ฤทธิ์ของ ECa-233 และ ASIATICOSIDE ต่อการทำงานของ
พี-กลัยโคโปรตีน

นางสาว นฤมล ร่วมสุข

EFFECTS OF ECa-233 AND ASIATICOSIDE ON

P-GLYCOPROTEIN FUNCTION

Miss Narumol Ruamsuk

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By   Miss Narumol Ruamsuk

Field of Study   Pharmacology

Thesis Advisor   Assistant Professor Suree Jianmongkol, Ph.D.

Thesis Co-Advisor   Associate Professor Thitima Pengsuparp, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

…………………………………………….. Dean of the Graduate School

(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…………………………………………….. Chairman

(Associate Professor Sopit Thamaree, Ph.D.)

……………………………………….……. Thesis Advisor

(Assistant Professor Suree Jianmongkol, Ph.D.)

…………………………………………….. Thesis Co-Advisor

(Associate Professor Thitima Pengsuparp, Ph.D.)

…………………………………………….. Examiner

(Associate Professor Mayuree Tantisira, Ph.D.)

…………………………………………….. External Examiner

(Associate Professor Nusara Piyapolrungroj, Ph.D.)

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดมาตรฐานบัวบก (ECa-233) และ asiaticoside (ATS) ต่อการทำงานของ P-glycoprotein (P-gp) และผลในการเพิ่มความเป็นพิษ ต่อเซลล์ของยา vinblastine (VBL) ในเซลล์เพาะเลี้ยง Caco-2 และ LLC-GA5-COL300 (human P-gp overexpressed-LLC-PK$_1$) ในการทดสอบการทำงานของ P-gp ทำโดยดูผลต่อการสะสม ของ Calcein-AM ในเซลล์ และการทดสอบความเป็นพิษต่อเซลล์ของ VBL ทำโดยวิธี MTT assay ผลการศึกษาพบว่า ECa-233 (1000 µg/mL) สามารถเพิ่มการสะสมของ Calcein ในเซลล์ LLC-GA5-COL300 ได้ 84 % และในเซลล์ Caco-2 ได้ 42% นอกจากนี้ยังพบว่า ECa-233 สามารถ เพิ่มการสะสมของ Calcein ในเซลล์ LLC-PK$_1$ ซึ่งมีการแสดงออกของ P-gp น้อยกว่าเซลล์ LLC-GA5-COL300 ได้ 55% จึงเป็นไปได้ว่าผลของ ECa-233 ไม่เกี่ยวข้องกับการทำงานของ P-gp แต่ มีผลต่อตัวขนส่งยาอื่นๆ ที่เกี่ยวข้องกับการขนส่ง Calcein เมื่อดูผลในการเพิ่มความเป็นพิษของ VBL เมื่อให้ร่วมกับ ECa-233 (1000 µg/mL) ในเซลล์ Caco-2 พบว่า ECa-233 มีผลเพิ่มการตาย ของเซลล์จาก VBL ได้อย่างมีนัยสำคัญ ค่า IC$_{50}$ ของ VBL ในกลุ่มที่ได้รับ ECa-233 ร่วมด้วยมีค่า ลดลง 5 เท่า นอกจากนี้ยังพบว่า ATS สามารถเพิ่มความเป็นพิษของ VBL ในเซลล์ Caco-2 ได้ อย่างมีนัยสำคัญ (2 เท่า) โดยไม่มีผลต่อการทำงานของตัวขนส่งยาออกนอกเซลล์ จึงอาจสรุปได้ ว่า ECa-233 และ ATS มีผลเพิ่มความเป็นพิษของ VBL ได้โดยอาจออกฤทธิ์ผ่านทางกลไกอื่นที่ไม่ เกี่ยวข้องกับการทำงานของ P-gp

สาขาวิชา____เภสัชวิทยา_____ ลายมือชื่อนิสิต_____

ปีการศึกษา___2552_____ ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก_____

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม_____

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NARUMOL RUAMSUK : EFFECTS OF ECa-233 AND ASIATICOSIDE ON P-GLYCOPROTEIN FUNCTION. THESIS ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., THESIS CO- ADVISOR : ASSOC. PROF. THITIMA PENGSUPARP, Ph.D., 78 pp.

In the present study, standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) were tested for their effects on P-gp function as well as their potentiative effect on vinblastine (VBL)-induced cytotoxicity in the in vitro cell culture models of Caco-2 and LLC-GA5-COL300 (human P-gp overexpressed-LLC-PK$_1$) cell lines. In this study, the P-gp activity was determined by calcein-AM accumulation assay whereas the cytotoxic activity was evaluated by an MTT assay. The results revealed that ECa-233 (1000 µg/mL) was able to increase the intracellular level of calcein in LLC-GA5-COL300 by 84 % and Caco-2 cells by 42%. In addition, ECa-233 also increased calcein accumulation in LLC-PK$_1$ by 55%. Because the expression of P-gp is significant lower in LLC-PK$_1$ cells than in LLC-GA5-COL300 cells, it was unlikely that P-gp was a major target of ECa-233 action. It was possible that ECa-233 could affect other membrane efflux pumps involving with calcein transport. Furthermore, the presence of ECa-233 (1000 µg/mL) in the co-treatment study with VBL in Caco-2 cells resulted in significant higher number of VBL-induced cell death. The apparent IC$_{50}$ of VBL decreased by 5 folds in the presence of ECa-233. Moreover, ATS could also significantly enhance the cytotoxicity of VBL in caco-2 cells (by 2 folds) without any modulating effects on efflux pumps. Taken together, the cytotoxic effects of VBL may be enhanced by ECa-233 and ATS through mechanisms other than P-gp modulations.

Field of Study : Pharmacology   Student's Signature

Academic Year : 2009   Advisor's Signature

Co-Advisor's Signature

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
ATS	asiaticoside
°C	degree Celsius
C. asiatica	*Centella asiatica*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
e.g.	example gratia (for example)
et al.	*et alii*, and others people
Etc.	et cetera (and other similar things)
EDTA	Ethylenediamine tetraacetic acid
FBS	fetal bovine serum
Fig	figure
g	gram (s)
h	hour (s)
HBSS	hanks' balanced salts
HCl	Hydrochloric acid
IC_{50}	concentration required to achieve 50% inhibition
kDa	kiloDalton
L	liter (s)
mg	milligram (s)
µg	microgram (s)
µl	microlitre (s)
µl	micromolar (s)
M	molar
M199	Medium 199
mg	milligram (s)
min	minute (s)

mL	mililitre (s)
mM	milimolar (s)
MDR	multidrug resistance
MRP	multidrug resistance-related proteins
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular wieght
nM	nanomolar (s)
nmole	nanomole (s)
NaCl	sodium chloride
NaOH	sodium hydroxide
PBS	phosphate-buffer saline
P-gp	permeability glycoprotein
rpm	round per minute
sec	second
SEM	standard error of mean

CHAPTER I

INTRODUCTION

Background and rationale

Multi-drug resistance (MDR) is one of the major reasons for the failure of chemotherapy in most cancer patients. Certain tumors are intrinsically resistant to treatment whereas others acquire resistance with exposure to structurally unrelated drugs. This phenomenon could result from overexpression of membrane efflux protein to pump the drugs out of the cells. Consequently, the intracellular concentration of these drugs decreased below its effective threshold level (Juliano and Ling, 1976). P-glycoprotein (P-gp), in particular, has been linked to MDR associated with a variety of cancers (Ambudkar *et al.*, 1999; Stouch and Gudmundsson, 2002). P-gp belongs to the ATP-binding cassette (ABC) superfamily of transporters that utilize the energy of ATP hydrolysis to translocate a wide range of substrates across a variety of cellular membranes. Examples of these compounds include anthracyclines (e.g., doxorubicin), *Vinca* alkaloids (e.g., vinblastine, vincristine), podophyllotoxins (e.g., etoposide) and taxanes (e.g., paclitaxel) (Teodori *et al.*, 2002; Krishna and Mayer, 2000). In addition to the MDR cancer cells, P-glycoprotein is also found in various normal epithelial tissues such as liver, intestine, kidney, and endothelia of the blood– brain barrier (Thiebaut *et al.*, 1987, Cordon-Cardo *et al.*, 1990).

A number of investigations have explored the compounds that can reverse MDR chemoresistant cells to become sensitive for chemotherapeutic agents. These compounds are called either chemosensitizers, modulators or MDR-reversing agents. These modulators have a broad spectrum of chemical structures, which cause difficulty in identifying the chemosensitizing properties of their structures. Generally, P-gp modulators required hydrophobic property with or without cationic functional groups at physiological pH (Stouch and Gudmundsson, 2002). The examples of these modulators include anthracyclines (doxorubicin), *Vinca* alkaloids (vincristine, vinblastine), podophyllotoxins (etoposide), and taxanes (taxol) (Sauna *et al.*, 2001, Sawicka *et al.*, 2004), verapamil (Tsuruo *et al.*, 1981), cyclosporine D analog (valspodar

or PSC 833) (Sikic, 1999), and novel hydrophobic peptides such as reversins 121 and 205 (Sharom *et al.*, 1999).

The clinical results of known P-gp modulators in overcoming MDR in chemotherapy have been quite disappointed, mainly because of the dose-limiting toxicity of these compounds. Hence, compounds of natural origin with MDR modulating property especially those from edible plants or herbs emerged as new potential MDR reversing agents. Certain dietary phytochemicals and herbal supplements, e.g., piperine (Bhardwaj *et al.*, 2002), curcumin (Anuchapreeda *et al.*, 2002) and capsaicin (Yi *et al.*, 2006) have been found to inhibit the P-gp function (Nabekura *et al.*, 2005). These natural compounds may be less toxic than the synthetic inhibitors although their clinical applications remain to be verified.

Centella asiatica is a pan-tropical plant in Thailand. This herbal plant has been used to relieve symptoms in various pathologic conditions including mental disorders, inflammation (Vogel *et al.*, 1990), circulatory problems (Dabral and Sharma, 1983) and immune system deficiencies (Lepetit, 2005). In addition, this plant contained the pharmacological activities in wound healing (Shukla *et al.*, 1999), antitumor (Babu *et al.*, 1995) and cognition enhancement in experimental rats (Kumar and Gupta, 2002; Gupta *et al.*, 2003). As known, the major components in *Centella asiatica* included triterpene saponins, in particular, asiaticoside and madecassoside, and their aglycone (asiatic acid and madecassic acid, respectively) (Eun and Lee, 1985; Bilbo *et al.*, 1995; Izu *et al.*, 1992). Asiaticoside at sub-cytotoxic concentration (0.5 mg/ml) could induce apoptosis, and enhance the cytotoxic effect of vincristine in cancer cells (Huang *et al.*, 2004).

Being natural compounds, the characteristic property and selectivity of the standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) toward different cell types are very limited, especially in the normal cells and the cells with high MDR activity. Extract from *Centella asiatica* and its ingredients including asiaticoside might be able to potentiate the effect of vincristine through the mechanism involving P-gp inhibition. In view of the molecular structure of the triterpene compounds found in this

plant, it was possible that the extracts and asiaticoside might be able to inhibit P-gp function. In this study, the inhibitory effects of the extract of *Centella asiatica* on P-gp function as well as its potentiative effects with cytotoxic agents in the cancer cells would be reported.

Objective

1. Determine the inhibitory effect of standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) against P-gp function.

2. Examine the potentiative effects of standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) on vinblastine-induced cytotoxicity.

Hypothesis

Standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) could enhance the cytotoxic effects of VBL in cells with high P-gp expression through inhibition of P-gp mediated drug efflux.

Expected Benefit and Application:

The information of the ECa-233 and asiaticoside of the *Centella asiatica* may be useful for further studies in the application of *Centella asiatica* extract in traditional medicine development.

