ผลของฟิลแลนทินและไฮโปฟิลแลนทินต่อการทำหน้าที่ของพี-ไกลโคโปรตีนและมัลติดรักรีซิสแทนซ์โปรตีน 2 ในเซลล์คาโค-2

เรืออากาศเอกหญิงปัญกร สิทธรศิรา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON FUNCTIONING OF P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE PROTEIN 2 IN CACO-2 CELLS

Flt. Lt Punyakorn Siththornsira

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ปัญกร สิทธรศิรา : ผลของฟิลแลนทินและไฮโปฟิลแลนทินต่อการทำหน้าที่ของพี-ไกลโคโปรตีนและมัลติดรักรีซิสแทนซ์โปรตีน 2 ในเซลล์คาโค-2 (EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON FUNCTIONING OF P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE PROTEIN 2 IN CACO-2 CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร.สุรีย์ เจียรณ์มงคล, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร.นนทิมา วรรธนะภูติ, 85 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาและเปรียบเทียบผลของฟิลแลนทินและไฮโปฟิลแลนทินต่อการทำหน้าที่ของ P-glycoprotein (P-gp) และ multidrug resistance protein 2 (MRP2) ในแบบจำลองเซลล์คาโค-2 ตลอดจนศึกษาผลของสารทั้งสองต่อการทำหน้าที่ของ P-gp เมื่อเซลล์ได้รับสารเป็นระยะเวลานาน ทั้งนี้ในการศึกษาการทำงานของโปรตีนดังกล่าวจะวัดจากการสะสมของ substrate ที่มีความจำเพาะต่อ P-gp และ MRP2 ในเซลล์คาโค-2 ที่เพาะเลี้ยงเป็นเวลา 21 วัน ซึ่งในสภาวะที่ใช้ในการทดลองนี้เซลล์มีการแสดงออกให้เห็นถึงการทำงานของทั้ง P-gp และ MRP2 ผลที่ได้จากการศึกษาพบว่าไฮโปฟิลแลนทินมีผลยับยั้งการทำงานของ P-gp ในระดับที่ใกล้เคียงกับฟิลแลนทิน โดยทั้งฟิลแลนทินและไฮโปฟิลแลนทินไม่มีผลต่อการทำงานของ MRP2 นอกจากนี้การเปลี่ยนแปลงสภาวะของการให้สารในการทดลอง ยังมีผลทำให้ฤทธิ์ในการยับยั้งการทำงานของ P-gp ของสารหายไป จึงเป็นไปได้ว่าฤทธิ์ของฟิลแลนทินและไฮโปฟิลแลนทินที่มีต่อการทำงานของ P-gp เป็นไปในลักษณะผันกลับได้ แต่การให้ฟิลแลนทินหรือไฮโปฟิลแลนทินเป็นระยะเวลานาน 2 หรือ 7 วันกับเซลล์คาโค-2 ไม่มีผลทำให้การทำหน้าที่ของ P-gp เปลี่ยนแปลงไป ดังนั้นจึงเป็นไปได้ว่าฟิลแลนทินและไฮโปฟิลแลนทินอาจทำให้เกิดปัญหาอันตรกริยาระหว่างยาจากคุณสมบัติในการยับยั้งการทำหน้าที่ของ P-gp โดยตรง

ภาควิชา....เภสัชวิทยาและสรีรวิทยา..... ลายมือชื่อนิสิต.....
สาขาวิชา.....เภสัชวิทยา.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา..2553... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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PUNYAKORN SITHTHORNSIRA : EFFECTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN ON FUNCTIONING OF P-GLYCOPROTEIN AND MULTIDRUG RESISTANCE PROTEIN 2 IN CACO-2 CELLS. ADVISOR : ASST. PROF. SUREE JIANMONGKOL, Ph.D., CO-ADVISOR : ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., 85 pp.

The purpose of this study was to investigate and compare the effects of phyllanthin and hypophyllanthin on the function of P-glycoprotein (P-gp) and multidrug resistance protein 2 (MRP2), using the *in vitro* model of the Caco-2 cells. Furthermore, prolonged exposure to phyllanthin and hypophyllanthin on function of P-gp was determined. The activities of P-gp and MRP2 were assessed by measuring the intracellular accumulation of their specific substrate. Under the conditions used in this study, the Caco-2 cells cultured for 21 days expressed the function of both P-gp and MRP2 efflux pumps. The results demonstrated that hypophyllanthin was able to inhibit the P-gp function in comparable potency with phyllanthin, but the inhibitory effects of both compounds against MRP2 were not observed. By varying the treatment conditions, the inhibitory action of both compounds against P-gp function could be loss. This finding suggested the reversibility of the inhibitory effect of these two compounds. Nevertheless, prolonged exposure of the Caco-2 cells to either phyllanthin or hypophyllanthin for 2 or 7 days had no significant effects on the variation of P-gp function. Hence, it was possible that phyllanthin and hypophyllanthin might be able to cause problems regarding P-gp-mediated drug interactions through the direct inhibition of this efflux pump.

