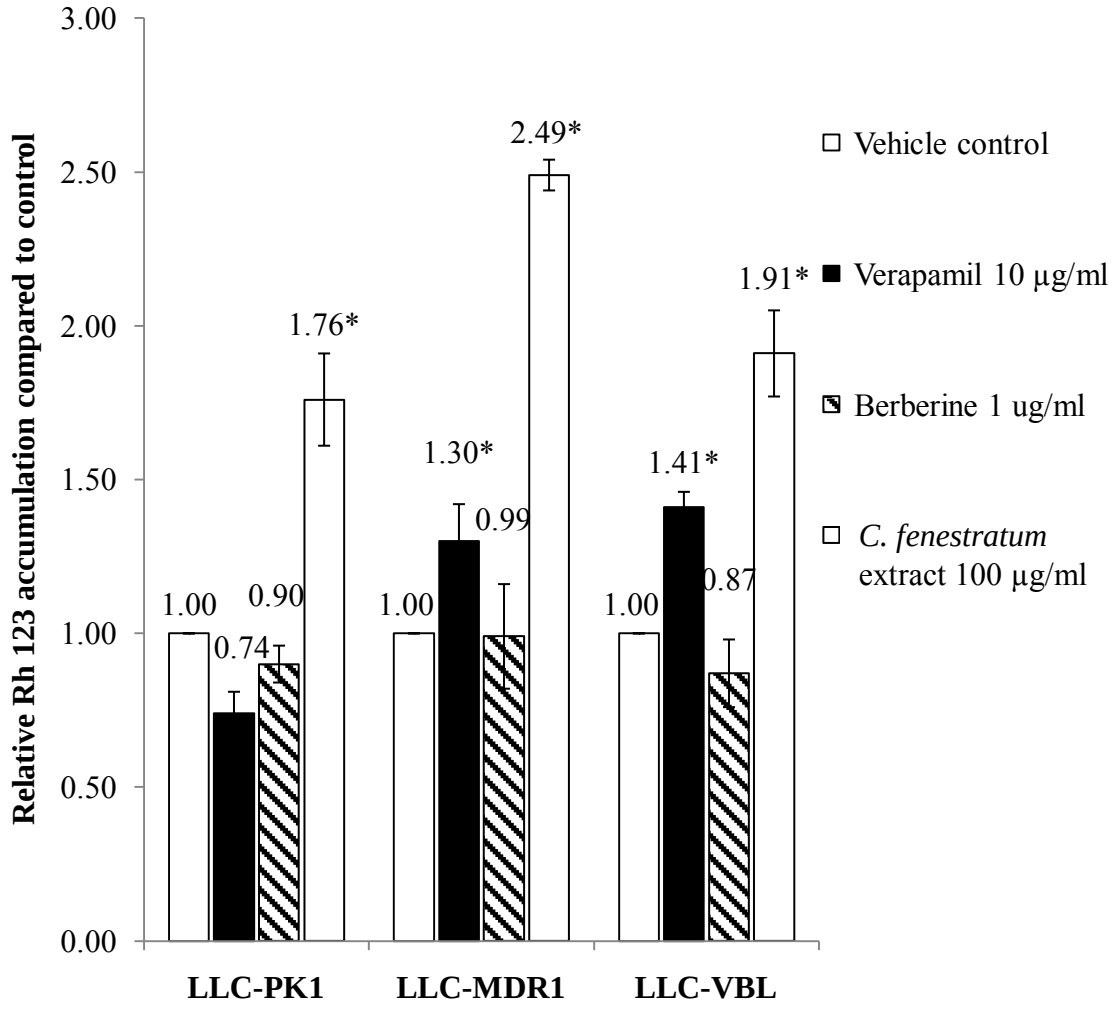


Figure 17. Effects of *C. fenestratum* extract and berberine on the Rh 123 accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells. Cells were treated with vehicle control (PBS), verapamil 10 µg/ml (positive control), *C. fenestratum* extract 100 µg/ml and berberine 1 µg/ml. Data were shown as mean ± SEM, from three independent experiments. (* = significantly different from the control, $P < 0.05$).

Under a normal condition, Rh 123 is pumped out of the cells by Pgp and accumulated when Pgp is blocked (Quesada *et al.*, 1996). Our results showed that *C. fenestratum* extract could inhibit Pgp function since they significantly increased Rh 123 accumulation, similar to verapamil, a Pgp inhibitor. However, berberine had no effect on Rh 123 accumulation.

Interestingly, in LLC-PK1 cells, a wild type cell, *C. fenestratum* extract could also increase Rh 123, this may be due to the fact that the Rh 123 is also a substrate of other transporter, MRP, besides Pgp (Vasconcelos *et al.*, 2007; Versantvoort *et al.*, 1996). MRP or multidrug resistance-associated protein is a member of ABC transporter protein similar to Pgp. The MRP is found in kidney cells, and LLC-PK1 cells are a epithelial kidney cells (Silverman, 1999). Therefore, *C. fenestratum* extract may inhibit MRP function of LLC-PK1 cells.

Taken together, our findings about the effect of *C. fenestratum* extract on Rh 123 accumulation and VBL cytotoxicity suggested that the *C. fenestratum* extract can inhibit Pgp function.

4.5.3. Effects of *C. fenestratum* stem extract and berberine on ATPase activity of Pgp in LLC-PK1, LLC-MDR1 and LLC-VBL cells

Pgp, an ATP-dependent efflux transporter, pumps substrates out of cells by using energy from ATP hydrolysis (Aanismaa and Seelig, 2007). To study the interaction of *C. fenestratum* extract or berberine with Pgp, Pgp-ATPase activity was examined, using plasma membranes isolated from LLC-PK1, LLC-MDR1 and LLC-VBL cells. The ATPase activity was measured by monitoring phosphate release during ATP hydrolysis. The Pgp-ATPase activity is calculated from ATPase activity of sample minus with ATPase activity of vanadate-sample. Vanadate is the specific inhibitor of Pgp-ATPase which is able to inhibit ATPase activity in Pgp by traping ADP in nucleotide binding site of Pgp (Loo and Clarke, 2002).

Figure 18A showed the effect of *C. fenestratum* extract and berberine on Pgp-ATPase activity in LLC-PK1, LLC-MDR1 and LLC-VBL cells. The *C. fenestratum* extract significantly decreased Pgp-ATPase activity in the Pgp-overexpression cells (LLC-MDR1 and LLC-VBL) when compared with the control by approximately 0.62- and 0.47 -fold, respectively, as shown in Table 18.

On the other hand, berberine, a major compound, had no effect on Pgp-ATPase activity in these three cells when compared with the control. Moreover, verapamil, a high-affinity drug-substrate of Pgp or competitive Pgp inhibitor (Scarborough, 1995), could significantly increase Pgp-ATPase activity in both of Pgp-overexpression cells; LLC-MDR1 and LLC-VBL cells, by approximately 1.79- and 1.65-fold of each control, respectively, as demonstrated in Table 18. These results correlated with the inducing of Pgp-ATPase activity of verapamil in human lymphoblastic leukemia cell line resistant to VBL; CEC/VBL_{100}, by approximately 1.82-fold of control (Shepard *et al.*, 1998).

In addition, the effect of *C. fenestratum* extract and berberine on verapamil-stimulated ATPase activity of the Pgp in LLC-MDR1 and LLC-VBL cells were also determined. Both *C. fenestratum* extract and berberine significantly decreased verapamil-stimulated ATPase activity in LLC-MDR1 and LLC-VBL cells when compared with verapamil as shown in Figure 18B. The relative ATPase activity in the presence of *C. fenestratum* extract when compared with verapamil was 0.32- and 0.36-fold in LLC-MDR1 and LLC-VBL cells, respectively, as shown in Table 19. In addition, the relative ATPase activity in the presence of berberine when compared with verapamil was 0.53- and 0.55-fold in LLC-MDR1 and LLC-VBL cells, respectively, as shown in Table 19.