Research Plan

ECa-233 and ATS

↓

Cytotoxic effects (MTT assay)

↓ Non-toxic dose

Effect on P-gp function (Calcein-AM acuumulation)

↓

Potentiative effects on vinblastine-induced cytotoxicity (MTT assay)
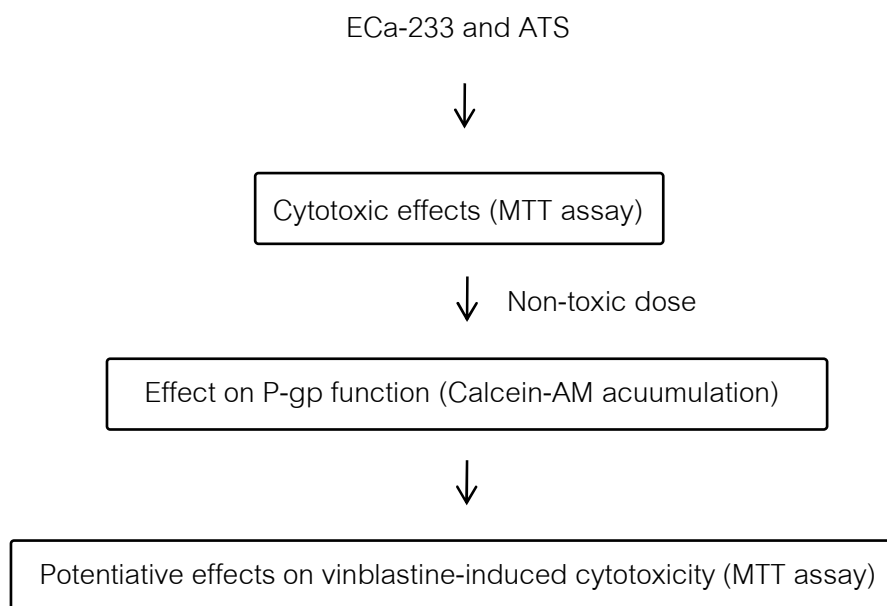
CHAPTER II

LITERATURE REVIEWS

1. P-glycoprotein (P-gp)

Drug transporters consist of uptake and efflux transporters, indicating intracellular or extracellular transport directions. Most efflux transporters belong to the ATP-binding cassette (ABC) superfamily of membrane proteins, including multidrug resistance (MDR) such as ABCC1 (p-gp) and ABCC2 (multidrug resistance-related proteins, MRP) and ABCG2 (breast cancer resistance protein, BCRP). These transporters have influence on the intracellular concentration of numerous compounds in a variety of cells and tissues. Being an efflux pump, these transporters play a major role as defense mechanism against penetration of xenobiotics such as bilirubin, arsenic (Hoffmann and Kroemer, 2004) or transmembrane transport of various endogenous compounds such as aldosterone, corticosterone, cortisol, progesterone (Fujise *et al*., 2002). The pump is fueled by ATP hydrolysis, leading to phosphorylation of transporter protein. Consequently, it can transport its substrates out of the cells.

1.1 Structure of the multidrug resistance P-glycoprotein

P-glycoprotein (P-gp) is the best known efflux pump responsible for multidrug resistance in chemotherapy. P-gp is a plasma membrane protein that acts as an ATP-dependent efflux pump, therefore, it reduces the intracellular accumulation of various structurally unrelated anticancer drugs. Its presence in organ systems that influence drug absorption (intestine), distribution to site of action (central nervous system and leukocytes), and elimination (liver and kidney), as well as several other tissues and overexpression in tumoral cell lines confers an MDR phenotype. P-gp is also found on the apical surface of pancreatic ductules and on the luminal surface of capillary endothelial cells in the brain and testes (Matheny *et al*., 2001; Stavroskaya, 2000; Larsen *et al*., 2000).

P-gp is encoded by a small family of two genes in human (*MDR1* and *MDR2*), and three genes in rodents (*mdr1*a or *mdr3*, *mdr1*b or *mdr1* and *mdr2*).

However, only P-gp encoded with *MDR1* genes involve in the MDR phenotype (Endicott and Ling, 1989; Gottesman and Pastan, 1993). The *mdr1* P-gp is a 170kDa membrane protein which consists of two duplicated halves. Each contains six predicted hydrophobic membrane spanning segments followed by a highly conserved intracellular ATP-binding site (Chen *et al*., 1986), as shown in Figure 1. P-gp can be phosphorylated on several sites (Hamada *et a*l., 1987) through several kinases, including protein kinase C and the cAMP-dependent protein kinase A (Chambers *et al*., 1992; Mellado and Horwitz, 1987). Phosphorylation of P-gp appears to modulate its activity and the level of drug resistance. P-gp detection has become an important tool in the investigation of MDR.
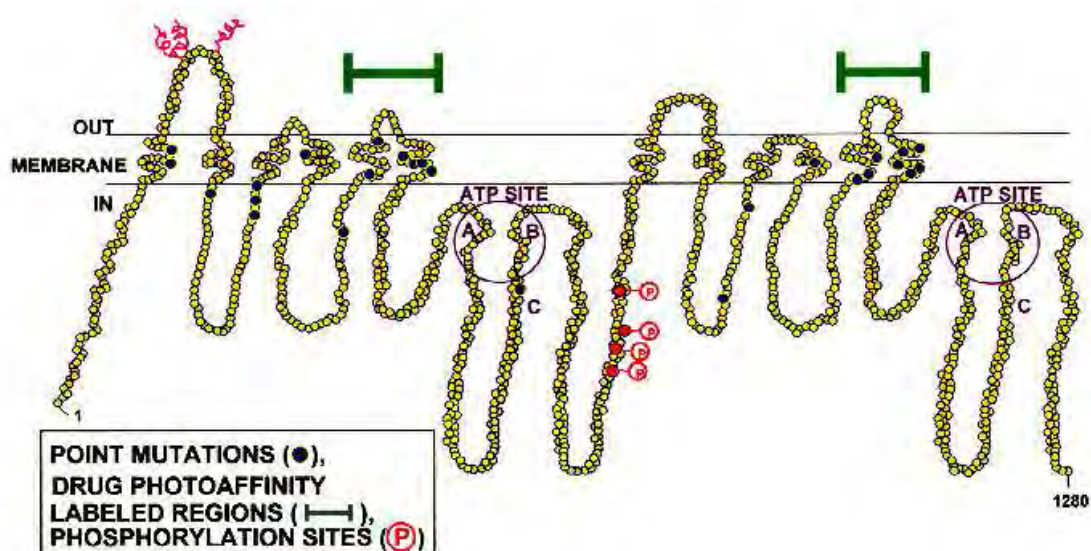
Figure 1: The basic structural unit of ABC transporters (P-glycoprotein, P-gp) contains 1280 amino acids, consists of six transmembrane domains followed by an ATP-binding cassette. In plasma membrane transporters (such as the MDR transporter illustrated), two of these units are fused so that the transporter contains 12 transmembrane domains and two ATP-binding sites and the first extracellular loop is heavily N-linked carbohydrates (Lee *et al*., 1998).

1.2 Agents that interact with P-gp

Several chemicals with different molecular structure can bind to P-gp and become P-gp substrates or P-gp inhibitors. Furthermore, a number of compounds can affect the expression of P-gp (Matheny *et al.*, 2001).

1.2.1 P-gp substrates

Generally, the known P-gp substrates share certain common features including hydrophobicity and a positive charge at pH 7 (Sarkadi and Miller, 1997; Ambudkar *et al.*, 1999). Examples of P-gp substrates are anticancer drugs (*Vinca* alkaloids, anthracyclines, and epipodophyllotoxins), antibiotics, and other cytotoxic agents (Table 1) (Cascorbi, 2006; Sauna *et al.*, 2001).

Table 1: Typical substrates of P-gp (Cascorbi, 2006)

Groups of compound	Drugs
Anticancer drugs	docetacel, doxorubicin, etoposide, imatinib, paclitaxel, teniposide, vinblastine, vincristine
Steroids	dexamethason, Methylprednisolone
Immunosuppressants	cyclosporine, sirolimus, tacrolimus
HIV protease inhibitors	amprenavir, indinavir, nelfinavir, saquinavir, ritonavir
Antibiotics	erythromycin, ofloxacin
β-blockers:	bunitrolol, carvedilol, celiprolol, tanilolol;
Ca^{2+}-channel blockers:	diltiazem, verapamil,
Cardiac drugs	digoxin, digitoxin, quinidine
HMG-CoA inhibitors	atorvastatin, lovastatin
H_1-Antihistamines	fexofenadine, terfenadine
Antiemetics	ondansetron
Fluorescent dyes	rhodamin 123
Miscellaneous	amitriptyline, colchicine, itraconazole, lansoprazole, loperamide, losartan, morphine, phenytoin, rifampicin

1.2.2 P-gp inhibitors

Several non-cytotoxic compounds have been reported as chemosensitizers or MDR modulators. These compounds have been evaluated both *in vitro* and in clinical trials for their ability to reverse the P-gp mediated multi-drug resistance (MDR) in cancer. It is possible that the MDR modulators can interfere with substrate binding, ATP hydrolysis, ATPase activity, and inhibition of protein kinase C (PKC) (Ambudkar *et al.*, 1999; Wang *et al.*, 2003).

The molecular structure of P-gp inhibitors contains planar ring with basic nitrogen atom in an extended side chain of the aromatic ring (Figure 2). Moreover, the chemosensitizing activity increases if the nitrogen atom is incorporated into a non aromatic ring (Wiese and Pajeva, 2001). The examples of MDR reversing agents (Table 2) include verapamil (Tsuruo *et al.*, 1981), reversins 121 and 205 (Sharom *et al.*, 1999), and the cyclosporine D analog, Valspodar (PSC 833) (Sikic, 1999). However, subsequent animal and clinical studies have yielded disappointing results, mainly because of the dose-limiting toxicity of these compounds.

Table 2: Examples of MDR reversing agents (Krishna and Mayer, 2001)

Category	Examples
calcium channel blockers	verapamil, nifedipine, diltiazem, beperidil
calmodulin inhibitors	trifluperazine, prochlorperazine, *trans*-flupenthixol
coronary vasodilators	dipyridamole, amiodarone
indole alkaloids	vindoline, reserpine
quinolines	chloroquine, quinine
lysomotropic agent	nigericin, monexin
hormones and antihormones	progesterone, tamoxifenm
cephalosporins	cefoperazone, ceftriaxone
anticancer drug analogs	N-acetyldaunorubicin, C-20'-vinblastine
cyclosporins	cyclosporine A
protein kinase inhibitors	staurosporine, H-87
surfactants and lipids	cremophor-EL, Tween 80, PS
antibodies	MRK16, UIC-2
cyclosporine A analogs	PSC-833
cyclopropyldibenzosuberane	LY335979
acridonecarboxamide	GF120918
diketopiperazine	XR9051
diarylimidazole	OC144-093

Verapamil

Cyclosporine

PSC-833

XR-9576
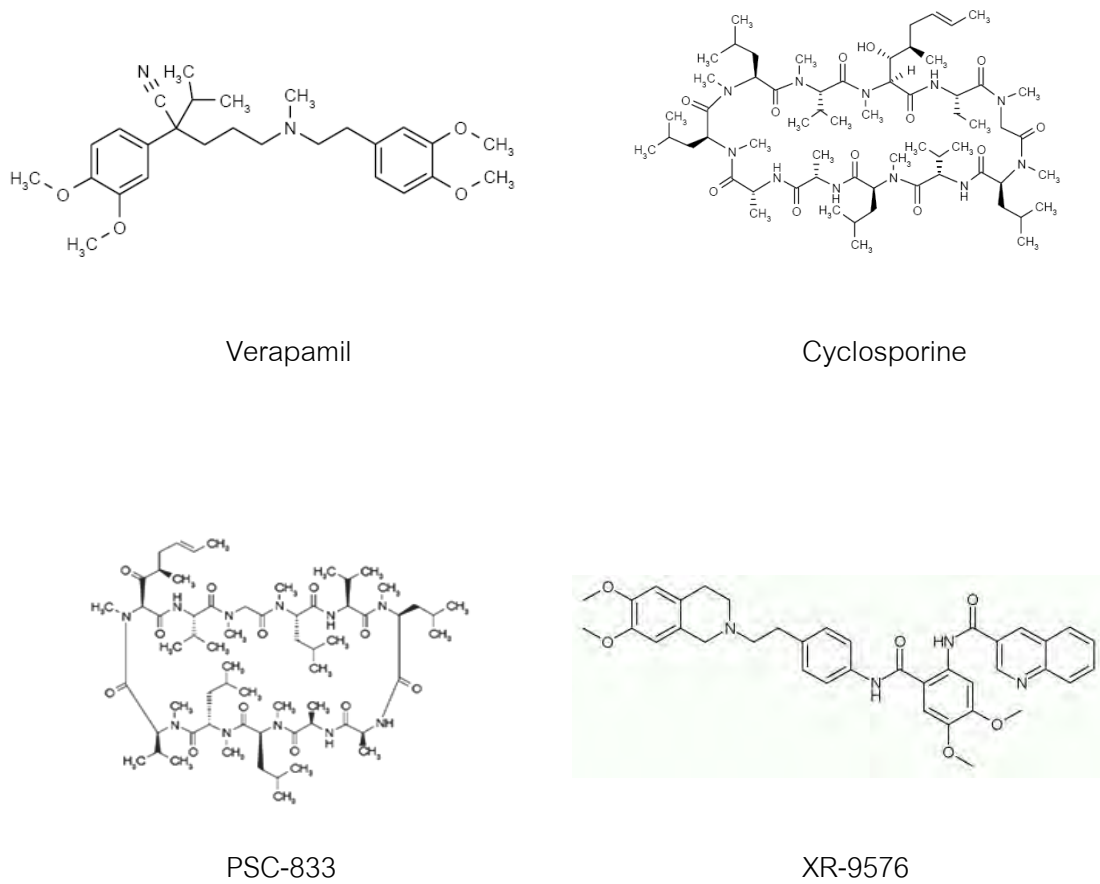
Figure 2: Some structures of P-gp modulators
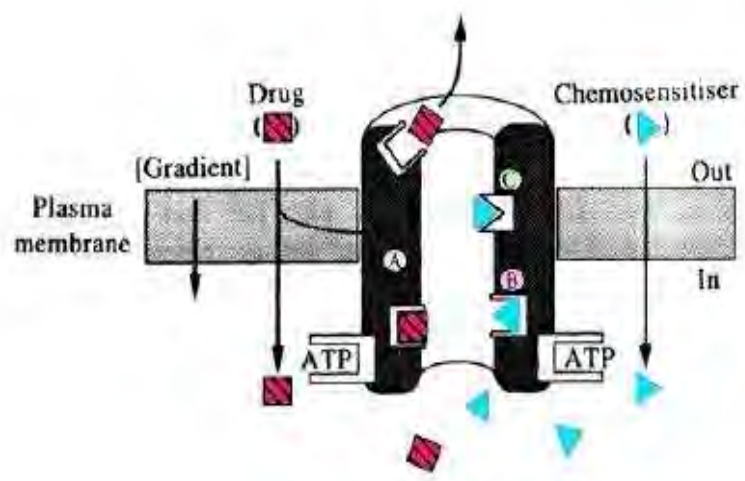
Figure 3: Functional representation of P-gp (Gottesman and Pastan, 1993).