Department : Pharmacology and Physiology Student's Signature .....
Field of Study : Pharmacology Advisor's Signature .....
Academic Year : 2010 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
ABCB1	=	ATP-binding cassette, subfamily B
ANOVA	=	analysis of variance
ATP	=	adenosine triphosphate
ADP	=	adenosine diphosphate
BSA	=	bovine serum albumin
ºC	=	Degree celsius
Caco-2	=	human colon adenocarcinoma
Calcein-AM	=	Calcein Acetoxymethyl Ester
CDCF	=	5(6)-carboxy-2',7'-dichlorofluorescein
CDCFDA	=	diacetate ester of CDCF
Cox	=	cyclooxygenase
CYP	=	Cytochrome P450
DMEM	=	Dulbecco's Modified Eagle's Medium
DMSO	=	Dimethyl sulphoxide
e.g.	=	example gratia (for example)
et al.	=	et alii, and others people
EDTA	=	ethylenediaminetetraacetic acid
Etc.	=	Et cetera (and other similar things)
FBS	=	fetal bovine serum
Fig	=	figure
g	=	gram
h	=	hour
HBSS	=	Hank s' balanced salts
IR	=	Infrared
HPLC	=	High-performance liquid chromatography
KB-V1	=	human black cervix carcinoma
kDa	=	kiloDalton

L	=	liter
LS180	=	Intestinal human colon adenocarcinoma
MDR1	=	multidrug resistance protein
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
MRP	=	Multidrug resistance-associated protein
MW	=	Molecular weight
NBD	=	nucleotide-binding domain
NEAA	=	non essential amino acid
NMDA	=	*N*-methyl *D*-aspartate
PBS	=	Phosphate buffered saline
P-gp	=	Permeability glycoprotein
pH	=	Measure of the acidity or basicity of a solution
PXR	=	pregnane X receptor
rpm	=	round per minute
sec	=	second
SEM	=	Standard error of mean
µg	=	microgram
µl	=	microliter
µM	=	micromolar
µmole	=	micromole
v/v	=	volume by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Phyllanthus amarus Schum. et Thonn. or "Luk Tai Bai" in Thai name (family Euphorbiaceae) has been known as a medicinal plant in Thailand for the treatment of several diseases including flu, hepatitis, viral infection, bacterial infection, and cancer (Nair and Abraham, 2008). A number of studies on *P. amarus* revealed that the extracts and its constituents contained various pharmacological actions such as anti-inflammatory effects, anticancer, hepatoprotective and chemoprotective actions (Kassuya et al., 2006; Rajeshkumar et al., 2002; Chirdchupunseree and Pramyothin, 2010; Sharma et al., 2010). Two major pharmacological active lignans isolated from *P. amarus* were phyllanthin and hypophyllanthin (Khatoon et al., 2006; Murugaiyah and Chan., 2007; Annamalai and Lakshmi, 2009). Recent studies in both *in vitro* and *in vivo* models suggested that phyllanthin actively protected against hepatotoxicity induced by carbon tetrachloride, galactosamine and ethanol (Syamsundar et al., 1985; Chirdchupunseree and Pramyothin, 2010). In addition, it was suggested that these two lignans were markedly accounted for the hepatoprotective actions of *P. amarus* (Shim et al. 2000; Khatoon et al., 2006). Hence, these two compounds might have values as new drug entities for further development.

In the processes of drug discovery and development, in addition to drug efficacy, drug safety and potential drug interactions are major issues for investigation. Problems in drug interactions may involve interference either at the metabolizing enzymes or at the efflux transporter proteins. The inhibitory actions of both phyllanthin and hypophyllanthin on cytochrome P450 were demonstrated in a number of studies (Kumar and Kuttan, 2006; Appiah-Opong et al., 2008; Taesotikul et al., 2011). Furthermore, Leite et al. (2006) reported that phyllanthin and other lignans purified from *P. amarus* were able to increase the accumulation of rhodamine in vincristine-resistant Lucena-1 cell line, suggesting the inhibitory action on P-glycoprotein (P-gp) activity. However, there was no report regarding the effect of hypophyllanthin, another major lignan found in *P. amarus,* on P-gp function. It

was possible that hypophyllanthin, of which the core structure is similar to that of phyllanthin, could inhibit the P-gp activity.

P-gp is the main energy-dependent efflux transporter protein in the ABC transporter superfamily (Higgins, 1992). P-gp can be found at the apical site of various normal epithelial cells and tissues such as intestine, kidney proximal tubule and lungs (Fojo et al.,1987; Fromm, 2003; Thuerauf and Fromm, 2006). In addition to P-gp, multidrug resistance protein 2 (MRP2) is another efflux transporter in the ABC superfamily, which can be found at the apical membrane of intestine and other tissues similar to P-gp. Depending upon the anatomical localization, the presence of P-gp and MRP2 plays important roles in pharmacokinetic behaviors of several drugs and xenobiotics by influencing absorption, distribution and elimination (Fromm, 2004). Changes of their activity can potentially alter pharmacokinetic of a number of drugs. Consequently, the pharmacodynamic responses to the drug are possibly affected due to the changes in drug levels.