However, *C. fenestratum* extract and berberine had no effect on ATPase activity in wild type LLC-PK1 cells. The values of Pgp-ATPase activity in LLC-PK1 cells were much lower than those in LLC-MDR1 and LLC-VBL cells as shown in Table 18. This may due to LLC-PK1 cells had less Pgp protein levels than the other cells.

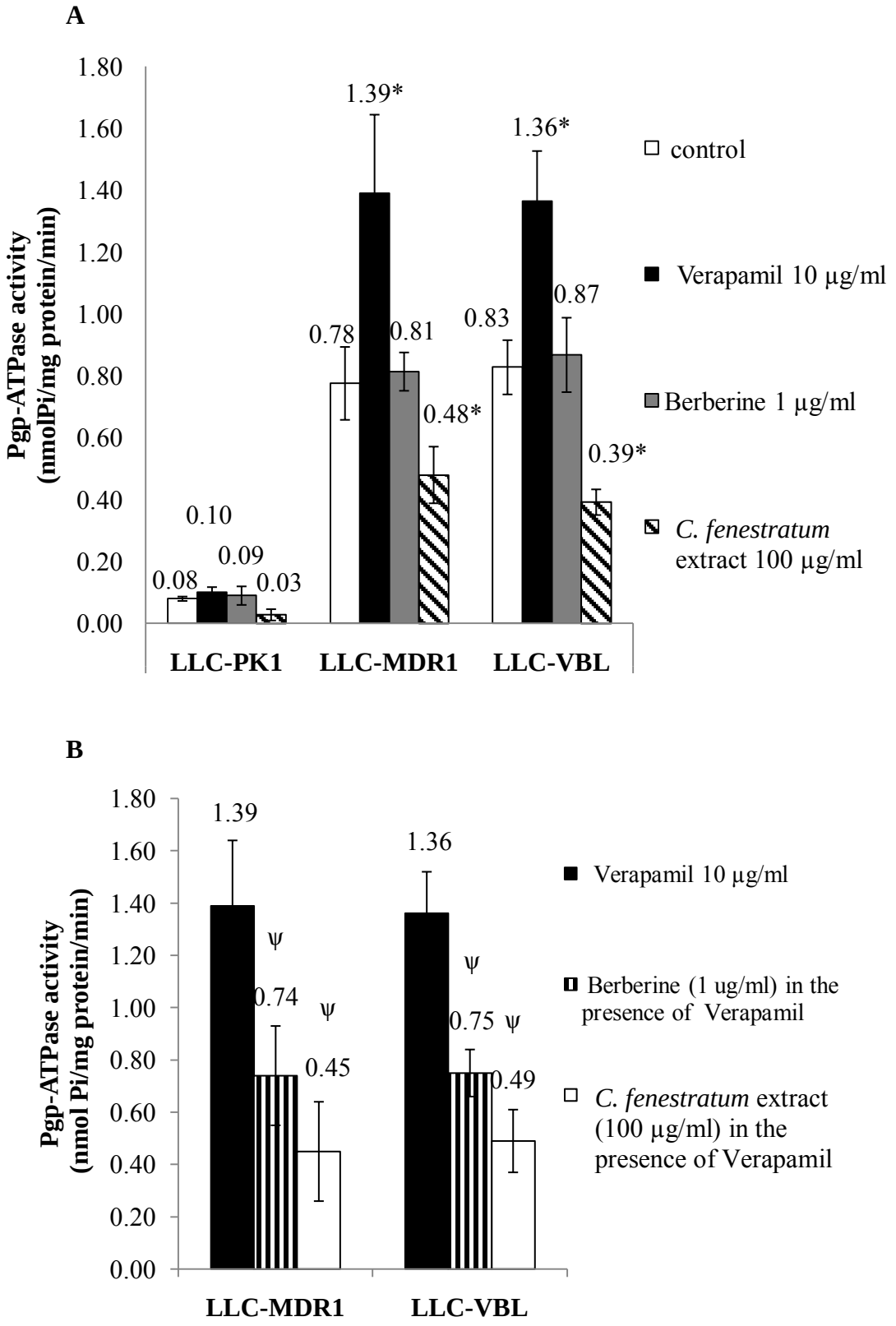


Figure 18. Effects of verapamil, *C. fenestratum* extract and berberine on Pgp-ATPase activity in LLC-PK1, LLC-MDR1 and LLC-VBL cells. **(A)** Effects of *C.*

fenestratum extract and berberine on the Pgp-ATPase activity in LLC-PK1, LLC-MDR1 and LLC-VBL cells. **(B)** Effects of *C. fenestratum* extract and berberine on the verapamil-stimulated ATPase activity in LLC-MDR1 and LLC-VBL cells. Plasma membrane vehicles 10 μg were incubated with 3 mM of ATP in the presence or absence of verapamil 10 μg/ml, *C. fenestratum* extract 100 μg/ml and berberine 1 μg/ml. The reaction was started by incubation at 37 °C for 1 hr. Data was shown as mean ± SEM, from five independent experiments, each carried out in triplicate. * = significantly different from the control, $P < 0.05$; ψ = significantly different from each positive control, $P < 0.05$.

4.6. Effect of *C. fenestratum* stem extract and berberine on Pgp expression

After the effect of *C. fenestratum* stem extract and berberine on Pgp function were evaluated in all three cells as described above. Their effect on Pgp expression was investigated in LLC-MDR and LLC-VBL cells by Western blot analysis. Western blot analysis was used to determine the level of Pgp expression, using rabbit polyclonal Pgp antibody and HRP-conjugated rabbit IgG and ECL for signal detection. Figure 19A showed the Pgp expression levels in LLC-PK1, LLC-MDR1 and LLC-VBL cells cultured in the presence or absence of *C. fenestratum* extract (100 μg/ml) and berberine (1 μg/ml) for 48 hr. This incubation time (48 hr) was correlated with the cytotoxicity assay which used to determine the non-toxic concentrations of *C. fenestratum* extract and berberine. Therefore, at 48 hr all cells still alive when they were treated with *C. fenestratum* extract and berberine. In addition, Figure 19B showed the relative Pgp expression levels compared to the control in LLC-MDR1 and LLC-VBL cells.

From Figure 19A and 19B, there were no change of Pgp expression levels in both cells after treated with *C. fenestratum* extract and berberine. Therefore, *C. fenestratum* extract and berberine had no effect on Pgp expression. Previous study by Suzuki's group reported that berberine and palmatine, major compounds found in *C. fenestratum* extract, slightly up-regulated the mRNA of *Mdr1a* and *Mdr1b*, but they had no effect of on protein expression (Suzuki *et al.*, 2010).