2. Natural products as a potential MDR modulator

More recently, certain dietary phytochemicals and herbal supplements, such as piperine (Bhardwaj *et al.*, 2002), curcumin (Anuchapreeda *et al.*, 2002) sulforaphane and erucin (Kristin and Elizabeth, 2007), quinine (Avendano and Menendez, 2002), ecteinascidins (Fricker, 2001) and capsaicin (Yi *et al.*, 2006) and capsaicin (Yi *et al.*, 2006), have been found to inhibit the P-gp function. These natural compounds may be less toxic than the synthetic inhibitors, although their clinical applications remain to be verified.

Like capsaicin and curcumin, terpenoids such as (R)-(+)-citronellal, abietic acid and glycyrrhetic acid may inhibit P-gp-mediated transport. They can interact with P-gp substrates in the intestinal absorption process by increases in the apical-to-basolateral transport and decreases in the basolateral-to-apical transport and efflux ratio (Yoshida *et al.*, 2006) and triterpenes such as pisosterol has been shown to have antitumor activity against seven tumor cell lines, especially leukemia and melanoma cells (Montenegro *et al.*, 2004) .
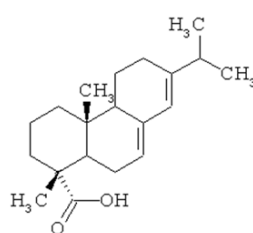
Figure 4: Some structures of natural products

3. Cell cultures for determination of P-gp activity

3.1 Caco-2 cells

Caco-2 cells, a human colonic adenocarcinoma cell line (Pinto *et al.*, 1983), is widely used across the pharmaceutical industry as an *in vitro* model of the human small intestinal mucosa to predict the absorption of orally administered drugs. When cultured on microporous filter, Caco-2 differentiates spontaneously to enterocytes under conventional condition upon reaching confluence after 14-21 days (Pascale, 1998). The cells in the monolayer possess of brush border on the apical surface, tight junction between adjacent cells which prevent the diffusion of solutes and expression hydrolyses and trypical microvillar transporter. Several active transport systems are expressed in Caco-2 cells (e.g., sugars, amino acids, dipeptides, bile acids, vitamins and hormones) and enzymes found in the brush border membranes (e.g., amimopeptidase, alkaline phosphatase, sucrase, dipeptidyl aminopeptidase, and γ-glutanyl transpeptidase) (Gan and Thakker, 1997). In addition, Caco-2 cells have expression of energy-dependent drug efflux pumps such as P-glycoprotein, multidrug resistant associated protein and lung cancer associated resistance protein. Thus, Caco-2 cells seem to be suitable with well to develop barrier properties in drug absorption studies (Ferrec *et al.*, 2001) and serving as the basis for the creation of innovative new models that are contributing to our understanding of drug efflux transporters such as P-glycoprotein (ABCB1) and BCRP (ABCG2).

3.2. LLC-PK$_1$ and LLC-GA5-COL300 cell lines

The LLC-PK$_1$ cell line was derived from the kidney of a normal, healthy male pig (*Sus scrofa*) (Hull *et al.*, 1976). This cell line has a structure and function similar to those of renal proximal tubular cells (Decorti *et al.*, 2001). The LLC-PK$_1$ line is often used as a model for epithelial tissue, as well as in a wide spectrum of pharmacologic and metabolic research investigations. As a model for human P-gp, LLC-GA5-COL300 cells was human *MDR1* cDNA -transfected epithelial cells, which was derived from a clone of LLC-PK$_1$ cells stably transfected with a cDNA encoding the human P-gp (Ueda *et al.*, 1992). A highly polarized epithelium formed by LLC-GA5-COL300 cells that

expressed human P-glycoprotein specifically on the apical surface showed a multidrug-resistant phenotype (Ueda *et al*., 1992). LLC-GA5-COL300 cells have P-gp activity more than parental cells as determined by transepithelial transport and cellular accumulation of P-gp substrate (Saeki *et al*., 1993).

4. *Centella asiatica*

4.1 *Centella asiatica*

Centella comprises some 50 species including the most ubiquitous species *Centella asiatica*. This perennial creeper flourishes abundantly in moist areas in tropical and sub-tropical regions (James and Dubery, 2009). *C. asiatica*, also known as Gotu kola or Indian pennywort (Bruneton, 1995), is a medicinal plant that has probably been used since prehistoric times for various medicinal and cosmetic purposes. This plant is listed as a drug in the Indian Herbal Pharmacopoeia, the German Homeopathic Pharmacopoeia (GHP), the European Pharmacopoeia, and the Pharmacopoeia of the People's Republic of China (Schaneberg *et al*., 2003). According to World Health Organisation (WHO) monographs, Herbal *Centella* should not contain less than 2% of the triterpene ester glycosides asiaticoside and madecassoside (World Health Organisation, 1999). (Figure 5)

Figure 5: *Centella asiatica*

C. asiatica contains several active constituents, of which the most important are the triterpenoid glycoside (saponines). In addition, Centella contains other components including phytosterols (campesterol, stigmasterol and sitosterol) and a volatile oil consisting of vallerin, camphor, cineole and an unidentified terpene acetate that comprises 35% of the total oil content. Vitamins B and C, flavonoids, tannins, several amino acids, sugars, an alkaloid named hydrocotyline and the element (Ca, Mg and Na) also have been found in this plant (Leung and Foster, 1998).

The triterpenoid glycosides (Figure 6) include asiaticoside, asiaoside, Asiatic acid, madecassic acid, madecassoside, oxyasiaticoside, brahminoside, brahmoside and centelloside.
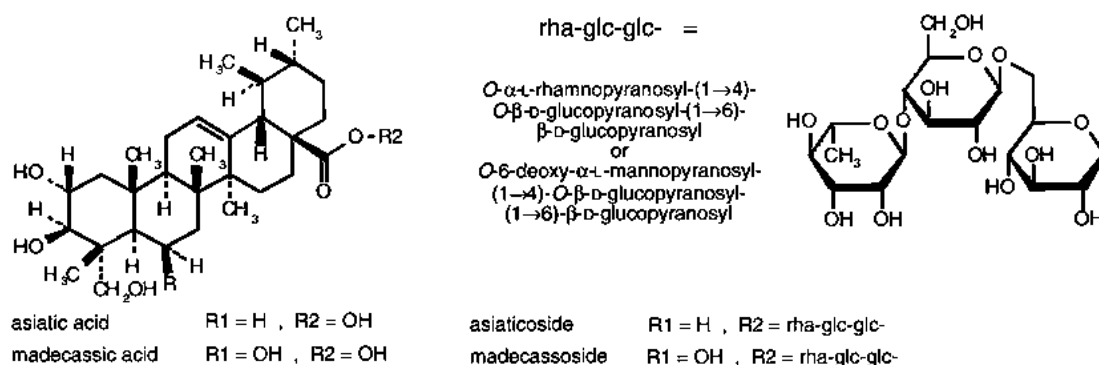
Figure 6: Major chemical constituents in *Centella asiatica*

When considering the molecular structure of triterpene compounds found in *C. asiatica* compared with compounds that MDR modulator was found that the main structure together with the planar aromatic ring. It was possible that the extracts and asiaticoside might be able to inhibit P-gp function.

4.2 Pharmacological activities of *C. asiatica* and asiaticoside.

Antibacterial

The essential oil of *C. asiatica* showed a broad spectrum of antibacterial activities against Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Shigella sonnei*)

organisms. Activity against Gram-positive bacteria was greater than against Gram-negatives. Germacrene compounds in the essential oil are known to be strong antimicrobial and antitumour agents (Oyedeji and Afolayan, 2005).

Anti-inflammatory

Asiaticoside was reported to reduce acute skin reactions in radiotherapy treated rat (Chen *et al.*, 1999)

Antioxidant

Oral treatment with 50 mg/kg/day of crude methanol extract of *C. asiatica* for 14 days significantly increased the anti-oxidant enzymes, like superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHPx), and anti-oxidants like glutathione (GSH) and ascorbic acid decreased in lymphoma-bearing mice (Jayashree *et al.*, 2003)

Antipsoriatic

A cream formulation of *C. asiatica* was found to be successful in treatment of psoriasis in 7 patients (Natarajan and Paily, 1973).

Anti-tumor

Methanolic extract of *C. asiatica* (100 µg/ mL) showed 100% cytotoxicity to 2 tumor cell lines (Dalton's ascites tumour cells and Ehrlich ascites tumour cells). The acetone fraction of *C. asiatica* extract, a partially purified fraction (3.5 and 8 µg/mL), inhibited the proliferation of mouse lung fibroblast cells after exposure for 6-7 days at 37°C. Both the crude extract and the acetone fraction of *C. asiatica* significantly reduced the development of murine solid tumors when administered simultaneously with tumour transplantations or given 10 days prior to tumour transplantation. The latter finding suggested a mechanism which involved stimulation of the immune system. The crude extract also significantly reduced ascites tumour growth and increased the life span of tumor bearing mice. The mechanism might involve inhibition of DNA synthesis

(Babu *et al*., 1995). Asiaticoside at sub-cytotoxic concentration could induce apoptosis, and enhance the cytotoxic effect of vincristine in cancer cells (Huang *et al*., 2004).

Anxiety

C. asiatica is used in Ayurvedic medicine for the treatment of anxiety. Triterpenoids have been shown to soothe anxiety and boost mental function in mice. After assessing baseline measurements of acoustic startle response (ASR), mood self-rating scale, heart rate, and blood pressure, 40 healthy subjects were randomized to receive 12 g non-standardized *C. asiatica* dissolved in 300 mL grape juice or placebo. Evaluations were recorded at 30, 60, 90, and 120 min after beginning therapy. *C. asiatica* significantly decreased ASR amplitude compared to placebo at 30 and 60 min; heart rate, blood pressure, and mood did not change (Bradwejn *et al*., 2000).

Diabetic microangiopathy

Diabetes is characterized by increased skin blood flow and decreased venous return, resulting in blood pooling. Forty-eight patients with diabetic microangiopathy were randomized to one of three treatment groups for 6 months: 60 mg total triterpenic fraction of *C. asiatica* (TTFCA) twice daily, placebo, or no treatment. Using laser doppler flowmetry measurements, the researchers concluded those taking TTFCA had significant reductions in skin blood flow at rest after 3 and 6 months compared to baseline values. In addition, VAR scores (decrease of skin blood flow on standing) increased significantly from 6.4 % to 23.9 % at 3 months and 25.9 % at 6 months. During the investigation period, pO_2 increased while pCO_2 values decreased significantly in the *C. asiatica* group. Fasting blood sugar and hemoglobin A1C values did not change (Cesarone *et al*., 2001).

Gastric ulcer (World Health Organisation, 1999)

Extract of *C. asiatica* effectively treated stress-induced stomach and duodenal ulcers in humans (Kartnig, 1988; Shin *et al*., 1982). Oral administration of *C. asiatica* extract to rats produced a dose-dependent reduction in stress-induced gastric ulceration, and the antiulcer activity was similar to that of famotidine (Chatterjee *et al*.,

1992). The mechanism of action could be associated with a central nervous system-depressant activity of *C. asiatica*, owing to an increase in the concentration of GABA (γ-aminobutyric acid) in the brain (Chatterjee *et al*., 1992).

Keloid and scar management

Studies have shown that *C. asiatica* increased collagen synthesis in vitro and extracellular matrix accumulation in vivo (Maquart *et al*., 1990, 1999). It enhanced tensile strength in wound tissue (Suguna *et a*l., 1996), and facilitated the wound healing process. The latter activity was attributed to the active ingredient, asiaticoside, presented in *C. asiatica* (Shukla *et al*., 1999a). In the treatment of keloids, asiaticoside compared favorably with compression bandaging and provided more lasting results than intralesional cortisone or radiation therapy (Bosse *et al*., 1979). In addition to its oral use, *C. asiatica* has been used as a topical cream in a comprehensive scar management program. Observationally, it was found to improve scar maturity from an average of six months without treatment to three months with treatment (Widgerow *et al*., 2000).