The substrates of P-gp and MRP2 efflux pumps are diverse with broad spectrum of chemical structures (Takano et al., 2006). It has been reported that certain substrates of P-gp could be transported out of the cells by MRP2 (Dietrich et al., 2003). The examples of these substrates are vinblastine (Flanagan et al., 2002), ritonavir (Guttman et al., 1999) and tetramethylpyrazine (Wang et al., 2010). In this regard, it was possible that phyllanthin, a reported P-gp inhibitor, could also inhibit MRP-2 function. Any compounds that can interact with more than one type of transporters might escalate problems regarding drug interactions (Chan et al., 2004). It might be beneficial to investigate further that either phyllanthin or hypophyllanthin was able to influence the activity of MRP2.

Long term uses of either phyllanthin or hypophyllanthin are usually required to ensure the hepatoprotective outcome. Prolonged exposure can possibly affect the function of efflux transporters, in particular P-gp, which may be useful for prediction of drug interactions. Recently, it was reported that indomethacin heptyl ester could down regulate the function and expression of P-gp in the Caco-2 cells upon 48 and 72 h exposure (Zrieki et al., 2008). Conversely, it has been reported that incubation of the Caco-2 cells with capsaicin for 48 and 72 h led to an increased P-gp activity through the up-regulation of P-gp

protein and MDR1 mRNA levels (Han et al, 2006). Hence, the effect of prolonged exposure to both phyllanthin and hypophyllanthin on the activity of P-gp should also be determined

This study was aimed to investigate the inhibitory effect of hypophyllanthin on the function of P-gp. In addition, the inhibitory effect of phyllanthin and hypophyllanthin on the function of MRP2 was determined. Furthermore, the effect of phyllanthin and hypophyllanthin on the function of P-gp after prolonged exposure was also evaluated.

Hypothesis

1. Similar to phyllanthin, hypophyllanthin could inhibit the P-gp function.
2. Phyllanthin and hypophyllanthin were able to inhibit the MRP2 function.
3. Either phyllanthin or hypophyllanthin might affect the P-gp function after prolonged exposure.

Objectives

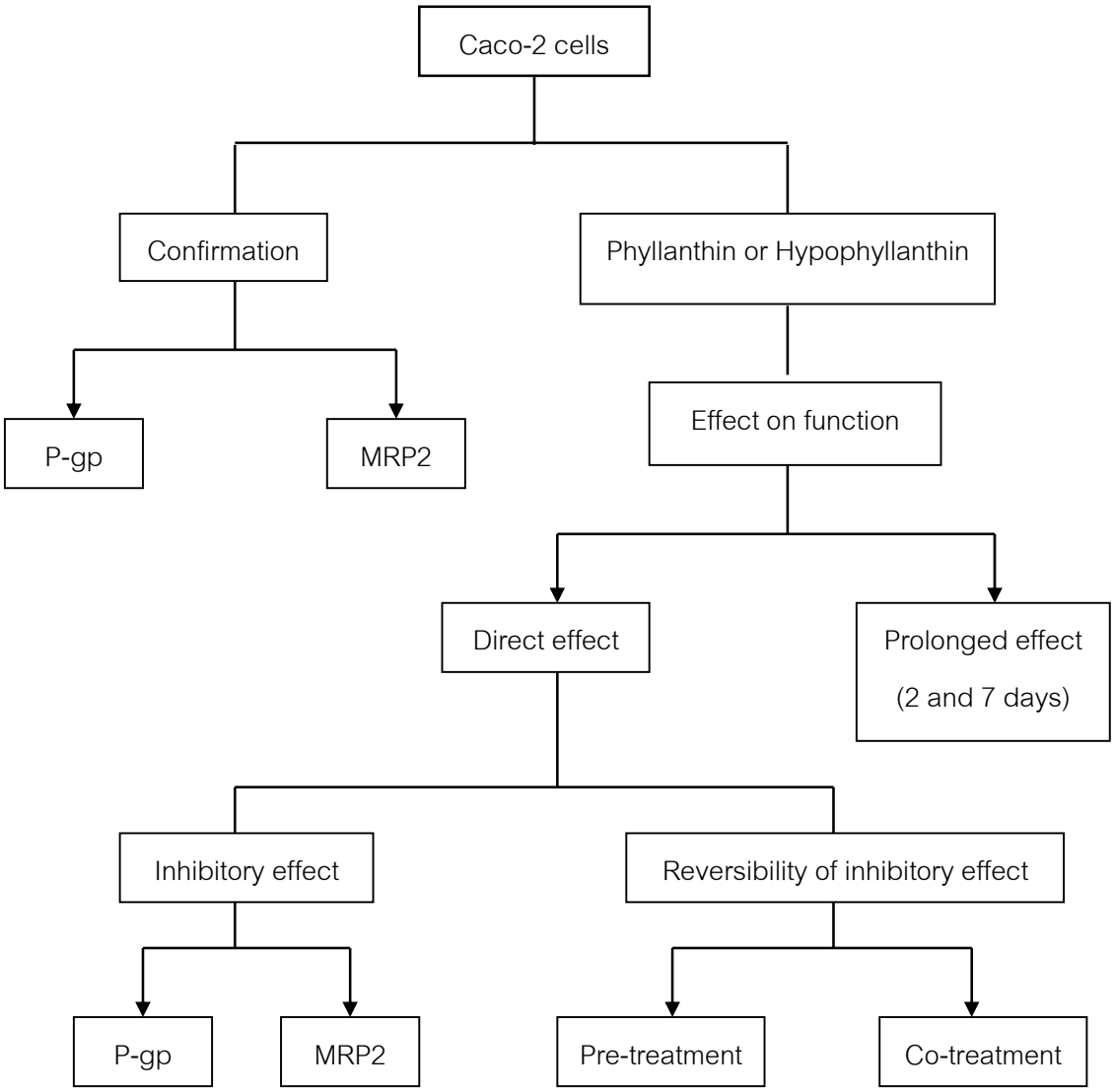
The specific objectives of this study were:

1. To determine and compare the effects of phyllanthin and hypophyllanthin on the function of P-gp and MRP2 in the *in vitro* Caco-2 cells model.
2. To determine the effects of prolonged exposure to either phyllanthin or hypophyllanthin on P-gp function in the Caco-2 cells.

Scope of study

The present study determined the inhibitory effects of phyllanthin and hypophyllanthin, the major lignans of *P. amarus* against the function of P-gp and MRP2 as well as their prolonged effects on P-gp function in the Caco-2 cells culture model. The Caco-2 cell is a human intestinal Caco-2 cell line which contains the characteristic of differentiated intestinal cells (Kedinger et al., 1987) with ability to express P-gp and MRP2 transporter function (Bailey et al., 1996). In this study, the Caco-2 cells were evaluated for the function of P-gp and MRP2. Then, the inhibitory effects of phyllanthin and hypophyllanthin against the function of P-gp and MRP2 were tested. The reversibility of inhibitory action was also investigated by varying the exposure condition between the specific substrate and the test inhibitors. In addition, the effect of prolonged exposure to either phyllanthin or hypophyllanthin on the function of P-gp was evaluated.

Experimental design



CHAPTER II

LITERATURE REVIEW

Phyllanthus amarus

Phyllanthus amarus Schum. et Thonn. *(P. amarus)* is one of the herbal plants in the family Euphorbiaceae. *P. amarus* (synonyms *P. niruri*) is known as “Luk Tai Bai” in Thai name. This plant is found in all parts of Thailand. It is a small erect annual herb that grows up to 10-60 cm high with has terete stem and spreading leaf branches above. Leaves and flowers are very numerous thereby flowers arise in leaf axis (Figure 1).

Figure 1: *Phyllanthus amarus*

This plant has been used as traditional medicine for treatment of several diseases such as hepatitis, colds, flu, tuberculosis, liver diseases, anemia, jaundice and liver cancer; bacterial infections, diabetes and hypertension (Nair and Abraham, 2008).

The chemical constituents in *P. amarus* primarily include lignans such as phyllanthin, hypophyllanthin (Khatoon et al., 2006; Pramyothin et al., 2007; Murugaiyah and Chan., 2007; Annamalai and Lakshmi, 2009); flavonoids such as quercetin, astragalin, quercertrin, isoquercitrin and rutin; tannin such as geraniin. It also contains minor compounds such as hydrolysable tannins like phyllanthusiin D (Houghton et al.,1996) amariin (Foo, 1993), amarulone (Rao and Bramley, 1971), amarinic acid and alkaloids.

Phyllanthin and hypophyllanthin (Figure 2) are the major pharmacological active lignans of *P. amarus*. These two lignans have been reported to be present only in *P. amarus* but not or less present in the other *P. species* (Khatoon et al., 2006; Tripathi et al., 2006).

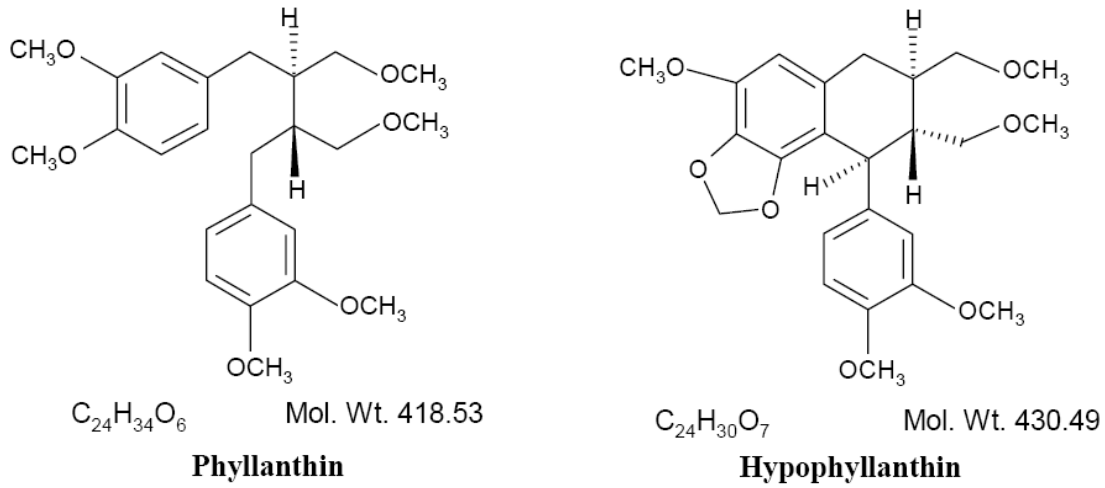


Figure 2: Chemical structures of phyllanthin and hypophyllanthin.