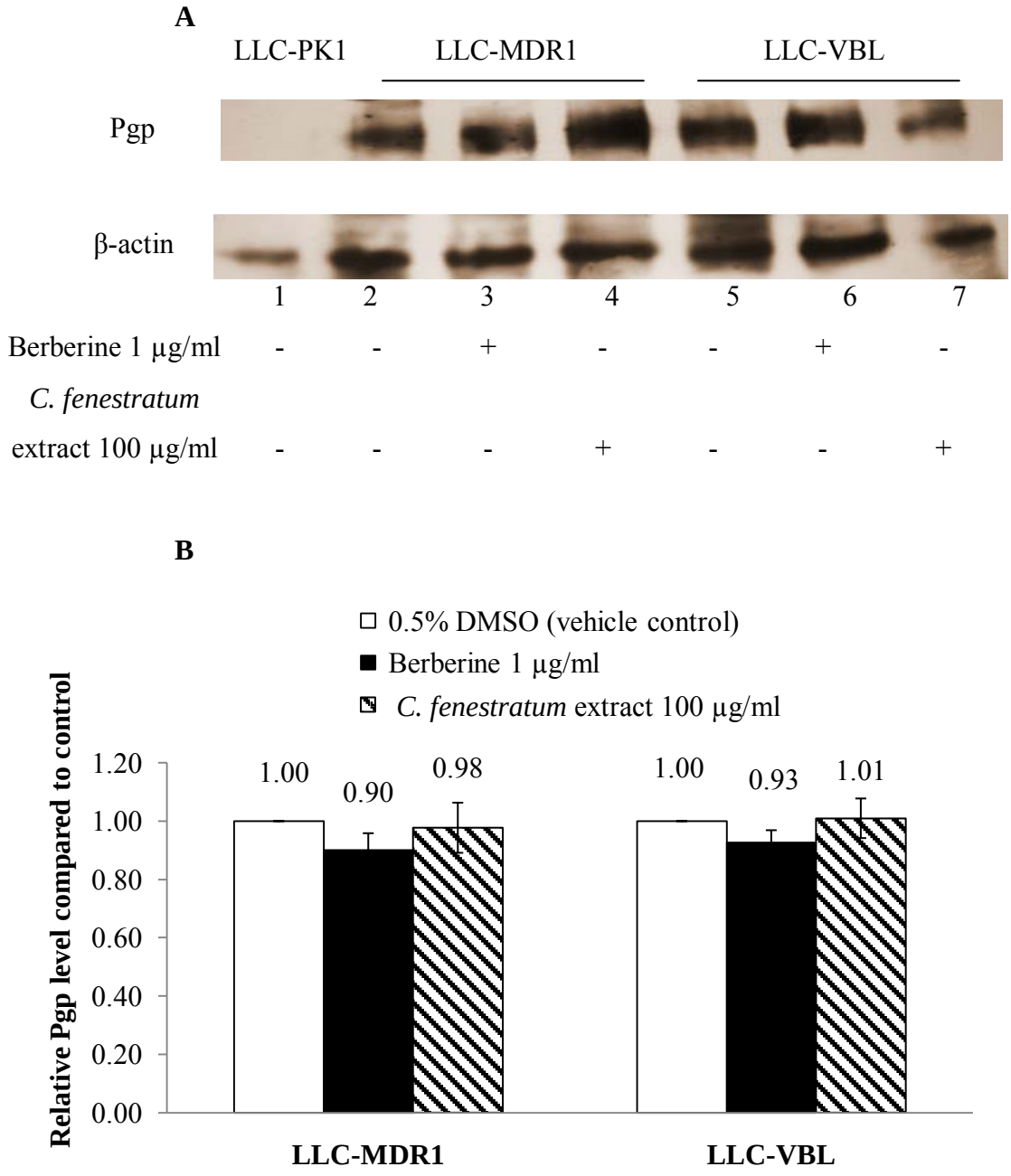


Figure 19. Effects of *C. fenestratum* extract and berberine on Pgp expression levels in LLC-PK1, LLC-MDR1 and LLC-VBL cells **(A)** The representative of Pgp expression levels in LLC-PK1, LLC-MDR1 and LLC-VBL cells cultured in the presence or absence of *C. fenestratum* extract 100 µg/ml and berberine 1 µg/ml. The Pgp levels were evaluated by Western blot analysis. **(B)** The relative Pgp expression levels were compared to that of vehicle control in LLC-MDR1 and LLC-VBL cells. Data were shown as mean ± SEM, from three independent experiments.

CHAPTER V
DICCUSSION AND CONCLUSION

There was several reports indicated drug-herb interaction through cytochrome P450 and/or P-glycoprotein (Yang *et al.*, 2006; Zhou *et al.*, 2007). Since Pgp can mediate drug-herb interaction and the information about drug and *C. fenestratum* interaction have not been reported yet. In this study, we investigated the effect of *C. fenestratum* stem extract on Pgp functions and *MDR1* expression in porcine renal epithelial (LLC-PK1) and *MDR1* - transfected (LLC-MDR1) and vinblastine-induced *MDR1* - transfected (LLC-VBL) cell lines.

In the first study, *C. fenestratum* stem ethanolic extract were prepared. *C. fenestratum* stems were macerated with 80% ethanol everyday for 5 days. The percent yield of crude extract was 19.7 % (w/w) and the crude ethanolic extract had similar TLC-fingerprint pattern comparing to the authentic *C. fenestratum* extract. The berberine contents of *C. fenestratum* extract were 10.0 ± 0.002 % w/w, determined by TLC-densitometer. The percent yield of crude extract and the amount of berberine determined in this study were higher than those in the previous report of Rojsanga's group which were 18.41 ± 0.16 and 3.37 ± 0.30 % w/w, respectively, using same system (Rojsanga *et al.,* 2006). This may result from different sources of crude drugs.

Next, the cell models were evaluated for their baseline Pgp levels by determining Pgp expression using Western blot analysis and Pgp functions using Rh 123 accumulation assay and vinblastine (VBL) cytotoxicity assay. The Pgp-overexpression cells; LLC-MDR1 and LLC-VBL, contained high Pgp levels and were resistant to vinblastine cytotoxicity. The Pgp levels in LLC-VBL cells were similar to those in LLC-MDR1 cells. Whereas, the wild type cell, LLC-PK1 cells had undetectable Pgp level and sensitized to vinblastine cytotoxicity. From previous report (Booth-Genthe *et al.*, 2006), the IC_{50} value of vinblastine in LLC-PK1 cell was 3.71 ng/ml that was similar to ours. In this study, the IC_{50} values of vinblastine determined in LLC-MDR1 and LLC-VBL cells were higher than in LLC-PK1 cells, approximately 3.76– and 6.72- fold, respectively, when compared with wild type cell.

Furthermore, Rh 123 accumulation in LLC-PK1 cells were higher than in LLC-MDR1 and LLC-VBL cells for 2.26- and 1.79- fold, respectively. Therefore, we chose the LLC-PK1, LLC-MDR1 and LLC-VBL cells to evaluate the effect of the *C. fenestratum* extract and berberine on Pgp functions and Pgp expression.

From the IC_{50} values of vinblastine, LLC-VBL cells were more resistant to VBL than LLC-MDR1 cells, whereas, the Pgp levels of both cells were similar because there were other cellular mechanisms of resistance to VBL besides the Pgp, *e.g.*, modifications in tubulin or microtubule-associated protein, and modifications in the regulation of programmed cell death or apotosis (Chen *et al.*, 2000). Therefore, the LLC-VBL cells which were cultured with VBL at the concentration of 1.1 pM were modified all of these mechanisms and were highest resistant to VBL.

Before studying the effect of *C. fenestratum* extract and berberine on Pgp functions and expression in those three cells, non-toxic concentrations of the *C. fenestratum* extract and berberine were determined using MTT assay. The non-toxic concentrations of the *C. fenestratum* extract and berberine chosen from this study were 100 and 1 μg/ml, respectively. From Table 4, *C. fenestratum* extract was more toxic to LLC-PK1 cells than to LLC-MDR1 and LLC-VBL cells, whereas, berberine had no difference in its toxicity to those three cells. The *C. fenestratum* extract contained various Pgp substrate compounds, such as berberine, plamatine and jatrorrhizine, therefore, they were pumped out of Pgp-overexpressing cells; LLC-MDR1 and LLC-VBL. However, berberine can be a substrate of other drug transporters than Pgp, such as multidrug resistance-associated protein (MRP1) (Shitan *et al.*, 2007), the human organic cation transporter 1 (OCT1, SLC22A1) and the human organic cation transporter 2 (OCT2, SLC22A2) (Nies *et al.*, 2008) which can be found in all three cells. Therefore, the toxicity of berberine was not different in all three cells.