Triterpenoids strengthen the skin, increase the concentration of antioxidants in wounds, and restore inflamed tissues by increasing blood supply. Because of these properties, *C. asiatica* has been used externally for burns, psoriasis, prevention of scar formation following surgery, recovery from an episiotomy following vaginal delivery of a newborn, and treatment of external fistulas.

Asiaticoside accelerated the healing of superficial postsurgical wounds and ulcers by accelerating cicatricial action (Morisset *et al.*, 1987). Asiaticoside stimulates the epidermis by activating the cells of the malpighian layer in porcine skin, and by keratinization *in vitro* (May, 1968). Topical application of asiaticoside promoted wound healing in rats and significantly increased the tensile strength of newly formed skin (Morisset *et al.*, 1987; Rosen *et al.*, 1972).

Venous hypertensive angiopathy

Forty patients with severe venous hypertension, ankle swelling, and lipodermatosclerosis were randomized to receive either total triterpenic fraction of *C. asiatica* (TTFCA) 60 mg twice daily or placebo for 8 weeks. After trial conclusion, patients taking the herbal extract experienced a significant decrease in skin flux and rate of ankle swelling compared to baseline values. In addition, patients in the active group reported rapid clinical improvement, reflected by a reduction in the analogue scale line score (e.g., symptoms of edema, pain, restless limbs, swelling, and change in skin condition/color) from 9.5 at baseline to 4.5 after eight weeks (Cesarone *et al*., 2001). In another study using Laser Doppler evaluation, subjects taking 60 mg TTFCA twice daily

Venous insufficiency

When blood vessels lose their elasticity, blood pools in the legs and fluid leaks out of the blood vessels, causing the legs to swell (venous insufficiency). In a double-blind study, 94 people with venous insufficiency were randomized to one of three treatment groups: titrated extract of *C. asiatica* (TECA) at a daily dose of 60 or 120 mg or placebo for 3 months. Individuals who took *C. asiatica* at either dose demonstrated significant clinical improvements in limb heaviness, edema, and global evaluation of efficacy. Venous distension, measured by plethysmography, was significantly better in the active group at 40 mmHg, 50 mmHg and 60 mmHg, compared to deteriorating placebo values (Pointel *et al*., 1987).

Other indications

C. asiatica may also have potential application for scleroderma (Sasaki *et al*., 1972), alcohol-induced liver cirrhosis (Darnis *et al*., 1979), leg ulcers (Huriez, 1971), and as adjunctive treatment in leprosy (Chaudhury *et al*., 1987). Animal studies indicate it may have potential for prevention of aspirin- or ethanol-induced gastric ulcers (Sairam *et al*., 2001; Cheng and Koo, 2000).

4.3 Side effects and toxicity

Alcoholic extracts of *C. asiatica* have shown no toxicity at doses of 350 mg/kg when given i.p. to rats.1 Reported adverse effects include GI upset and nausea. Topical use of the extract has led to reports of rash (Eun and Lee, 1985). Three cases of jaundice with elevated liver enzymes were reported in Argentina following dosing of *C. asiatic*. Patients had taken *C. asiatic* (standardization and dose unknown) for 20-60 days, and recovered on discontinuation of the herb (Jorge and Jorge, 2005).

CHAPTER III

MATERIAL AND METHODS

Materials

1. Chemicals

Standardized extract of *Centella asiatica* (ECa-233) were kindly provided by Associate Professor Rutt Suttisri and Associate Professor Ekarin Saifah, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of the Pharmaceutical Sciences, Chulalongkorn University. ECa-233 contained triterpenoid glycosides of at least 80% with the ratio between madecassoside and asiaticoside at 1.50 ± 0.50.

Asiaticoside (ATS) was kindly provided by Assistant Professor Chamnan Patarapanish, Department of Food and Pharmaceutical Chemistry, Faculty of the Pharmaceutical Sciences, Chulalongkorn University. On the day of the experiment, ECa-233 and ATS were dissolved in 100% DMSO and further diluted with culture medium. The final concentration of DMSO in each experiment was less than 0.5%. In addition, the solvent control was also conducted in each experiment.

The following chemicals were purchased from Sigma Chemical Company (St. Louis, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Bradford reagent, calcein acetoxymethyl ester (calcein-AM), dimetyl dulphoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), potassium chloride, sodium phosphate dibasic anhydrous, streptomycin sulfate, trypan blue, trypsin, Triton x-100, vinblastine sulfate salt, verapamil, colchicine, Hank's balanced salt solution and non essential amino acids.

The following chemicals were purchased from Merck (Darmstadt, Germany); methanol, potassium dihydrogen phosphate, potassium hydroxide, sodium bicarbonate and sodium chloride.

Fetal bovine serum (FBS) was purchased from Hyclone (USA) for cultured LLC-PK$_1$ and LLC-GA5-COL300 cells and from Biochrom AG (USA) for cultured Caco-2 cells.

M199 medium and Dulbecco's Modified Eagle's Medium were purchased from Gibco Life Technologies, Inc. (Grand Island, NY, USA).

All other chemicals and solvents used throughout this study were the highest grade commercially available reagent or analytical grade reagents.

2. Equipments

The following instruments were major equipment used in this study:

1. Inverted microscope; Axiovert 135 (Zeiss, Germany)

2. Multilabel microplate reader (Perkin Elmer VICTOR^3 Wallac 1420, Germany)

3. Tissue culture plates (12-well, 24-well, 96-well) and flasks (Corning, USA)

3. Cell cultures

Caco-2 cells (Figure 7) (epithelial human colon adenocarcinoma cell line, ATCC no. HTB-37) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% nonessential amino acids and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.
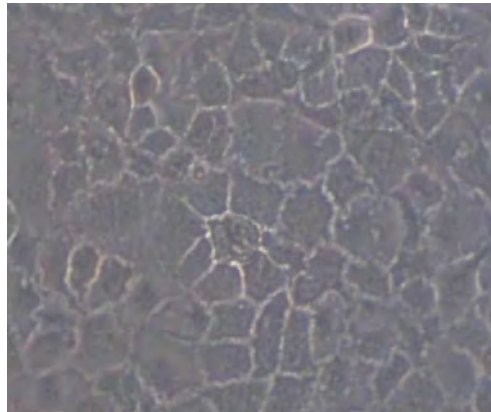
Figure 7: The morphology of Caco-2 cells in the 75th passage, 72 hr after subculture under normal culture condition. The picture was taken by an inverted microscope with phase contrast optics in combination with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany), 10X objective.

LLC-PK$_1$ cells (Figure 8) (a porcine kidney epithelial cell line, ATTC no. CL-101) was cultured in M199 supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.
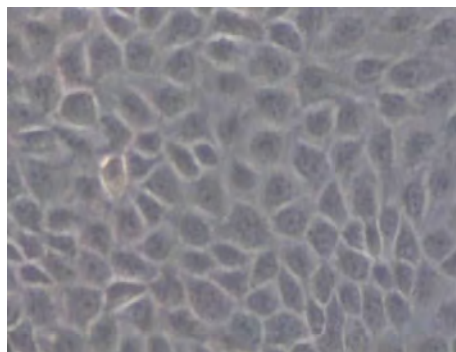
Figure 8: The morphology of LLC-PK$_1$ cells in the 25th passage, 72 hr after subculture under normal culture condition. The picture was taken by an inverted microscope with phase contrast optics in combination with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany), 10X objective.

LLC-GA5-COL300 cell line (Figure 9) [from Riken Cell Bank (Ibaraki, Japan)], was human *MDR1* cDNA -transfected epithelial cells, which was derived from a clone of LLC-PK$_1$ cells stably transfected with a cDNA encoding the human P-gp. LLC-GA5-COL300 cells were maintained in M199 with 1% penicillin-streptomycin, 10% (v/v)

fetal bovine serum (FBS) and 300 ng/mL of colchicine in a humidified atmosphere of 5% CO_2 at 37 °C. (Tanigawara *et al*., 1992; Ueda *et al*., 1992). Before each experiment, the medium were replaced with fresh and colchicine-free medium for 6 hr.
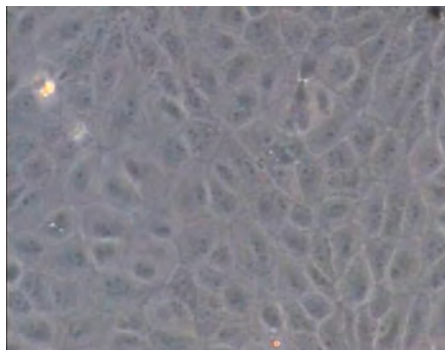
Figure 9: The morphology of LLC-GA5-COL300 cells in the 25th passage, 72 hr after subculture under normal culture condition. The picture was taken by an inverted microscope with phase contrast optics in combination with microcomputer-assisted image capture system (Pinnacle 8, Pinnacle system, Germany), 10X objective.

In this study, the cells were grown to 80-90% confluence while changing for the fresh medium every other day (48 hr). The subcultures were done when the cultures reached 80-90% confluence. The culture medium was aspirated and the cells were trypsinized in 0.25% trypsin-EDTA solution for 7 min (for LLC-PK$_1$ and LLC-GA5-COL300 cells) and 2 min (for Caco-2 cells) at 37 °C. Then, the trypsinization reaction was stopped by adding the fresh culture medium. The cell suspension was centrifuged at 1,500 rpm for 4 min at 37 °C. The pellets were collected and resuspended in fresh medium for further experiment. For the cytotoxicity studies, Caco-2 cells were seeded at 1×10^4 cells/ well and LLC-PK$_1$ and LLC-GA5-COL300 cells were plated at 2×10^3 cells/ well in 96 wells plate. For uptake study, Caco-2 cells were seeded in 24 wells plate at 8×10^4 cells/ cm^2 and LLC-PK$_1$ and LLC-GA5-COL300 cells were plated at 8×10^4 cells/ cm^2 and 10×10^4 cells/ cm^2.

4. Methods

4.1 The cytotoxicity studies

4.1.1 Cytotoxic effects of ECa-233 and ATS

After seeding for 24 hr, various concentrations of ECa-233 (0-1000 μg/ mL) or ATS (0-100 μg/mL) or 0.5% DMSO (vehicle control) were added and incubated for another 72 hr. The cell viability was evaluated by the MTT assay (Mueller *et al*., 2004; Holst and Oredsson, 2005).

4.1.2 The potentiative effects of ECa-233 and ATS on vinblastine-induced cytotoxicity

After seeding for 24 hr, the cells were treated with vinblastine (VBL) at various concentrations (ranging from 0-1000 nM) and incubated for 72 hr. At the end of incubation period, the cell viability was evaluated by the MTT assay (Mueller *et al*., 2004; Holst and Oredsson, 2005). In parallel experiments, the concentration-response relationship of VBL-induced cytotoxicity was performed in the presence of test materials at non-cytotoxic concentration, including ECa-233 or ATS or verapamil (positive control). In these experiments 0.5% DMSO was also used as vehicle control group.

4.1.3 MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay is one of the established methods to assess cell viability (Chiba *et al*., 1998). The assay is dependent on the cellular reduction of yellow colored MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. At the end of the incubation, culture mediums were replaced with MTT solution (0.4 mg/ mL in culture medium without supplement). The plates were incubated for 4 hr in a 5% CO_2 at 37 °C. After removal of the MTT containing medium, the formazan crystal were dissolved in DMSO, and read immediately at 570 nm by using a Multilabel microplate reader (Perkin Elmer VICTOR[3] Wallac 1420, Germany). The cellular reduction of MTT which represents cell viability was expressed as the percentage of the values obtained from control group.

4.1.4 The viability assay with trypan blue staining

In brief, cells monolayers were incubated with ECa-233 (1000 μg/ mL) or ATS (100 μg/ mL) in HBSS for 1, 2 and 4 hr at 37 ºC in 5% CO_2. At the end of incubation,

the cells were trypsinized in 0.25% trypsin-EDTA solution for 2 min at 37 °C. Then, the trypsinization reaction was stopped by adding the fresh culture medium. An aliquot of cell suspension was mixed with an equal volume of 0.4% trypan blue. Then the mixture was placed in a Hemocytometer for cell counting under a microscope. Viable cells exclude trypan blue whereas dead cells were stained with blue color.