Pharmacological actions of *P. amar*us and its major lignan constituents

1.) Antiviral activity

P. amarus showed inhibitory activity on HIV replication both in vitro and in vivo studies. The water and alcohol extract of *P. amarus* blocked HIV-1 attachment and the HIV-1 enzymes integrase, reverse transcriptase and protease to defferent levels. In addition, a gollotannin containing fraction and the isolated ellagitannins, geraniin and corilagin were exhibited the strong mediators for these antiviral activities. Administration of *P. amarus* to volunteers could reduce HIV replication by more than 30% (Notka et al., 2003; Notka et al., 2004). Administration of *P. amarus* extract decreased the progression of FMuLv-induced erythroleukemia. Treatment with *P. amarus* induced the expression of p53 and p45NFE2 and decreased the expression of Bcl-2 in the spleen of infected mice (Harikumar et al., 2009).

2.) Antibacterial activity

The methanolic extract of *P. amarus* showed significant concentration dependent antibacterial activity particularly against gram-negative microbes (Mazumder et al., 2006). Ethanolic extracts of *P. amarus* were effective against extended spectrum beta-lactamase producing *Escherichia coli* isolated from stool samples of HIV sero-positive patients with or without diarrhea (Akinjogunla et al., 2010).

3.) Hypoglycemic, hypocholesterolemic and anti-hypertensive activity

The aqueous extracts of the leaf and seed of *P. amarus* produced a dose-dependent decrease in the fasting plasma glucose and cholesterol, and weight reduction in treated mice (Adeneye et al., 2006). In addition, aqueous extract of the leaves of *P. amarus* was reported to reduce blood pressure in rabbits (Amaechina and Omogbai, 2007).This hypotensive action might be related to myocardial depression, muscarinic receptor mediated vascular smooth muscle relaxation and the calcium channel ion blockade.

4.) Antinephrotoxic activity

The leaf and seed aqueous extracts of *P. amarus* (100–400 mg/kg/day) were reported to significantly attenuate the increases in serum creatinine and blood urea nitrogen levels and tubulonephrosis in acetaminopjen-mediated nephrotoxic Wistar rats (Adeneye and Benebo, 2008). Similar effects were also shown in the gentamicin model of acute renal injury. The nephroprotective effect of *P. amarus* could be due to the inherent antioxidant and free-radical-scavanging principle(s) contained in the extract (Adeneye and Benebo, 2008).

5.) Anti-inflammatory activity

In mice model, the hexane extract (HE), the lignan-rich fraction (LRF), or the isolated lignans (phyltetralin, nirtetralin, niranthin) exhibited high anti-inflammatory properties by inhibiting carrageenan-induced paw edema and neutrophil influx (Kassuya et al., 2005). The hexane extract and niranthin decreased the specific binding of [(3)H]-PAF to mouse cerebral cortex membranes. Moreover, niranthin exhibited anti-inflammation and anti-allodynia which probably mediated through its direct antagonistic action on the platelet activating factor (PAF) binding sites (Kassuya et al., 2006).

6.) Anticarcinogenic and antimutagenic activity

Aqueous extract from *P. amarus* showed an antimutagenic effect against induction by 2-aminofluorene (AF2), 2-aminoanthracene (2AA) and 4-nitroquinolone-1-oxide (4-NQO) in *Salmonella typhimurium* strains TA98 and TA100, and in *Escherichia coli* WP2 uvrA/pKM101 (Sripanidkulchai et al., 2002). In addition, it also exhibited potent anticarcinogenic activity against 20-methylcholanthrene (20-MC) induced sarcoma development and increased the survival of tumor harboring mice. The extract was found to inhibit DNA topoisomerase II of *Saccharomyces cerevisiae* mutant cell cultures and inhibited cell cycle regulatory enzyme cdc25 tyrosine phosphatase. It was suggested that

the antitumor and anticancer activities of *P. amarus* might be related with the inhibition of metabolic activation of carcinogen as well as the inhibition of cell cycle regulators and DNA repair (Rajeshkumar et al., 2002).

7.) Hepatoprotective activity

Aqueous extract of *P. amarus* showed the protective effect on ethanol-induced rat hepatic injury (Pramyothin et al., 2007). Administration of aqueous extract of *P. amarus* protected against carbon tetrachloride (CCl_4) induced liver damage in female mice (Krithika and Verma, 2009). Ethanolic extract of *P. amarus* showed hepatoprotective effect against aflatoxin B1-induced liver damage in mice (Naaz et al., 2007). The modes of actions were possilbly linked to reduction of the intracellular reactive oxygen species, and increases in both enzymatic and non-enzymatic antioxidant levels (Naaz et al., 2007). In addition, the extract was found to enhance liver regeneration in partial hepatectomised albino rats which were had alcoholic liver injury (Chattopadhyay et al., 2006). The methanolic extract from leave of *P. amarus* had hepatoprotective effect against ethanol-induced oxidative damage in adult male Wistar albino rats, which related to a reduction of lipid peroxidation and an increase of the antioxidant defense mechanism. (Faremi et al., 2008).