The second study, the effects of *C. fenestratum* extract and berberine at non-toxic concentrations on the Pgp functions using MTT cytotoxicity assay, Rhodamine 123 accumulation assay and ATPase assay were performed. In the first assay, the vinblastine co-treatment could be used to measure Pgp function using MTT cytotoxicity assay since vinblastine is a cytotoxic drug and also known as Pgp substrate which can be pumped out of cells by Pgp (Bruggemann *et al.*, 1992). If the

cells contained Pgp, it would resist to vinblastine toxicity. Our result showed that LLC-PK1 cells, wild type cells lacking Pgp, were sensitive to vinblastine, whereas, Pgp-overexpression cells; LLC-MDR1 and LLC-VBL cells were resistant to vinblastine. In addition, LLC-VBL cells were more resistant to vinblastine than LLC-MDR1 cells which correlated with the level of Pgp determined in each cell. Moreover, the cytotoxicity of vinblastine in LLC-MDR1 and LLC-VBL cells were increased when treated with verapamil, the competitive Pgp inhibitor and positive control. Our results demonstrated that the *C. fenestratum* extract could potentiate the effect of vinblastine-induced cytotoxicity in LLC-MDR1 cells and more significantly in LLC-VBL cells, similar to verapamil. However, berberine, a major compound found in *C. fenestratum* extract could potentiate the effect of vinblastine-induced cytotoxicity only in LLC-VBL cells. This might be due to the concentration of berberine used in this experiment was too low (1 μg/ml), compared to that found in *C. fenestratum* extract (11 μg/ml). In order to confirm the effect on Pgp function, rhodamine 123, a fluorescence Pgp substrate was used in the accumulation assay. The cells contained Pgp would accumulate Rh 123 less than wild type. From our result, wild type LLC-PK1 cells could accumulate Rh 123 more than Pgp-overexpressed LLC-MDR1 and LLC-VBL cells for 2.26- and 1.79- fold, respectively, compared with wild type cells. Furthermore, verapamil, the Pgp inhibitor and a positive control could significantly increase Rh 123 accumulation in LLC-MDR1 and LLC-VBL cells, but it had no effect on LLC-PK1 cells. Interestingly, *C. fenestratum* extract could increase Rh 123 accumulation, whereas, berberine had no effect on the accumulation of Rh 123 in all three cells. These results were correlated with their effects on vinblastine cytotoxicity. As discussed earlier, berberine had slightly effect on vinblastine cytotoxicity and no effect on Rh 123 accumulation, this may be due to its non-toxic concentration (1 μg/ml) determined in this study were too low. In previous studies, the berberine had been demonstrated to be a Pgp substrate which was pumped out of the cells by Pgp at the concentrations of 10 μM or 3.3 μg/ml (Pan *et al.*, 2002; Shitan *et al.*, 2007). It was reported that berberine at the concentration of 10 μM or 3.3 μg/ml could increase Rh 123 accumulation (He and Liu, 2002). From our results of berberine contents in the *C. fenestratum* extract determination by TLC-densitometry, the non-toxic concentration of *C. fenestratum* extract used in the

vinblastine cytotoxicity assay was 100 μg/ml, contained berberine at the concentration of approximately 11 μg/ml (33 μM). Therefore, non-toxic concentration of berberine (1 μg/ml) was lower than the berberine content in the crude extract about 10-fold. Consequently, the result of Rh 123 accumulation and vinblastine cytotoxicity could confirm that the *C. fenestratum* extract can inhibit Pgp functions. *C. fenestratum* extract could also increase Rh 123 in LLC-PK1 cells, wild type cells, this may be due to the fact that the Rh 123 also a substrate of other transporters *e.g.*, MRP (Vasconcelos *et al.*, 2007; Versantvoort *et al.*, 1996). MRP or multidrug resistance-associated protein is a member of ABC transporter protein similar to Pgp. The MRP is found in kidney cells, and LLC-PK1 cells are epithelial kidney cells (Silverman, 1999). Therefore, *C. fenestratum* extract may also be able to inhibit MRP function of LLC-PK1 cells.

In order to find some mechanisms underlying the inhibitory effect on Pgp function of the *C. fenestratum* extract, the ATPase assay was performed. P-glycoprotein was well known as an ATP-dependent efflux transporter, it needed ATP hydrolysis to pump the substrate out of the cells catalyzed by ATPase enzyme which were part of P-glycoprotein molecule. ATPase activity was determined from ATP hydrolysis when substrate was pumped out of cells by Pgp (Aanismaa and Seelig, 2007). From the vehicle control experiment, the wild type LLC-PK1 cells had very low Pgp-ATPase activity due to containing very low Pgp level. On the other hand, the Pgp-overexpression cells; LLC-MDR1 and LLC-VBL cells, had high Pgp-ATPase activity. In addition, verapamil, a high-affinity drug-substrate of Pgp or competitive Pgp inhibitor (Scarborough, 1995), could significantly increase Pgp-ATPase activity in both Pgp-overexpression cells. Since binding of verapamil to Pgp caused conformational change to activate ATPase activity. Our result revealed that the *C. fenestratum* extract could significantly inhibit Pgp-ATPase activity in LLC-MDR1 and LLC-VBL cells, whereas, berberine had no effect on Pgp-ATPase activity of these cells. However, both *C. fenestratum* extract and berberine could significantly decrease the high-capacity verapamil-stimulated ATPase activity of the Pgp in LLC-MDR1 and LLC-VBL cells. Pgp is an unusual ATP-driven transporter, in that it has a low affinity for ATP and exhibits a high level of constitutive or basal ATPase activity. From the previous study, berberine exhibited the biphasic effect on Pgp-ATPase

activity (Najar *et al.*, 2010). It could stimulate Pgp-ATPase activity at low concentrations (1.67 μg/ml) and inhibit at high concentrations (8.33-33.33 μg/ml) due to the rate-limitation by ATP binding in transition states of ATPase reaction (Al-Shawi *et al.*, 2003; Najar *et al.*, 2010). Similar results were found with curcumin (Anuchapreeda *et al.*, 2002). Binding of drugs could stimulate ATP hydrolysis at the two nucleotide sites in an alternating fashion, leading to drug transport (Senior *et al.*, 1995). From our results showed that the *C. fenestratum* extract containing higher berberine contents (~11 μg/ml of berberine) inhibited Pgp-ATPase activity, whereas, berberine at low non-toxic concentration (1 μg/ml) had no effect. This may also due to the different cell types and condition to determine Pgp-ATPase assay. Since Pgp-ATPase activity correlated with the transport of Pgp substrates, it could be used for evaluating the function of Pgp. Verapamil (a high-affinity Pgp substrate) could stimulate the Pgp-ATPase activity upon its binding to Pgp. In addition, the Pgp contained multiple substrate-binding sites (Martin *et al.*, 2000), therefore, the verapamil-stimulated ATPase activity was used to confirm the effect of *C. fenestratum* extract and berberine on Pgp-ATPase activity and to evaluate the interaction of *C. fenestratum* extract and berberine on multiple overlapping substrate-binding sites within the Pgp (Garrigos *et al.*, 1997). The results also showed that *C. fenestratum* extract and berberine could inhibit verapamil-stimulated ATPase activity. The binding of *C. fenestratum* extract and berberine may cause change of the Pgp conformation and affect the binding of verapamil. These results suggested that the binding site of *C. fenestratum* extract and berberine may overlap with the verapamil binding site.