4.2 Determination of P-gp function

In this study, the function of P-gp was determined through the accumulation of calcein-AM. Calcein-acetoxymethylester (calcein-AM) is a non-fluorescent substrate of P-gp, highly lipid-soluble dye that rapidly penetrates the plasma membrane of cells. Once inside the cell, ester bonds are cleaved by endogenous esterases, transforming calcein-AM into hydrophilic and intensely fluorescent calcein. Cells expressing high levels of *MDR1* rapidly extrude non-fluorescent calcein-AM from the plasma membrane, thereby reducing accumulation of fluorescent calcein in the cytosol (Homolya *et al.*, 1993). The amount of mdr1 activity is therefore inversely proportional to the accumulation of intracellular calcein fluorescence (Hauser *et al.*, 1998; Jakob *et al.*, 1998; Homolya *et al.*, 1993). On the day of the experiment, cells monolayers were pre-incubated with the test material. Then, calcein-AM (400 nM) was added into the culture, and incubated at 37 ºC for another 30 min. At the end of incubation, the cells were chilled on ice and washed with ice-cold PBS three times. Cells were, then, lysed in 0.1% Triton X-100 for 30 min. The lysate was determined for calcein by spectrofluoremeter (Multilabel microplate reader (Perkin Elmer VICTOR[3] Wallac 1420, Germany)) with excitation at 485 nm and emission at 535 nm. In addition, the protein content was also determined by Bradford method (Bradford, 1976). The intracellular calcein level was expressed in nmole/ mg of protein. In this experiment, verapamil (a known P-gp inhibitor) and indomethacin (a known MRP2 inhibitor) were used as positive control group.

4.3 Bradford Protein Assay (Bradford, 1976)

The Bradford assay, a colorimetric protein assay, is based on an absorbance shift in the dye coomassie (without protein, the solution is red-brown in its

acidic solution) when the previously red form coomassie reagent changes and stabilizes into coomassie blue by the binding of protein. At the end of lysate, sample was mixed with a bradford reagent and read at 595 nm by using a Multilabel microplate reader (Perkin Elmer VICTOR[3] Wallac 1420, Germany), using bovine serum albumin as a protein standard. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

5. Statistical analysis

The cell viability as measured by MTT assay was calculated based on those observed in the control group. The results were expressed as percent of control.

$$\text{Percent of control} = \frac{\text{Mean treatment OD x 100}}{\text{Mean control OD}}$$

Concentration-response curve of cell viability was plotted and performed regression analysis to estimate the IC_{50} value.

The percentage of viable cells in trypan blue staining was calculated from the average number of unstained cells in each quadrant of Hemocytometer, and multiply by 2×10^4 to find the number of viable cells/ mL.

$$\text{Percent of viable cells} = \frac{\text{Number of live cells x 100}}{\text{The total number of (dead + live cells)}}$$

All experiments were repeated at least three times and presented as means ± SEM. (standard error). In each experiment, all data were done in triplicate. Either one-way ANOVA followed by the Dunnett's test and Tukey's test or Student's unpaired t-test were performed for statistical comparisons, where appropriate. *P* value ≤ 0.05 was considered significant.

CHAPTER IV

RESULTS

1. The cytotoxic activity of the test materials

At 72-hours treatment, neither ECa-233 (up to 1000 μg/ mL) nor asiaticoside (ATS, up to 100 μg/ mL) had any cytotoxic effect on viability of LLC-PK$_1$ (Figure 10) and Caco-2 cells (Figure 11) as determined by the MTT assay.
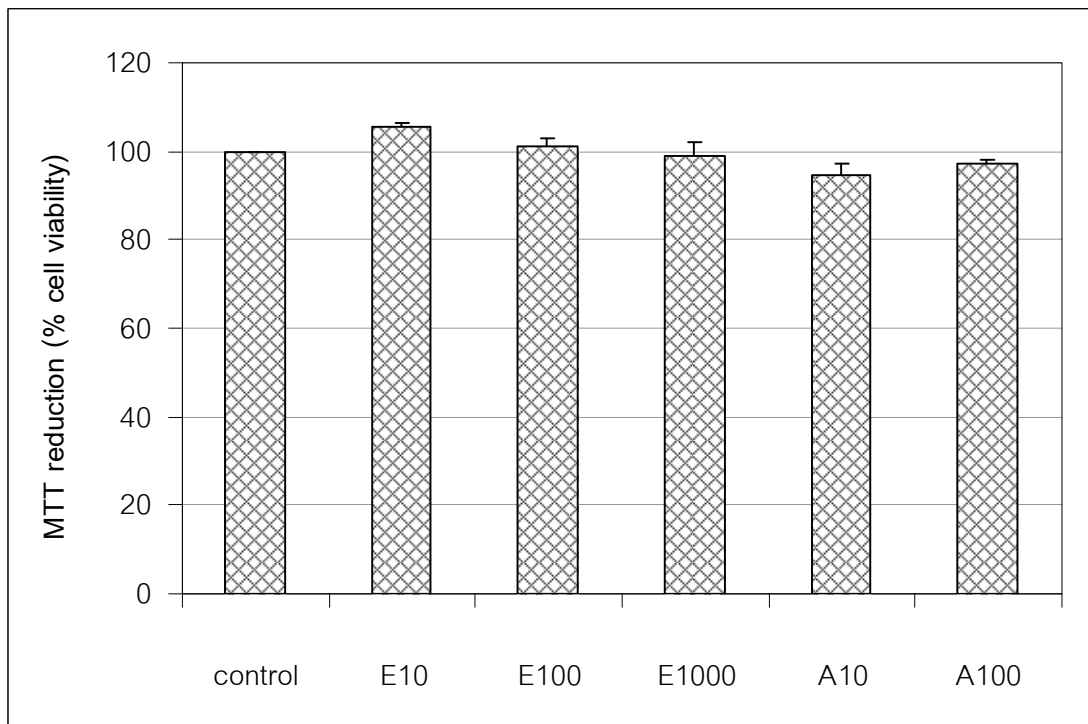
Figure 10: The cytotoxicity of ECa-233 (E; at concentrations of 10, 100, 1000 μg/ mL) and ATS (A; at concentrations of 10, 100 μg/ mL) in LLC-PK1 cells, using MTT reduction as the endpoint. The results were presented as the mean ± SEM of at least triplicate determination.
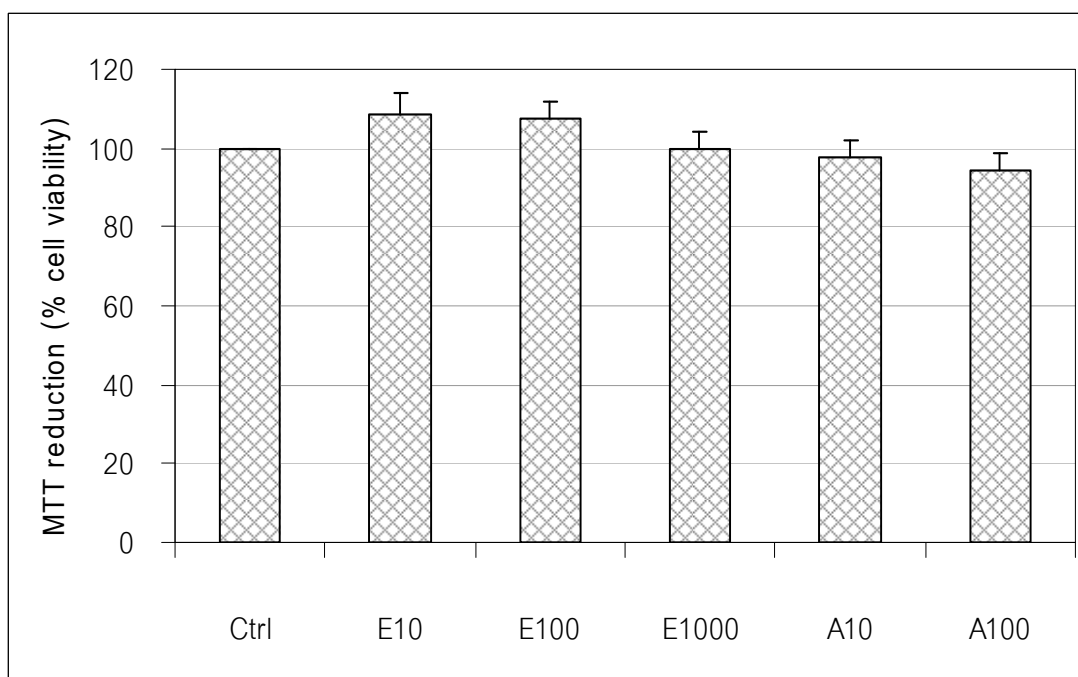
Figure 11: The cytotoxicity of ECa-233 (E; at concentrations of 10, 100, 1000 μg/ mL) and ATS (A; at concentrations of 10, 100 μg/ mL) in Caco-2 cells, using MTT reduction as the endpoint. The results were presented as the mean ± SEM of at least triplicate determination.

2. Effects of the test materials on P-gp function

2.1 P-gp function in P-gp overexpressed cells (LLC-GA5-COL300) and their parental cells (LLC-PK$_1$).

Prior to the accumulation of calcein-AM assays in LLC-GA5-COL300 and LLC-PK$_1$, the cytotoxic activity of the test materials was determined with trypan blue staining method. At 1, 2 and 4 hours treatment, ECa-233 (1000 μg/ mL) had no effect on viability of both cells types (Figure 12).

(a)
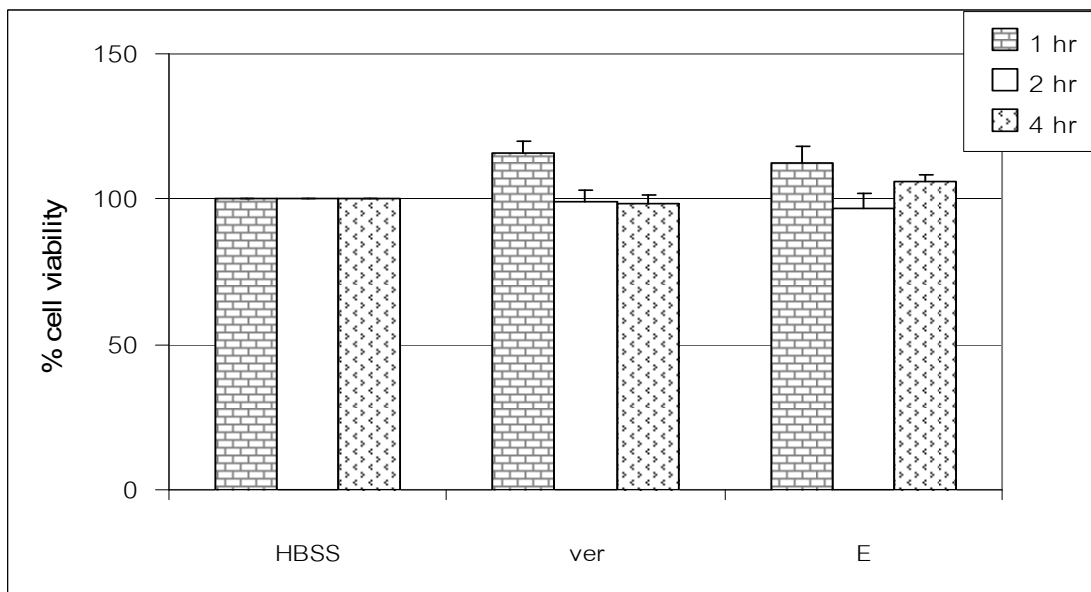
(b)
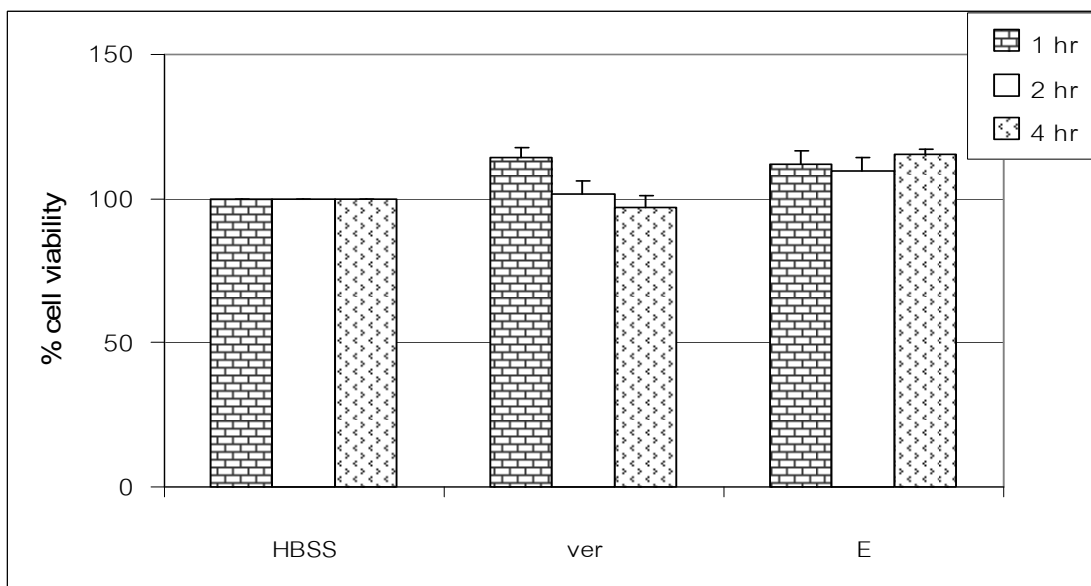
Figure 12: The viability of LLC-PK$_1$ cells (a) and LLC-GA5-COL300 cells (b) as determined by the trypan blue staining method. The cells were incubated with ECa-233 (1000 µg/ mL) or verapamil (100 µM) for 1, 2 and 4 hours prior to staining with trypan blue. The living cells and dead cells were counted under light microscope, followed by calculation for %cell viability. The results were presented as the means ± SEM of at least triplicate determinations.