Phyllanthin, one of the active lignins present in *P. amarus* was demonstrated its protective effect against carbon tetrachloride-induced toxicity in human hepatoma HepG2 cell line in a concentration-dependent manner (Krithika et al., 2009). It also restored the antioxidant capability of rat hepatocytes including the level of total glutathione, and activities of superoxide dismutase and glutathione reductase which showed protective effect on ethanol-induced rat liver cell injury (Chirdchupunseree and Pramyothin, 2010).

8.) Chemoprotective activity

Methanolic extract of *P. amarus* has chemoprotective activity against cyclophosphamide induced toxicity in mice (Kumar and Kuttan, 2005). In addition, the hydro-alcoholic extract of *P. niruri* (1000 mg/kg/day) given to Swiss albino mice with [7, 12-

dimethylbenz]-induced skin cancer could significantly reduce in tumor incidence, tumor yield, tumor burden and cumulative number of papillomas as compared to carcinogen treated control (Sharma et al., 2010).

9.) Inhibitory effect on efflux transporters

At present, reports on the effects of either phyllanthin or hypophyllanthin on the function of efflux transporters are quite limited. One report demonstrated that the cytotoxicity
of both phyllanthin and hypophyllanthin was potentiated in the presence of vinblastine in multidrug-resistant KB (KB-V1) cells. In addition, phyllanthin, but not hypophyllanthin, was found to inhibit the binding of vinblastine with membrane vesicles derived from this KB-V1 cell line. According to the authors, an interaction between phyllanthin and P-gp was proposed (Somanabandhu et al., 1993). Another study reported that the hexane extract of *P. amarus* along with its lignan constituents including phyllanthin, nirtetralin, niranthin and phyltetralin increased accumulation of rhodamine in vincristine-resistant Lucena-1 cell line, suggesting their inhibitory action on P-gp activities (Leite et al., 2006).

10.) Inhibitory effect on cytochrome P450 (CYP P450)

The aqueous extract of *P. amarus* showed inhibitory effect on several recombinant human CYP isoforms (Kumar and Kuttan, 2006; Appiah-Opong et al., 2008). Oral administration of the methanolic extract of this plant could inhibit CYP1A and CYP2B activities in the phenobarbitone-pretreated rats (Kumar and Kuttan, 2006). Recently, ethanolic and aqueous extracts of *P. amarus* exhibited inhibitory effects on various human CYP activities such as CYP1A4, CYP2D6, CYP2E1, and particularly CYP 3A4. Moreover, phyllanthin and hypophyllanthin were potent mechanism-based inhibitors of CYP 3A4 (Taesotikul et al., 2011).

P-glycoprotein (P-gp)

P-glycoprotein (P-gp) or *MDR1* (multidrug resistance) protein is a member of the ABC transporters and encoded by the human *ABCB1* gene. In human, the MDR isoforms are *MDR1* and *MDR3* whereas MDR isoforms in rodents are *mdr1a, mdr1b*, and *mdr2*. The human *MDR1* product (P-gp) and the mouse *mdr 1a/1b* function as drug efflux transporters (Marzolini et al., 2004). P-gp was the first identified transporter in multidrug resistance (MDR) to chemotherapy in cancer cells and was first isolated from colchicines-resistant Chinese hamster ovary cells by Juliano and Ling in 1976. P-gp functions as a transmembrane efflux pump, thereby it moves the drug molecules or substrates trapped within the cell membrane lipid bilayer to the extracellular domain (Figure 3).

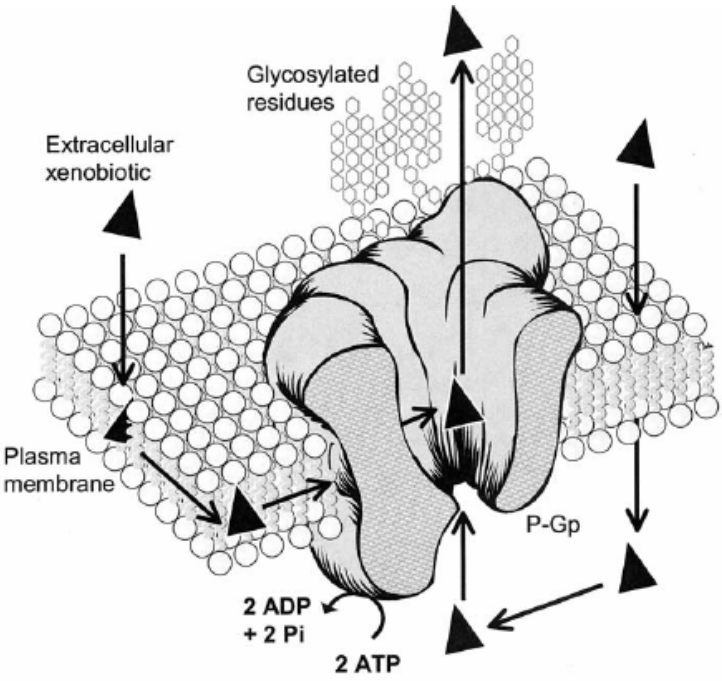


Figure 3: P-glycoprotein (P-gp). This model showed that P-gp–mediated efflux transport of drug substrates occur at the level of the plasma membrane or from the intracellular compartment. ATP, Adenosine triphosphate; ADP, adenosine diphosphate; Pi, inorganic phosphate (Marzolini et al., 2004).