Our results revealed that *C. fenestratum* extract could inhibit Pgp functions *in vitro* as demonstrating by increasing vinblastine cytotoxicity and Rh 123 accumulation, and inhibiting Pgp-ATPase activity in Pgp-overexpression cells; LLC-MDR1 and LLC-VBL cells, but it had no effect on wild type cells; LLC-PK1 cells. The inhibitory effect on Pgp function of *C. fenestratum* extract may be due to it could inhibit ATPase activity of Pgp. However, there were limited reports effect of berberine on Pgp function, whether it was an inducer or inhibitor of Pgp were debatable. According to previous studies, berberine decreased Rh 123 accumulation in oral cancer cell lines (OC2 and KB cells) and increased Paclitaxel-resistant cancer cell

lines, such as oral cancer, OC2, human gastric carcinoma, SC-M1 and human colon cancer cell line, COLO 205 (Lin *et al.*, 1999a; Lin *et al.*, 1999b). However, berberine was shown to inhibit rat intestinal Pgp which lead to increase bioavailability of digoxin and cyclosporine A (Qiu *et al.*, 2009). Besides, berberine could increase Rh 123 accumulation in bovine brain capillary endothelia cells; BCEC (He and Liu, 2002). These different results may due to the selection of cell types, concentrations, substrate types and the time of exposure.

From the previous chemical analysis of *C. fenestratum* extract, compounds other than berberine were other isoquinoline alkaloids, such as palmatine, jatrorrhizine, tetrahydropalmatine and crebanine (Rojsanga and Gritsanapan, 2005). Moreover, palmatine was reported to be able to increase Rh 123 accumulation and Pgp-ATPase activity. Those results suggested that palmatine was a Pgp substrate and may inhibit Pgp function. Therefore, compounds other than berberine in *C. fenestratum* extract, such as palmatine, may contribute to the inhibition effect on Pgp function.

Since *C. fenestratum* extract could inhibit Pgp function in the cells, its effect on Pgp expression using Western blot analysis was determined in our final study. The result suggested that both *C. fenestratum* extract and berberine had no effect on Pgp expression. From previous study, Suzuki's group reported that berberine and palmatine, the compounds found in *C. fenestratum* extract, slightly up-regulated the mRNA of *Mdr1a* and *Mdr1b*, but they had no effect on protein expression (Suzuki *et al.*, 2010). Table 5 summarized the effects of *C. fenestratum* extract and berberine on Pgp functions and expression examined from our study.

The results altogether indicated that *C. fenestratum* stem ethanolic extract was an inhibitor of P-glycoprotein function due to the inhibition of its ATPase activity without affecting protein expression.

Since the *C. fenestratum* stem ethanolic extract was shown to inhibit Pgp function *in vitro*, using it together with Pgp substrate prescription medicine is needed to be considered. It may increase the effect of the prescription medicine and cause serious side effects.

Table 5. Summary of the effects of *C. fenestratum* extracts and berberine on Pgp functions and expression

		Experiments			
		Pgp functions			**Pgp expression**
Cells	**Testing agent**	Vinblastine cytotoxicity	Rh 123 accumulation	ATPase activity	Western blot analysis
LLC-PK1 (wild type cells)	Verapamil	—	—	—	No study
	Berberine	—	—	—	—
	C. fenestratum extract	—	▲	—	—
LLC-MDR1 (Pgp over expression cells)	Verapamil	—	—	▲	No study
	Berberine	—	—	—	—
	C. fenestratum extract	—	▲	—	—
LLC-VBL (Pgp over-expression cells and induced cells with vinblastine)	Verapamil	▲	—	▲	No study
	Berberine	—	—	—	—
	C. fenestratum extract *	▲	▲	—	—

— = did not affect

▲ = increased with significantly different from the control ($P < 0.05$)

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APPENDICES

APPENDIX A
PREPARATION OF REAGENTS

Reagents for cell culture

Growth medium of LLC-PK1, LLC-MDR1 and LLC-VBL cells

Incomplete M199 Medium

M199 powder (1 package) was dissolved with ultrapure water and the 2.2 g of sodium bicarbonate was added. The mixing medium was adjusted pH to 7.2-7.4 with HCl and adjusted volume to 1,000 ml. They were further sterilized by filtration with 0.2 µM Bottle-Top Vacuum Filters and stored at 4 °C.

Complete M199 Medium

Before using, the incomplete M199 medium was supplement with 10% FBS, and 1% penicillin and streptomycin. Vinblastine (VBL) at the concentration of 1 pg/ml was added only to the LLC-VBL cells.

Phosphate buffer saline (PBS)

The PBS was made for 1 L, the ingredients including 8.00 g of NaCl, 0.20 g of KCl, 1.15 g of Na_2HPO_4, and 0.20 g of KH_2PO_4 were dissolved in 800 ml of ultrapure water and adjusted the pH to 7.2-7.4 with HCl. Then, the PBS solution was adjusted volume to 1,000 ml and sterilized by autoclaving for 20 min at 15 lb/sq and then stored at room temperature.

Reagents for protein determination

Bradford reagent for protein determination

For making 1 L of Bradford reagent

Coomassie Blue G250 (Brilliant blue G-250)	100	mg
95% MeOH	50	ml
85% Phosphoric acid	100	ml

After all ingredients were mixed well, the mixture was adjusted volume with ultrapure water to 1,000 ml. Then, the solution was filtered through Whatman filter paper no.93. The solution was kept in container protect from light at 4 ^{o}C.

Lowry assay reagent

Reagent A

2%(w/v) Na_2CO_3 in 0.1 N NaOH

NaOH	2	g
Na_2CO_3	10	g
Deionized distilled water	500	ml

Reagent B

Part A; $CuSO_4$	0.5	g
Distilled water	50	ml
Part B; Na-K tatrate	1	g
Deionized distilled water	500	ml

Before using 0.5 ml of part A and B were mixed with the final concentration 0.5%(w/v) $CuSO_4 \cdot 5H_2O$ and 1%$NaK(C_4H_4O_6) \cdot 4H_2O$ (Na-K tatrate).

Reagent C

Working solution was freshly prepared by mixing reagent A 50 ml and reagent B at the ratio of 50:1.

Reagents for Western blot analysis

50 % Acrylamide

For making 100 ml of 50% Acrylamide

Acrylamide	49.2	g
N,N'-Methylene bisacrylamide	0.8	g

Both compounds are dissolved in 50 ml of ultrapure water. The solution was stirred until completely solubilized, then adjusted volume to 100 ml and stored in dark bottles at room temperature.

Caution: Acrylamide is a neurotoxin. Use with extremely by care when handling solids and solutions containing acrylamide and bisacrylamide. Wear a mask and gloves when weighing out solid acrylamide.

10 % Ammonium persulfate (APS)

APS 100 mg were dissolved in 10 ml of ultrapure water. The solution was mixed and stored in dark at -20 ^{o}C.

Preparation of separating gel (main gel)

8% Gel separating Gel for 2 Gels

Ultra H_2O	5.7	ml
1.5 M Tris-HCl, pH 8.8	2.5	ml
50% Acrylamide	1.6	ml
10% SDS	0.1	ml
10% APS	0.1	ml
TEMED	10	µl
Total	10	ml

All the ingredients were thoroughly mixed and immediately pour between the glass plates. Before gel polymerization was complete, DDW was layered on the top of the separating gel (4-5 mm thick). The gels were leaved for approximately 20-30 min.

Preparation of stacking gel (top gel)

Once the separating gel has completely polymerized, DDW was removed from the top of the polymerized gel. To make stacking gel, the ingredients were

Ultra H_2O	3.25	ml
0.5 M Tris-HCl, pH 8.8	1.25	ml
50% Acrylamide	400	µl
10% SDS	50	µl
10% APS	40	µl
TEMED	5	µl
Total	5	ml

All the ingredients were thoroughly mixed and immediately pour gel between the glass plates. The combs were inserted between the two glass plates of two sets of gel apparatus. The gels were leaved for approximately 30-40 min to polymerize.