In order to investigate the potential effect of the test materials (ECa-233 and ATS) on P-gp function in LLC-GA5-COL300 and LLC-PK$_1$ cells, the accumulation of calcein-AM was determined. Intracellular non-fluorescent calcein-AM was converted into fluorescent calcein, which could be quantified with spectrofluorometry (Homolya *et al*., 1993). As shown in Figure 13, the levels of calcein-AM in LLC-PK$_1$ cells was approximately 3- fold higher than those in LLC-GA5-COL300 cells. These findings supported that LLC-GA5-COL300 cells had higher P-gp activity than LLC-PK$_1$ cells, resulting in the less accumulated calcein-AM being converted into fluorescent calcein in the LLC-GA5-COL300 cells.

In addition, the presence of verapamil (100 µM) increased the accumulation of calcein-AM in both LLC-PK$_1$ and LLC-GA5-COL300 cells (Figure 14). In LLC-PK$_1$, the presence of verapamil increased the level of intracellular calcein-AM by 1.5- fold ($P <$ 0.05). In LLC-GA5-COL300 cells, the effect of verapamil was markedly observed with the 3.8- fold ($P < 0.01$) increase in calcein-AM accumulation. These findings indicated that although LLC-PK$_1$ might contain P-gp activity, the level of P-gp expression was quite low in this cell type relatively to its expression in LLC-GA5-COL300. In this study, ECa-233 (1000 µg/ mL) had significant effects on the intracellular level of calcein-AM in LLC-PK$_1$ by approximately 1.5- folds (55% increase) ($P < 0.05$). In addition, treatment the LLC-GA5-COL300 cells with ECa-233 (1000 µg/ mL) significantly increased the intracellular level of calcein-AM by approximately 1.8- folds (85%) ($P < 0.05$), as shown in Figure 14. By contrast, ATS (100 µg/ mL) had no significant effect on calcein accumulation in both LLC-PK$_1$ and LLC-GA5-COL300 cells.
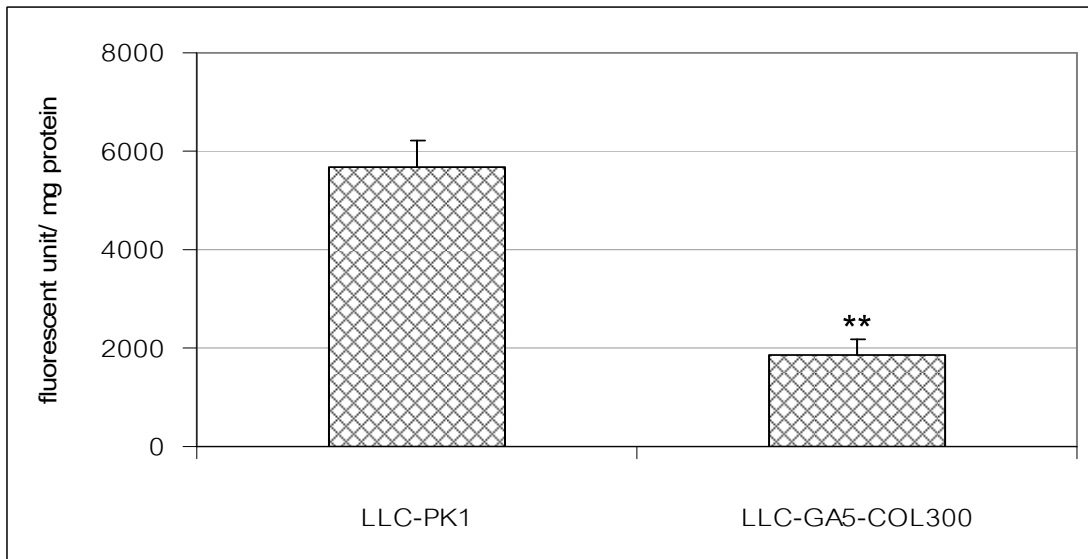
Figure 13: Accumulation of calcein-AM in LLC-PK$_1$ and LLC-GA5-COL300 cells, being cultured in 0.5% DMSO in HBSS. The data were presented as means ± SEM of at least triplicate determinations. ** P < 0.01 vs LLC- PK$_1$ cells.
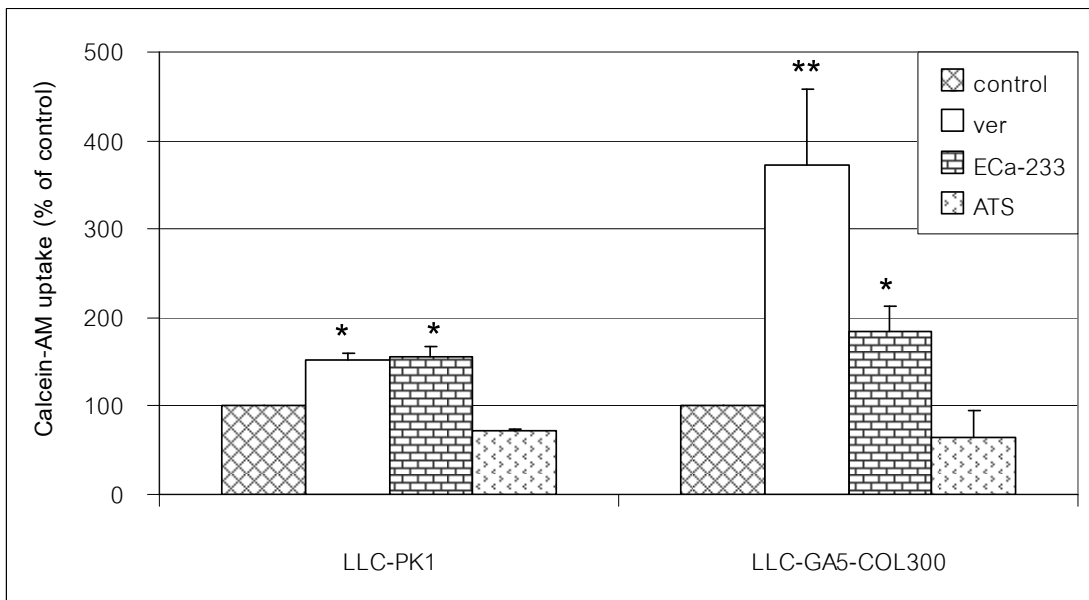
Figure 14: Effects of ECa-233 and ATS on the accumulation of calcein in LLC-PK$_1$ and LLC-GA5-COL300 cells. The cells were incubated with calcein-AM (400 nM) for 30 mins in the presence or absence of ECa-233 (1000 µg/ mL) or ATS (100 µg/ mL) or verapamil (100 µM; positive control group). The data were presented as means ± SEM of at least triplicate determinations. * P < 0.05, ** P < 0.01 vs control.

2.2 P-gp function in Caco-2 cells

The potential effect of the test materials (ECa-233 and ATS) on P-gp function was also investigated in the *in vitro* model of Caco-2 cells. As shown in Figure 15, the presence of either verapamil (100 μM) or indomethacin (10 μM) increased intracellular calcein by approximately 15-20%, reflecting the increased accumulated amount of calcein-AM in these cells. These findings suggested that Caco-2 cells cultured in this experimental condition possessed both the P-gp and MRP activity.

Treatment the Caco-2 cells with ECa-233 at the concentration of 1000 μg/ mL significantly increased calcein-AM accumulation approximately by 42% ($P < 0.01$). By contrast ATS (100 μg/ mL) had no significant effects on the intracellular level of calcein-AM in Caco-2 cells. The results suggested that ECa-233 might be able to inhibit P-gp activity or calcein efflux pump in Caco-2 cells.
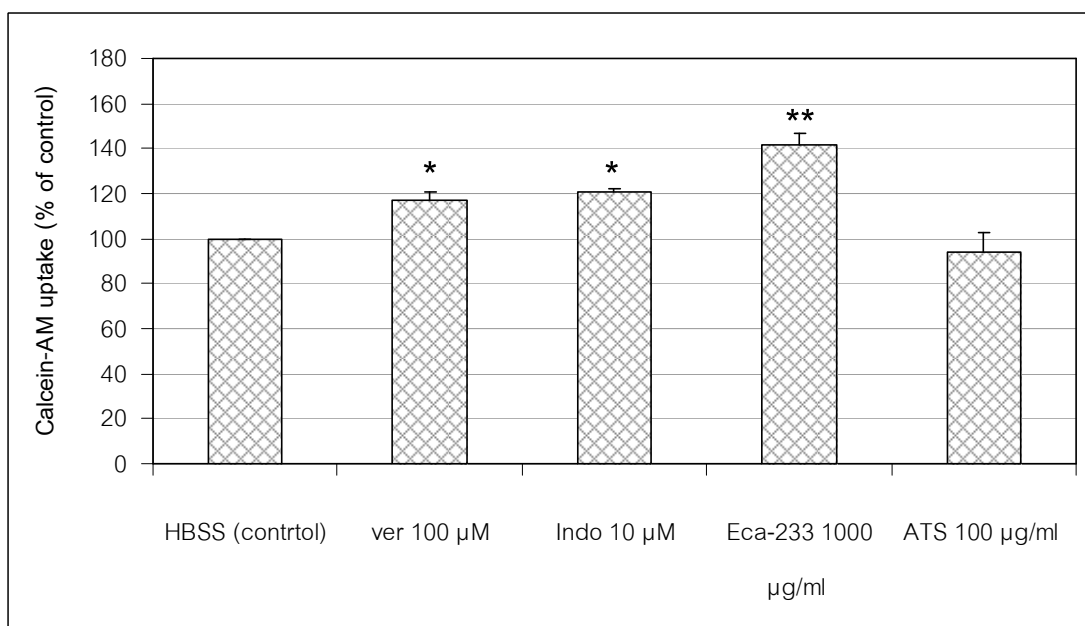

Figure 15: Effects of ECa-233 and ATS on the accumulation of calcein in Caco-2 cells. The cells were incubated with calcein-AM (400 nM) for 30 mins in the presence or absence of verapamil, indomethacin, ECa-233 and ATS. The data were presented as means ± SEM of at least triplicate determinations. * $P < 0.05$, ** $P < 0.01$ vs control.

3. The potentiative effect of the test materials on vinblastine (VBL)-induced cytotoxicity

ECa-233 and ATS were also determined for its capability to potentiate the cytotoxic effect of VBL in Caco-2 cells. As known, VBL is a substrate of P-gp/MRP efflux pump, the experiments hence were designed to measure the cell viability in the coexisting conditions between VBL and the test materials. In this study, the VBL-induced cytotoxicity was determined in the absence and presence of the test materials at the non-toxic concentration (ECa-233 at 1000 µg/ mL and ATS at 100 µg/ mL). After 72 hour of VBL treatment, the cells were determined for cytotoxicity with the MTT assay. As shown in Figure 16, cell viability decreased as the concentration of VBL increased. The IC_{50} value calculated from the regression analysis of the concentration-response curve of VBL was 75.9 ± 13.1 nM (Table 3). The presence of verapamil (20 µM which is a known P-gp and MRP inhibitor) decreased the apparent IC_{50} values of VBL in Caco-2 cells by approximately 29- fold. Indomethacin (10 µM) which is an MRP inhibitor reduced the IC_{50} values of VBL by 3- folds. In addition, co-treatment Caco-2 cells with VBL and the test materials (ECa-233 at 1000 µg/ mL and ATS at 100 µg/ mL) resulted in a shift of the the concentration response curve of VBL-induced cytotoxicity leftwardly (Figure 16). The apparent IC_{50} values as well as the folds of MDR reversal were shown in Table 3. ECa-233 at 1000 ug/mL appeared to be more effective than others in enhancing the cytotoxic effect of VBL. This stadardized extract incresed the cytoxicity of VBL by approximately 5- folds.
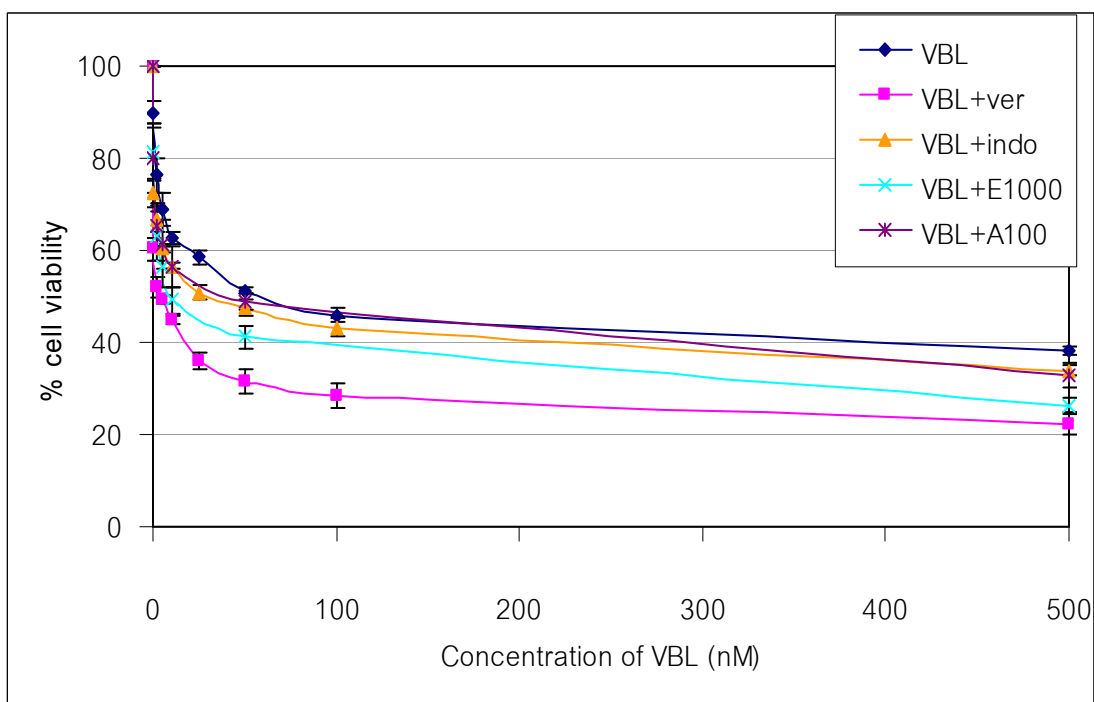
Figure 16: Concentration response curve of VBL- induced cytotoxicity in Caco-2 cells, as determined by the MTT assay. The cells were cultured with a full range of concentrations of VBL in the presence or absence of the test materials including ECa-233 (1000 µg/ mL) or ATS (100 µg/ mL) for 72 hour. In addition, verapamil (20 µM), and indomethacin (10 µM) were also applied as positive control groups. The results were presented as the mean ± SEM of at least triplicate determination.