1) Structure of P-gp

P-gp is a phosphorylated and glycosylated transmembrane protein with 1280 amino acids and a molecular weight of approximately 170 kDa. It is composed of two homologous halves, each of which contains six hydrophobic transmembrane domains (TMDs) and a relatively hydrophilic intracellular loop encoding an adenosine triphosphate (ATP) binding site (nucleotide – binding domain [NBD]) (Figure 4). Each NBD consists of two core consensus motifs or the Walker A and B motifs and an S signature of ABC transporters. These motifs are generally found in a wide range of ATPases, and are directly involved in the binding and hydrolysis of nucleotides. The two half molecules are separated by a highly charged 'linker region'. The TMDs appear to contain the drug-binding sites which form the passage for the membrane crossing of drug molecules. The ATP-binding domains act as ATPase converting ATP to ADP in order to provide the energy required for the action of P-gp (Zhou, 2008).

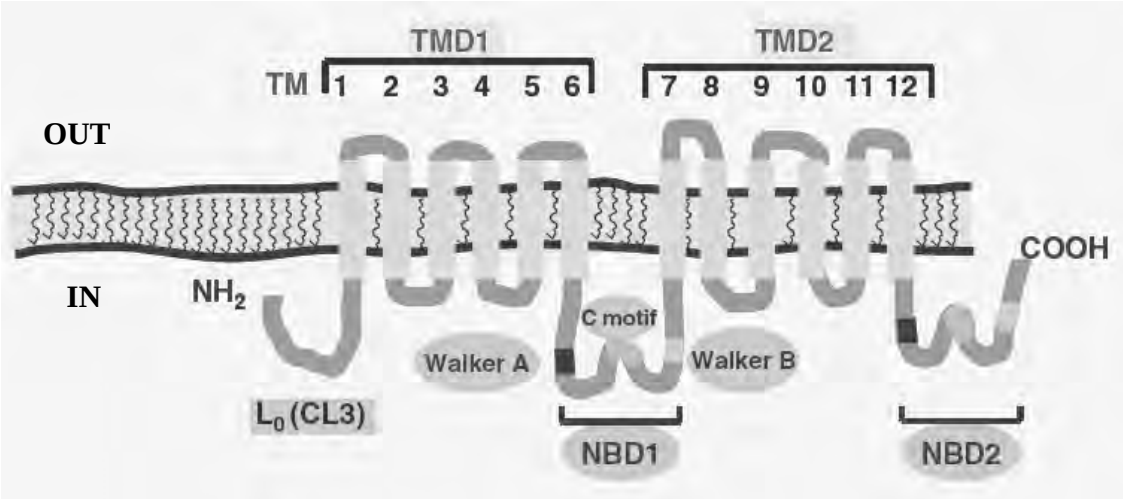


Figure 4: Topology of P-glycoprotein (Zhou, 2008).

2) Mechanism of P-gp Action

The action of P-gp involves two steps which are ATP hydrolysis and substrate transport out of the cells. Initially, the binding of ATP to both NBDs induces the formation of a putative nucleotide sandwich dimmer (Sauna and Ambudkar, 2007). Then, the drug that binds to P-gp drug-binding site within the cytoplasmic membrane leaflet is moved to the extracellular side of the membrane. There are three model proposed for the pumping action of P-gp including the ion translocating pump model, the vacuum cleaner model and the Flippase model (Figure 5). In the classical – ion-translocating pump model, P-gp forms a pore composed of the clustering of the 12 hydrophobic segments, and actively translocates (in an ATP-dependent manner) polar compounds out of the cell. At least two substrate molecules can simultaneously bind in the different overlapping regions. Then, P-gp expels the drugs directly into the extracellular medium due to the energy from ATP hydrolysis. In the vacuum cleaner model, drugs interact with the membrane lipids, then with P-gp. Next, P-gp complex turns inside the membrane and releases drugs in the extracellular medium. In the “ Flippase ” model, P-gp encounters drugs in the inner leaflet of the plasma membrane and flips them to the outer leaflet, subsequently, drug molecules diffuse into the extracellular medium (Sharom, 2008).