Application of samples

Once the stacking gel has completely polymerized, the combs were gently removed. The wells were flushed out thoroughly with electrophoresis buffer. The clips and sealing tapes were removed and set up the gel chamber. Electrophoresis buffer was filled out both inner and outer chamber. Before loading samples and protein marker, the air bubbles between layers were removed by gently rolling the chamber.

10X Electrophoresis and transfer buffer for Western blot analysis

To make 1 L of 10X of Electrophoresis and Transfer Buffer (2.5 M Tris, pH 8.3, 19.2 M glycine) for stock solution, the ingredients were

Tris-base	30.3 g
Glycine	144.2 g

All ingredients were dissolved in ultrapure water with continuously stirring. The solution was adjusted volume to 1,000 ml.

1X Electrophoresis buffer for Western blot analysis

To make 1 L of 1X Electrophoresis Buffer (250 mM Tris, 1.92 M glycine and 0.5% SDS), the ingredients were

10X Electrophoresis and Transfer Buffer	100 ml
Ultrapure water	890 ml
10% SDS	10 ml

1X Transfer buffer for Western blot analysis

To make 1 L of 1X Transfer Buffer (160 mM Tris, 0.25 M glycine and 20% methanol), the ingredients were

10X Electrophoresis and Transfer Buffer	80 ml
Ultrapure water	720 ml
Methanol	200 ml

Sample buffer for Western blot analysis

To make 5X sample buffer, for stock solution, the ingredients were

60 mM Tris HCl

25% Glycerol

2% SDS

14.4 mM 2-Mercaptoethanol

0.1% Bromphenol blue

All ingredients, except 2-mercaptoethanol, were dissolved in ultrapure water with continuously stirring. The solution was adjusted volume to 50 ml and then filtered with filter paper no. 93. 2-Mercaptoethanol at the concentration of 14.4 mM was later added and 5X sample was aliquoted into 1 ml/tube and stored at -20 ^{o}C.

RIPA lysis buffer

Ultra H_2O	3.25	ml
1 M NaCl	6.5	ml
10% Tritron-X	13	ml
10% Sodium deoxycholate	6.5	ml
100 mM NaF	6.5	ml
1 M Tris-Base, pH 8	3.25	ml
10% SDS	0.65	ml
200 mM Sodium orthovanadate	0.325	ml
100 mM PMSF	0.65	ml
Total	65	ml

2% SDS Laemmli buffer

Ultra H_2O	325	µl
1 M Tris-HCl, pH 6.8	25	µl
10% SDS	100	µl
Glycerol	50	µl

APPENDIX B
TABLES AND FIGURES OF EXPERIMENTAL RESULTS

Table 6. Data for construction of berberine calibration curve. Data presented as mean ± SD of three experiments.

Berberine (ng/lane)	**Peak area**
10	4,629 ± 134
20	8,179 ± 451
40	14,592 ± 634
60	20,071 ± 1,321
80	24,415 ± 1,719
C. fenestratum extract (400 ng/spot)	14,471 ± 719

Table 7. The relative of Pgp levels in LLC-PK1, LLC-MDR1 and LLC-VBL cells compared to LLC-PK1 cells. Data presented as mean ± SEM of three experiments. (* = significantly different from Pgp levels in wild type cell; LLC-PK1 cell, $P<0.05$).

Cell types	**Ratio of Pgp and actin levels [Relative Pgp levels compared to LLC-PK1 cell]**
LLC-PK1	0.29 ± 0.00 [1.00]
LLC-MDR1	1.71 ± 0.10 [4.11*]
LLC-VBL	1.31 ± 0.06 [4.60*]

Table 8. The Rhodamine 123 (Rh 123) accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells. Data presented as mean ± SEM of three experiments. (* = significantly different from Rh 123 accumulation in wild type cell; LLC-PK1 cell, $P<0.05$).

Cell types	**Rh 123 accumulation (nmole/mg) [fold of LLC-PK1]**
LLC-PK1	0.052 ± 0.007
LLC-MDR1	0.023 ± 0.002* [2.26]
LLC-VBL	0.029 ± 0.0003* [1.79]

Table 9. The percentage of cell viability of LLC-PK1, LLC-MDR1 and LLC-VBL cells incubated with vinblastine (VBL) at various concentrations for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

VBL concentrations (ng/ml)	**% cell viability (% of control)**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
1.28×10^{-3}	88.2 ± 10.8	96.7 ± 3.25	108.0 ± 6.20
6.40×10^{-2}	90.2 ± 8.00	95.7 ± 6.33	106.0 ± 6.70
0.32	92.5 ± 2.30	93.4 ± 3.58	101.0 ± 7.60
1.60	63.8 ± 4.50	85.5 ± 3.16	98.0 ± 7.50
8.00	-5.70 ± 1.40	57.8 ± 12.3	66.0 ± 7.30
40.0	-2.50 ± 7.40	3.10 ± 2.79	3.00 ± 5.70
200.0	-4.90 ± 3.80	-16.7 ± 4.89	-16.0 ± 3.00

Table 10. The IC_{50} values of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells. Data presented as mean ± SEM of three experiments. (* = significantly different from IC_{50} values in wild type cell; LLC-PK1 cell, $P<0.05$).

Cell types	**IC_{50} values of VBL (ng/ml) [fold of LLC-PK1]**
LLC-PK1	2.04 ± 0.15
LLC-MDR1	7.67 ± 1.18* [3.76]
LLC-VBL	13.71 ± 2.95* [6.72]

Table 11. The percentage of cell viability of LLC-PK1, LLC-MDR1 and LLC-VBL cells incubated with *C. fenestratum* extract at various concentrations for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

***C. fenestratum* extract (µg/ml)**	**% cell viability (% of control)**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
50	108.4 ± 5.30	103.5 ± 1.90	94.4 ± 2.30
100	85.3 ± 2.80	98.2 ± 1.90	91.2 ± 3.10
200	75.4 ± 3.10	86.0 ± 1.30	86.3 ± 2.30
300	68.4 ± 3.90	81.0 ± 2.10	69.9 ± 3.70
400	6.70 ± 8.30	70.0 ± 3.70	51.7 ± 6.10
500	2.60 ± 7.70	20.2 ± 4.80	18.1 ± 10.20

Table 12. The percentage of cell viability of LLC-PK1, LLC-MDR1 and LLC-VBL cells incubated with berberine at various concentrations for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

Berberine (µg/ml)	**% cell viability (% of control)**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
1.00×10^{-4}	103.5 ± 7.90	92.2 ± 12.7	94.1 ± 6.20
1.00×10^{-3}	92.1 ± 9.33	94.8 ± 9.03	87.5 ± 7.00
0.01	95.0 ± 7.98	94.7 ± 7.25	87.8 ± 6.30
0.10	92.6 ± 11.5	94.7 ± 4.71	87.1 ± 2.70
1.00	85.6 ± 4.41	85.8 ± 4.30	86.6 ± 5.60
10.0	59.2 ± 4.44	52.5 ± 2.70	47.1 ± 0.97
100.0	-6.30 ± 12.9	-36.9 ± 10.7	-9.80 ± 2.80

Table 13. The percentage of cell viability of LLC-PK1 cells incubated with VBL at various concentrations in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