Table 3: The apparent IC_{50} values of VBL in Caco-2 cells. Each value represented the mean ± SEM of at least three independent experiments.

Groups	IC_{50} of VBL (mean ± S.E, nM)	Fold reversal of MDR[a]
VBL	75.91 ± 13.07	
VBL + ver 20 µM	2.56 ± 1.05 *	29.60
VBL + Indo 10 µM	27.62 ± 6.41 *	2.75
VBL + ECa-233 1000 µg/ mL	14.26 ± 4.48 *	5.32
VBL + ATS 100 µg/ mL	33.04 ± 1.68 *	2.30

*$P < 0.05$ vs vinblastine group

[a] The fold reversal of MDR was defined as the ratio of IC_{50} values for VBL alone to that for VBL with verapamil, indomethacin, ECa-233 and ATS .

CHAPTER V

DISCUSSION AND CONCLUSION

Multi-drug resistance in chemotherapy could be the result of overexpression of membrane efflux transporters including P-glycoprotein (P-gp), multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP) (Chan *et al.*, 2004; Takano *et al.*, 2006; Dietric *et al.*, 2003) to pump the chemotherapeutic agents out of the cancer cell in an ATP-dependent manner (Cascorbi, 2006). Consequently, the intracellular drug accumulation decreases along with its effectiveness (Schinkel and Jonker 2003). In this study, the cytotoxicity of ECa-233 and ATS was determined with the MTT assay. The effects of the test materials on P-gp function were determined through intracellular accumulation of calcein-AM which is a known substrate of P-gp. The presence of P-gp inhibitor increases its intracellular accumulation (Okano *et al.*, 1996; Yang and Liu, 2004). In addition, P-gp function can be determined with the use of VBL-induced cytotoxicity testing. This approach was based on the principle that the presence of P-gp inhibitor would increase the accumulation of intracellular VBL, leading to an enhancement of its cytotoxicity.

A number of compounds with a broad spectrum of molecular structures are known to interact with P-gp and modulate its function (Matheny *et al.*, 2001). Generally, most P-gp substrates are hydrophobic, neutral or mildly positive lipophilic compounds with a planar structure (Gottesmann and Pastan, 1993). Certain dietary phytochemicals

and herbal supplements such as piperine (Bhardwaj *et al.*, 2002), curcumin (Anuchapreeda *et al.*, 2002) have been found to inhibit P-gp function. Triterpene compounds including asiaticoside have been known for their antitumor and antibiotic activities (Arntzen *et al.*, 1999; Anisimov *et al.*, 1981). In view of the molecular structure of the triterpene compounds, it was possible that these triterpene compounds including ATS which can be found in *Centella asiatica* might be able to modulate P-gp functions and potentiate the cytotoxic activity of certain drugs including VBL.

The concentrations of the test materials in this study were non lethal concentrations, using the criteria of more than 90% cells survival with either MTT assays or trypan blue staining method. To investigate the effect of the test materials on P-gp efflux transporter, the accumulation of calcein-AM was performed in LLC-GA5-COL300 which overexpress P-gp transporters and Caco-2 cells (Tanigawara *et al.*, 1992; Ueda *et al.*, 1992). As known, calcein-AM is non-fluorescent substrate of P-gp, and become fluorescent calcein after the cleavage by intracellular esterases (Troutman and Thakker, 2003; Legrand *et al.*, 1998; Perloff *et al.*, 2003). The expressions of P-gp along with MRP could also be observed in LLC-PK$_1$ cells (Takano *et al.*, 2006). However, the level of expression, in particular, of P-gp was considerably low in relative to *MDR1* –transfected LLC-GA5-COL300 cells. The expressions of P-gp in LLC-GA5-COL300 cells were 3- fold higher than those in its parental LLC-PK$_1$ cells, as determined by calcein-AM accumulation assay. In addition, the presence of verapamil increased the intracellular calcein-AM by 4 folds, suggesting the active P-gp activity in these LLC-GA5-COL300

cells. Treatment either LLC-GA5-COL300 or LLC-PK$_1$ cells with ECa-233 (1000 μg/ mL) had significant effects on calcein-AM accumulation. The effects of Eca-233 on calcein-AM uptake in these two cell types were slightly different. Hence, it was unlikely that Eca-233 could potently modulate P-gp function in these cells. Taken that LLC-PK$_1$ cells are derived from kidney epithelial cells, these cell type contains several membrane efflux pumps other than P-gp (Takano *et al.*, 2006). It was possible that ECa-233 affected the basal efflux pump activities involving calcein transport which existed in these cell types. Moreover, ATS (100 μg/ mL) had no effect on calcein accumulation in either LLC-PK$_1$ or LLC-GA5-COL300 cells. These findings suggested that ATS had no inhibitory actions against P-gp or other basal efflux pumps existed in these cell types.

The effect of ECa-233 against intracellular calcein-AM accumulation was also evaluated in Caco-2 cells. Caco-2 cells have been used as an *in vitro* model for absorptive intestinal barrier due to its expression of several membrane transporters including P-gp and MRP (Gutmann *et al.*, 1999). It has been reported that the variability of P-gp expression levels and function in Caco-2 cells depend on several factors such as culturing conditions, passage number, trypsinization of cells (Pascale *et al.*, 1998). Under the condition in this study, Caco-2 cells expressed P-gp and MRP efflux pumps, as shown by the decreases in calcein-AM accumulation in the presence of verapamil (P-gp and MRP inhibitor) and indomethacin (MRP inhibitor). ECa-233 could increase the calcein-AM accumulation significantly, suggesting the competition for calcein-AM uptake by components presented in ECa-233. However, ATS had no effects on the

efflux pump. It was possible that the ECa-233 might be able to inhibit P-gp activity or calcein efflux pump in Caco-2 cells. In addition, the effective pump modulating components in ECa-233 might not be ATS.

In this study, the cytotoxicity of vinblastine (VBL), which is a known P-gp substrate, was determined with the MTT assay in the presence of the test materials (ECa-233 and ATS). This approach was based on the principle that the presence of P-gp inhibitor such as verapamil would increase the accumulation of intracellular VBL, leading to an enhancement of its cytotoxicity. It was reported that verapamil could completely overcome vincristine resistance either P388 or P388/VCR cells by enhancing vincristine (VCR) cytotoxicity (Tsuruo *et al.*, 1981). VBL and VCR are anticancer *Vinca* alkaloids, acting by prevention of mitosis in metaphase (Richard *et al.*, 1976). The results showed that verapamil was able to significantly enhance VBL-induced cytotoxicity of Caco-2 cells, suggesting the existent of P-gp activity in these cells. These data were in agreement with those reported in other studies, although the apparent IC_{50} value of VBL (76 nM) obtained in this study was less than those reported in others (McDevitt and Callaghan, 2007). For example, it was reported that the IC_{50} value of VBL in Caco-2 cultured for 19 to 27 days was 26.5 μM (Stephens *et al.*, 2001). This discrepancy might result from the cultured conditions such as seeding density and cultured period (Pascale *et al.*, 1998). It has been known that the functional expression of P-gp in Caco-2 cells increased in time-dependent manner (Hosoya *et al.*, 1996). The

function of P-gp as a transporter protein increased significantly from day 17 to day 27 (Hosoya *et al.*, 1996).

The result in this study demonstrated that ECa-233 and ATS were able to significantly enhance the cytotoxicity of VBL. In addition, ECa-233 at 1000 µg/ml could apparently increase the cytotoxicity of VBL by approximately 5 folds. It was possible that the test materials which were applied at non cytotoxic concentrations might increase VBL accumulation within the cells and enhanced its cytotoxic action. According to the results, inhibition of P-gp activity might not be a major cause responsible for enhancing the VBL-cytotoxicity of these test materials. Considering that Caco-2 cells express several types of membrane transporters, it was possible that the potentiative effects of ECa-233 on VBL-induced cytotoxicity might result from the effects on transporters other than P-gp. Moreover, the results also suggested that the potentiative effect of ATS (about 2- folds) was not related to VBL efflux pump. Hence, other potentiative mechanisms of the test material, which involve with the mechanism of action of *Vinca* alkaloids should be further investigated. It was noteworthy to mention that MTT could be a substrate of efflux transporters including MDR1 and possibly MRP (Kati-Sisko *et al.*, 2004). Hence, the presence of MDR1 or MRP inhibitors might retain MTT molecules inside the cells, leading to erroneous conclusions about inhibitor toxicity (Kati-Sisko *et al.*, 2004).

In conclusion, it was likely that ECa-233 and ATS were able to enhance the cytotoxic effects of VBL through mechanisms other than P-gp modulations. *Centella*

asiatica could give rise to food/herb-drug interaction so it may be prudent to avoid

consuming P-gp substrate drugs with its. The findings may be useful for further studies

in the application of *Centella asiatica* extract in traditional medicine development.

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APPENDICES

APPENDIX A

TABLES OF EXPERIMENTAL RESULTS

Table 4: The percentage of MTT reduction of cultured LLC-PK$_1$ cells treated with the standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) for 72 hr prior. Each value represented the mean ± SEM of at least three independent experiments.

Group	Concentration (µg/mL)	% MTT reduction (mean ± S.E.) (Compared to control)
Control		100.00 ± 0.00
ECa-233 (E)	10	105.67 ± 0.94
	100	101.34 ± 1.80
	1000	98.91 ± 3.17
Asiaticoside (A)	10	94.51 ± 2.74
	100	97.26 ± 0.97

Table 5: The percentage of MTT reduction of cultured Caco-2 cells treated with standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS) for 72 hr prior. Each value represented the mean ± SEM of at least three independent experiments.

Group	Concentration (µg/mL)	% MTT reduction (mean ± S.E) (Compared to control)
Control		100.00 ± 0.00
ECa-233 (E)	10	108.79 ± 5.48
	100	108.79 ± 5.48
	1000	99.61 ± 4.76
Asiaticoside (A)	10	97.91 ± 4.17
	100	94.42 ± 4.18

Table 6: The percentage of cell viability in LLC-PK$_1$ and LLC-GA5-COL300 cells treated with the standardized extract of *Centella asiatica* (ECa-233)

for 1, 2 and 4 hr prior. Each value represented the mean ± SEM of at least three independent experiments.

Group	LLC-PK$_1$			LLC-GA5-COL300		
	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Eca-233 1000 µg/mL	112.00 ± 6.10	96.95 ± 5.12	106.06 ± 2.47	111.66 ± 4.72	109.72 ± 4.46	115.57 ± 1.57
verapamil 100 µM	115.96 ± 3.80	99.27 ± 3.95	98.22 ± 2.85	114.42 ± 3.40	101.47 ± 4.46	97.18 ± 4.06

Table 7: The accumulation of calcein in LLC-PK$_1$ and LLC-GA5-COL300 cells treated with the standardized extract of *Centella asiatica* (ECa-233)

and asiaticoside (ATS). Each value represented the mean ± SEM of at least three independent experiments.