VBL concentrations (ng/ml)	**% cell viability of LLC-PK1 (% of control)**			
	Vehicle control	**Verapamil 10 µg/ml (positive control)**	**Berberine 1 µg/ml**	***C. fenestratum* extract 100 µg/ml**
1.28×10^{-3}	88.2 ± 10.8	100.8 ± 4.60	79.0 ± 4.22	102.6 ± 2.01
6.40×10^{-2}	90.2 ± 8.00	99.3 ± 4.50	80.0 ± 2.76	104.4 ± 2.56
0.32	92.5 ± 2.30	104.6 ± 10.7	78.0 ± 5.28	101.4 ± 4.75
1.60	63.8 ± 4.50	35.4 ± 8.80	61.0 ± 5.42	78.2 ± 4.10
8.00	-5.70 ± 1.40	-20.2 ± 1.70	-32.0 ± 11.4	-31.0 ± 7.10
40.0	-2.50 ± 7.40	-19.2 ± 3.10	-47.0 ± 13.5	-25.4 ± 6.64
200.0	-4.90 ± 3.80	-17.0 ± 3.20	-16.0 ± 10.7	-8.40 ± 6.58

Table 14. The percentage of cell viability of LLC-MDR1 cells incubated with VBL at various concentrations in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

VBL concentrations (ng/ml)	**% cell viability of LLC-MDR1 (% of control)**			
	Vehicle control	**Verapamil 10 µg/ml (positive control)**	**Berberine 1 µg/ml**	***C. fenestratum* extract 100 µg/ml**
1.28×10^{-3}	96.7 ± 3.25	106.0 ± 2.73	98.3 ± 2.90	106.0 ± 2.74
6.40×10^{-2}	95.7 ± 6.33	102.0 ± 1.57	94.9 ± 2.47	99.0 ± 3.12
0.32	93.4 ± 3.58	104.0 ± 3.15	93.5 ± 3.59	102.0 ± 1.76
1.60	85.5 ± 3.16	93.6 ± 5.03	89.9 ± 3.01	80.6 ± 5.15
8.00	57.8 ± 12.3	24.4 ± 15.5	47.1 ± 7.77	37.0 ± 4.59
40.0	3.10 ± 2.79	-18.2 ± 11.5	-27.8 ± 17.1	-10.3 ± 5.81
200.0	-16.7 ± 4.89	-24.9 ± 6.80	-44.6 ± 10.4	-12.3 ± 2.31

Table 15. The percentage of cell viability of LLC-VBL cells incubated with VBL at various concentrations in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml for 48 hr determined by MTT assay. Data presented as mean ± SEM of three experiments.

VBL concentrations (ng/ml)	**% cell viability of LLC-VBL (% of control)**			
	Vehicle control	**Verapamil 10 µg/ml (positive control)**	**Berberine 1 µg/ml**	***C. fenestratum* extract 100 µg/ml**
1.28×10^{-3}	108.0 ± 6.20	101.5 ± 4.27	110.6 ± 14.7	114.0 ± 4.80
6.40×10^{-2}	106.0 ± 6.70	99.8 ± 2.45	102.6 ± 12.6	102.0 ± 2.67
0.32	101.0 ± 7.60	98.4 ± 7.21	97.0 ± 6.70	99.7 ± 5.47
1.60	98.0 ± 7.50	99.3 ± 6.90	88.0 ± 3.10	82.0 ± 4.08
8.00	66.0 ± 7.30	37.2 ± 7.48	56.6 ± 20.6	41.2 ± 6.91
40.0	3.00 ± 5.70	-40.8 ± 16.1	-32.5 ± 22.9	-27.2 ± 11.1
200.0	-16.0 ± 3.00	-42.1 ± 10.3	-58.4 ± 28.4	-27.0 ± 11.1

Table 16. The IC_{50} values for cytotoxic effect of VBL in LLC-PK1, LLC-MDR1 and LLC-VBL cells in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml. Data presented as mean ± SEM with N ≥ 3. (* = significantly different from VBL control in the same cell type, $P<0.05$).

Treatment	**IC_{50} values (ng/ml) [fold of control]**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
VBL (control)	2.04 ± 0.15	7.67 ± 1.18	13.71 ± 2.96 [1]
Verapamil 10 µg/ml (positive control)	1.40 ± 0.18	5.71 ± 0.77	6.86 ± 0.77* [0.50]
Berberine 1 µg/ml	3.25 ± 1.29	7.35 ± 0.38	10.1 ± 1.80* [0.74]
C. fenestratum extract 100 µg/ml	3.17 ± 0.21	5.23 ± 0.25	6.95 ± 0.90* [0.51]

Table 17. The relative of control values for Rh 123 accumulation in LLC-PK1, LLC-MDR1 and LLC-VBL cells when treated with vehicle control (PBS) or verapamil 10 µg/ml (positive control) or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml. Data presented as mean ± SEM with N = 3. (* = significantly different from vehicle control, $P<0.05$).

Treatment	**Rh 123 accumulation (nmole/mg) [Relative of control]**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
Vehicle control	0.052 ± 0.007 [1.00]	0.023 ± 0.002 [1.00]	0.029 ± 0.003 [1.00]
Verapamil 10 µg/ml (positive control)	0.038 ± 0.003 [0.74]	0.029 ± 0.002 [1.30 *]	0.041 ± 0.002 [1.41 *]
Berberine 1 µg/ml	0.047 ± 0.007 [0.90]	0.022 ± 0.003 [0.99]	0.026 ± 0.005 [0.87]
C. fenestratum extract 100 µg/ml	0.092 ± 0.009 [1.76 *]	0.056 ± 0.001 [2.49 *]	0.055 ± 0.002 [1.91 *]

Table 18. Effects of verapamil, berberine and *C. fenestratum* extract on Pgp-ATPase activity in LLC-PK1, LLC-MDR1 and LLC-VBL cells, Data presented as mean ± SEM with N=5. (* = significantly different from each control , $P < 0.05$).

Treatment	**Pgp-ATPase activity (nmol Pi/mg protein/min) [Fold of control]**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
Control	0.08 ± 0.01 [1.00]	0.78 ± 0.12 [1.00]	0.83 ± 0.09 [1.00]
Verapamil 10 µg/ml	0.10 ± 0.02 [1.25]	1.39 ± 0.25* [1.79]	1.36 ± 0.16* [1.65]
Berberine 1 µg/ml	0.09 ± 0.03 [1.13]	0.81 ± 0.06 [1.05]	0.87 ± 0.12 [1.05]
C. fenestratum extract 100 µg/ml	0.03 ± 0.02 [0.38]	0.48 ± 0.09* [0.62]	0.39 ± 0.04* [0.47]

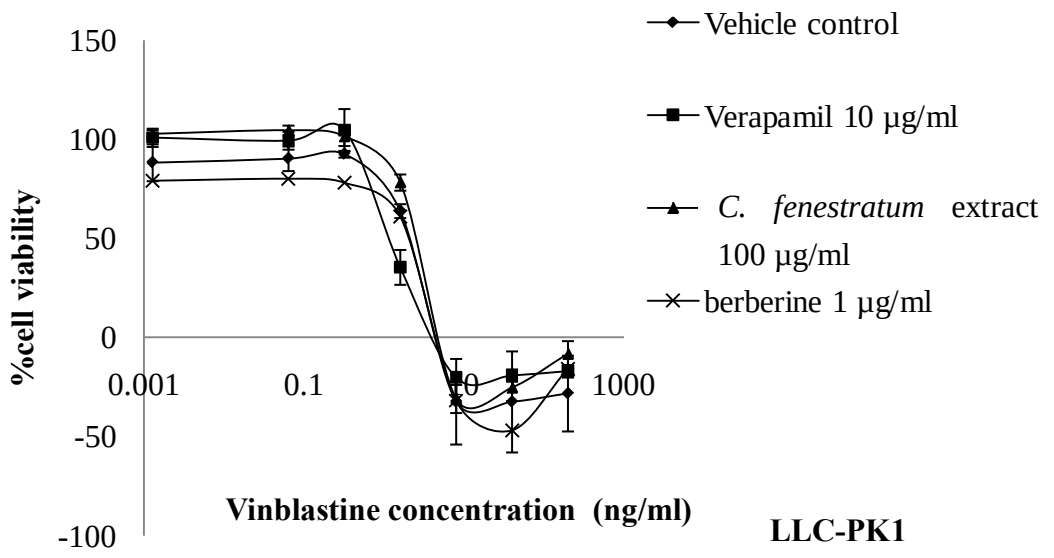
Table 19. Effects of berberine and *C. fenestratum* extract on the Pgp-ATPase activity in LLC-PK1, LLC-MDR1 and LLC-VBL cells when co-treated with verapamil 10 µg/ml. Data presented as mean ± SEM with N=5. (*= significantly different from each positive control, $P < 0.05$).

Treatment	**Pgp-ATPase activity (nmol Pi/mg protein/min) [Relative fold of Verapamil control]**		
	LLC-PK1	**LLC-MDR1**	**LLC-VBL**
Verapamil 10 µg/ml	0.10 ± 0.02 [1.00]	1.39 ± 0.25 [1.00]	1.36 ± 0.16 [1.00]
Berberine 1 µg/ml co-treated with Verapamil 10 µg/ml	0.08 ± 0.03 [0.8]	0.74 ± 0.19* [0.53]	0.75 ± 0.09* [0.55]
C. fenestratum extract 100 µg/ml co-treated with Verapamil 10 µg/ml	0.06 ± 0.01 [0.6]	0.45 ± 0.19* [0.32]	0.49 ± 0.12* [0.36]

Table 20. The relative of Pgp levels in LLC-MDR1 and LLC-VBL cells treated with berberine or *C. fenestratum* extract compared to that of vehicle control. These cells were cultured in the presence or absence of berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml. Data presented as mean ± SEM of three experiments.

Treatment	**Ratio of Pgp and actin [Relative Pgp levels compare to vehicle control]**	
	LLC-MDR1	**LLC-VBL**
0.5 % DMSO (vehicle control)	1.20 ± 0.35 [1.00]	1.14 ± 0.33 [1.00]
Berberine 1 µg/ml	1.05 ± 0.23 [0.90]	1.05 ± 0.29 [0.93]
C. fenestratum extract 100 µg/ml	1.12 ± 0.22 [0.98]	1.22 ± 0.48 [1.01]

A


150
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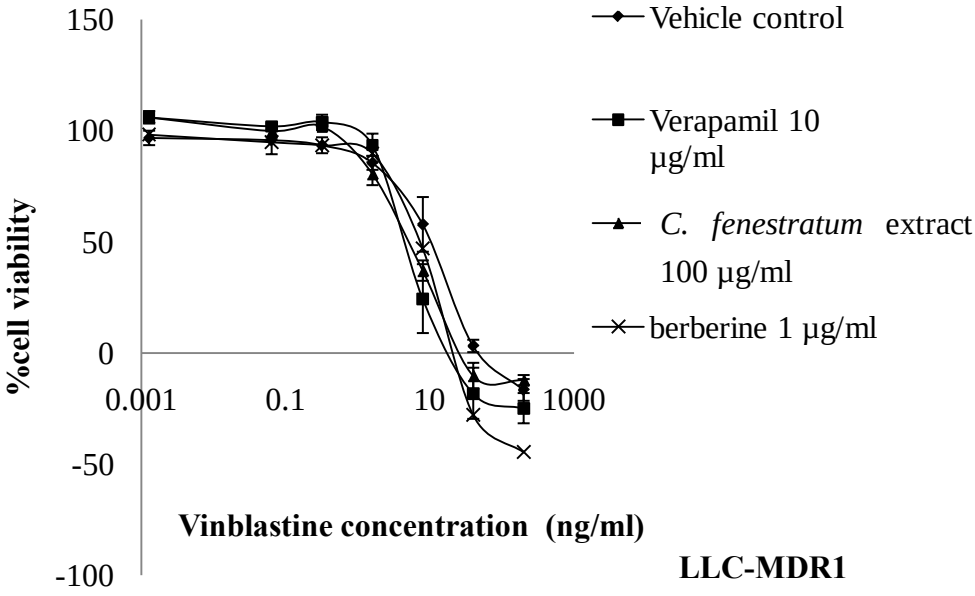
Vinblastine concentration (ng/ml)
Vehicle control
Verapamil 10 µg/ml
C. fenestratum extract 100 µg/ml
berberine 1 µg/ml
LLC-PK1

B


150
100
50
0
-50
-100
%cell viability
0.001
0.1
10
1000
Vinblastine concentration (ng/ml)
Vehicle control
Verapamil 10 µg/ml
C. fenestratum extract 100 µg/ml
berberine 1 µg/ml
LLC-MDR1

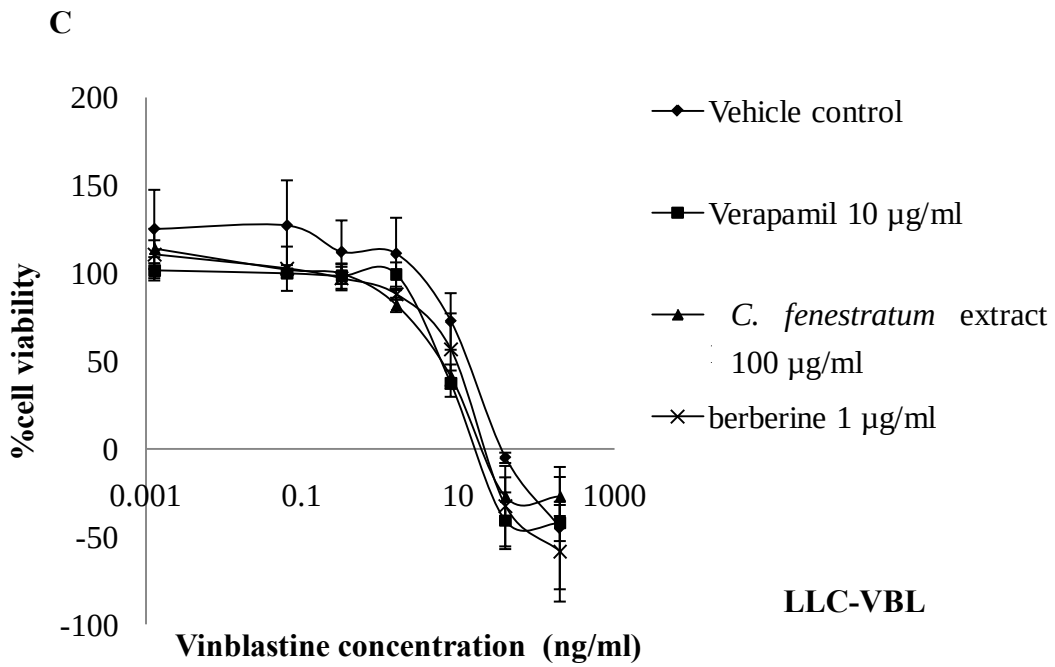


Figure 20. Concentration-dependent curves demonstrating the effect on the VBL-cytotoxicity in LLC-PK1 (A), LLC-MDR1 (B) and LLC-VBL (C) cells. They were tested in the presence and absence of verapamil (positive control) 10 µg/ml or berberine 1 µg/ml or *C. fenestratum* extract 100 µg/ml and incubated for 48 hr. Data were shown as percent of cell viability comparing with vehicle control cells (0.5% DMSO) measured by MTT assay. Each point presented the mean ± SEM values for more than three independent experiments performed in triplicate.

VITA

Miss Nareerat Thongda was born on January 15, 1986 in Bangkok, Thailand. She received her Bachelor in Biochemistry and Biochemical Technology from the Faculty of Sciences, Chiang Mai University, in 2008. Since graduation, she entered the Master's degree program in Biomedicinal Chemistry at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.