Group	LLC-PK$_1$		LLC-GA5-COL300	
	fluorescent unit / mg protein	% of control	fluorescent unit / mg protein	% of control
Contrtol	5675.50 ± 540.28	100.00 ± 0.00	1858.74 ± 321.56 **	100.00 ± 0.00
Eca-233 1000 µg/mL	8828.11 ± 1204.97	155.55 ± 11.89 *	3435.27 ± 922.22	184.82 ± 27.98 *
AST 100 µg/mL	4124.99 ± 339.88	72.68 ± 9.50	1185.30 ± 365.14	63.77 ± 31.59
verapamil 100 µM	8598.57 ± 975.98	151.50 ± 8.85 *	6931.93 ± 1157.85	372.94 ± 84.89 **

* *P* < 0.05, ** *P* < 0.01 vs control

Table 8: The Calcein-AM accumulation of cultured Caco-2 cells treated with standardized extract of *Centella asiatica* (ECa-233) and asiaticoside (ATS). Each value represented the mean ± SEM of at least three independent experiments.
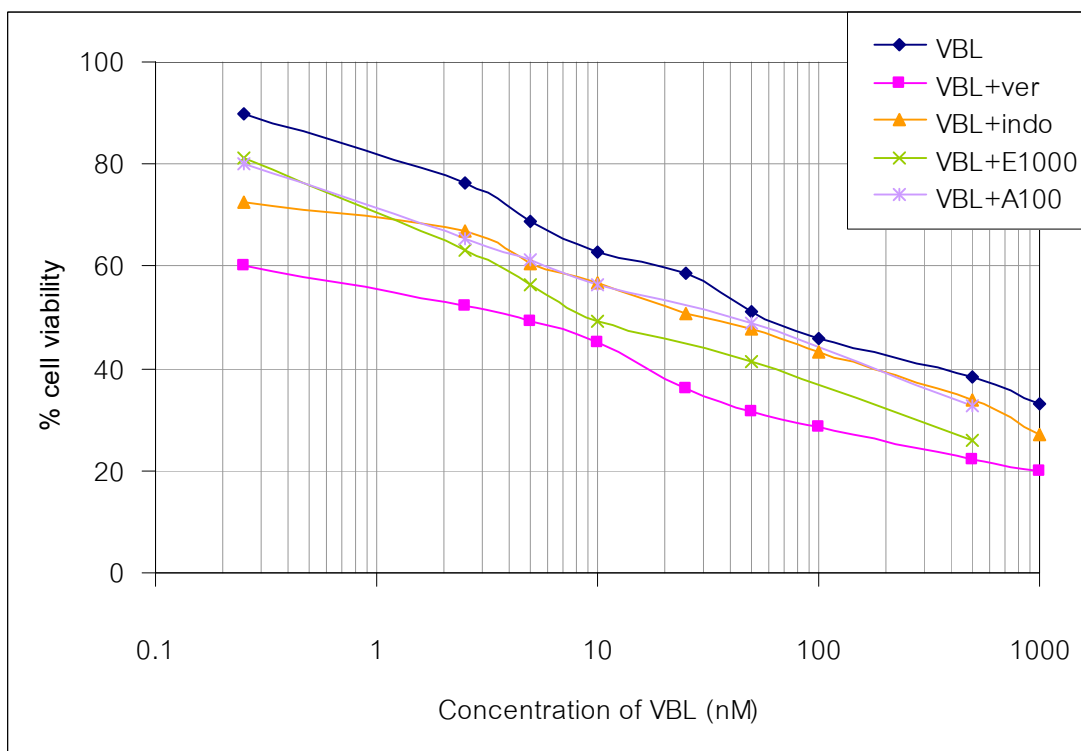
Group	fluorescent unit/mg protein	% of control
Contrtol	18633.00 ± 1007.22	100.00 ± 0.00
Eca-233 1000 µg/mL	26412.75 ± 1446.52	142.02 ± 4.49 **
AST 100 µg/mL	14426.91 ± 549.28	94.26 ± 8.27
verapamil 100 µM	21785.97 ± 1119.82	117.23 ± 8.05 *
Indomethacin 10 µM	21564.18 ± 1726.64	120.84 ± 2.90 *

* $P < 0.05$, ** $P < 0.01$ vs control

Figure 17: Concentration response curve of VBL-induced cytotoxicity in Caco-2 cells, as determined by the MTT assay. Concentration of VBL are expressed as the log10 of the nM concentration. The cells were cultured with a full range of concentrations of VBL in the presence or absence of the test materials including ECa-233 (1000 μg/mL) or ATS (100 μg/mL) for 72 hr. In addition, verapamil (20 μM), and indomethacin (10 μM) were also applied as positive control groups. The results were presented as the mean ± SEM of at least triplicate determination.

Table 9: The percentage of MTT reduction of cultured Caco-2 cells co-treated with VBL (at various concentrations) and either ECa-233 (1000 µg/mL) or ATS (100 µg/mL) for 72 hr prior. Each value represented the mean ± SEM of at least three independent experiments.

| Conc. of VBL (nM) | % MTT reduction (mean ± S.E, Compared to control) | | | | |
| | Group | | | | |
	VBL	VBL + ver	VBL + Indo	VBL + ECa-233	VBL+ATS
0.0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.25	89.89 ± 2.40	60.24 ± 2.47	72.41 ± 2.87	81.22 ± 5.60	80.11 ± 7.51
2.5	76.26 ± 3.80	52.11 ± 2.33	66.88 ± 2.73	63.23 ± 5.27	65.39 ± 4.65
5.0	68.85 ± 3.48	49.38 ± 0.83	60.37 ± 0.95	56.24 ± 4.45	61.37 ± 5.38
10.0	62.67 ± 1.14	44.94 ± 0.75	56.58 ± 0.56	49.16 ± 3.03	56.57 ± 4.52
25.0	58.51 ± 1.42	36.11 ± 1.74	50.89 ± 1.43	-	-
50.0	51.30 ± 0.81	31.49 ± 2.55	47.56 ± 1.94	41.19 ± 2.58	48.98 ± 1.80
100.0	45.80 ± 1.55	28.48 ± 2.51	43.29 ± 1.83	-	-
500.0	38.19 ± 0.75	22.34 ± 2.47	33.93 ± 1.19	26.12 ± 1.88	32.89 ± 2.61
1000.0	33.09 ± 0.33	19.90 ± 2.65	26.91 ± 1.77	-	-

Table 10: The regression analysis of concentration response curve of VBL-induced cytotoxicity and its square of regression factor (R^2) (Figure 17).

Group	Equation	R^2
VBL	y = -6.9843Ln(x) + 80.245	0.9893
VBL + ver 20 µM	y = -7.2141Ln(x) + 60.06	0.9820
VBL + Indo 10 µM	y = -5.7353Ln(x) + 69.668	0.9995
VBL + ECa-233 1000 µg/ mL	y = -6.2949Ln(x) + 65.273	0.9918
VBL + ATS 100 µg/ mL	y = -5.4822Ln(x) + 70.055	0.9932

APPENDIX B

PREPARATION OF REAGENTS

Preparation of M199 (1,000 mL)

1. Dissolve the following ingredients in 1,000 mL of distilled water.

    a. M199   1 pack

    b. NaH_2CO_3 2.20 g

2. Adjust pH of this solution to 7.2-7.4 by adding 1 N HCl or 1 N NaOH.

3. Sterile by filtration through 0.22 µm acrocap filter.

4. Store in a refrigerator at 4 °C (Stable for 1 month).

5. Mix with 10% fetal bovine serum (FBS) + 1% penicillin-streptomycin before use (89 mL of medium + 10 mL of FBS + 1 mL penicillin-streptomycin).

Preparation of DMEM (1,000 mL)

1. Dissolve the following ingredients in 1,000 mL of distilled water.

    a. M199   1 pack

    b. NaH_2CO_3 3.70 g

2. Adjust pH of this solution to 7.2-7.4 by adding 1 N HCl or 1 N NaOH.

3. Sterile by filtration through 0.22 µm acrocap filter.

4. Store in a refrigerator at 4 °C (Stable for 1 month).

5. Mix with 10% FBS + 1% penicillin-streptomycin before use (89 mL of medium + 10 mL of FBS + 1 mL penicillin-streptomycin).

Preparation of phosphate buffer saline solution (1,000 mL)

1. Dissolve the following ingredients in 1,000 mL of distilled water.

    a. Potassium chloride   0.20 g

    b. Sodium chloride   8.00 g

    c. Potassium phosphate monobasic (anhydrous)   0.20 g

    d. Sodium phosphate dibasic (anhydrous)   1.15 g

2. Adjust pH of this solution to 7.2-7.4 by adding 1 N HCl or 1 N NaOH.

3. Sterile by autoclave 121 °C, 20 min

4. Store in a refrigerator at 4 °C

Preparation of 0.25% Trypsin, 0.038% EDTA (100 mL)

1. Dissolve 0.25 g of trypsin and 0.038 g of EDTA in phosphate buffer saline solution and adjust volume to 100 mL.

2. Sterile by syringe filtration through 0.22 µm.
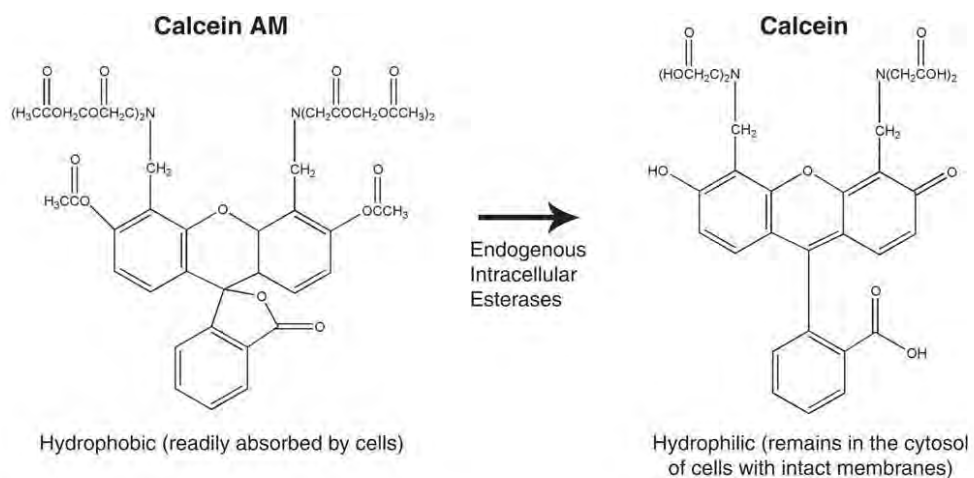
3. Store in a refrigerator at 4 °C.

APPENDIX C

Molecular structures of Calcein-AM (Sigma-Aldrich, 2006),
and verapamil hydrochloride (Sigma-Aldrich, 2004)

Calcein-AM (Sigma-Aldrich, 2006)

Empirical: $C_{46}H_{46}N_2O_{23}$

Structure: Calcein O, O'-diacetate tetrakis (acetoxymethyl) ester
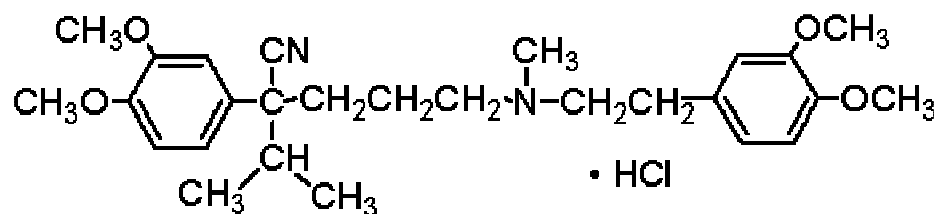

(From Sigma-Aldrich, 2006)

Molecular weight: 994.87

Solubility: soluble in DMSO

Storage temperature: stored at -20ºC and protect from light

Verapamil hydrochloride (Sigma-Aldrich, 2004)

Empirical: $(CH_3O)_2C_6H_3CH_2CH_2N(CH_3)(CH_2)_3C[C_6H_3(OCH_3)_2][CH(CH_3)_2]CN \cdot HCl$

Structure: 5-[N-(3,4-Dimethoxyphenylethyl) methylamino]-2-(3,4-dimethoxyphenyl) -2-

isopropylvaleronitrile hydrochloride

(From Sigma-Aldrich, 2004)

Molecular weight: 491.06

Solubility: methanol: 50 mg/mL, clear, colorless

Storage temperature: store at room temperature

BIOGRAPHY

Miss Narumol Ruamsuk was born on March 7, 1983 in Bangkok, Thailand. She received the Bachelor of Science from Suan Dusit Rajabhat University in 2005. She entered the master's degree program in Interdisciplinary program in pharmacology, Graduate School, Chulalongkorn University in 2006.

She has attended and presented poster at the 32nd Pharmacological and Therapeutic Society of Thailand Meeting, March 25-26, 2010 at Thammasat University, Pathumthani, Thailand. The subject presented was potentiative effects of standardized extract of *Centella asiatica* on vinblastine-induced cell death.