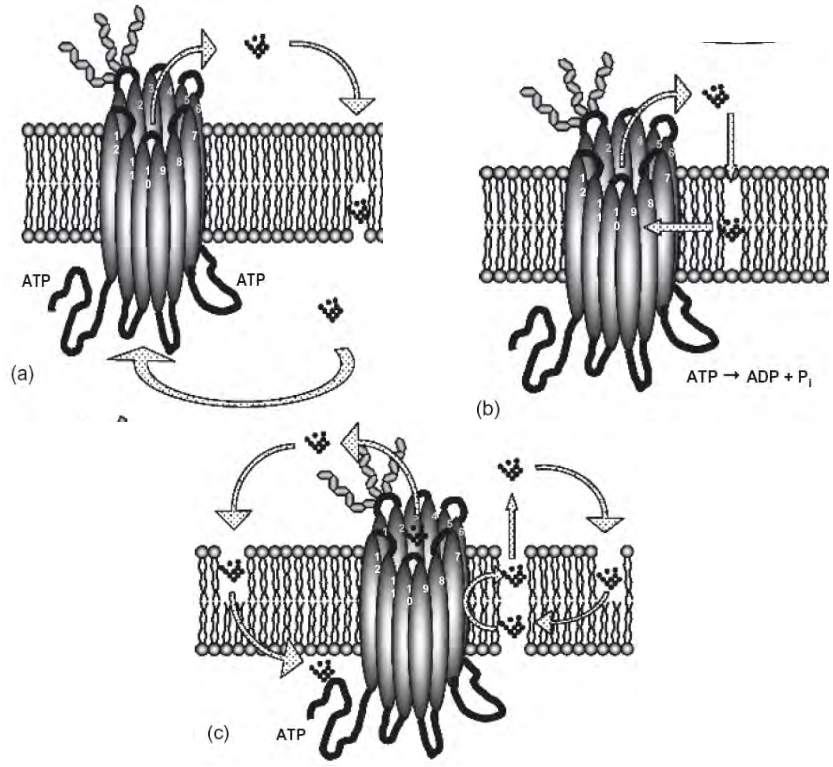


Figure 5. Different functional models of P-gp. (a) The pump model. (b) The vacuum cleaner model. (c) The " Flippase " model (Grandjean – Forestier et al., 2009).

3) Physiological function and localization of P-gp

The expression of P-gp can be found in the apical site of various epithelial or endothelial barriers in several tissues such as intestine, kidney, liver and blood vessels in the brain. In addition, P-gp is found to overexpress in the multi-drug resistant cancer cells, which can be accounted for a reduced efficacy in chemotherapy (Fojo et al.,1987; Fromm, 2003; Thuerauf and Fromm, 2006). Depending on its anatomical localization, P-glycoprotein functions to limit drug entry into the body after oral intake, to promote drug elimination into bile and urine, and to limit drug penetration into sensitive tissues such as the brain, testis, lymphocytes and fetal circulation (Figure 6) (Fromm, 2004).

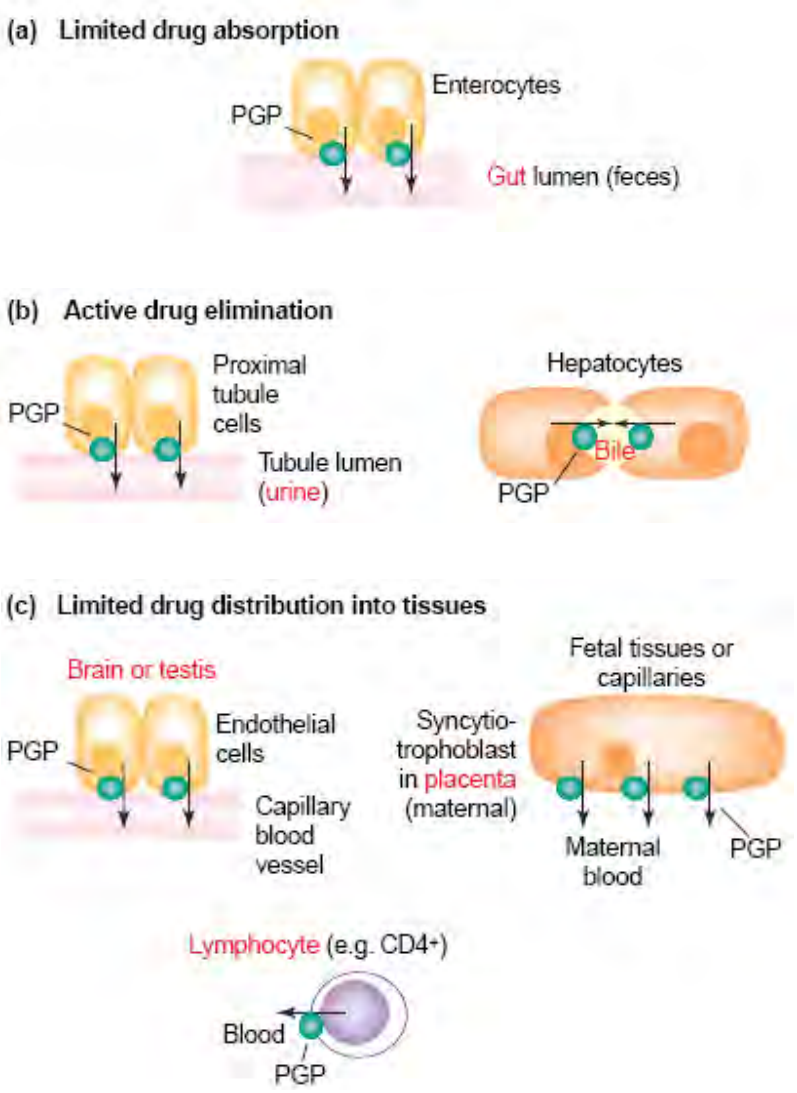


Figure 6. P-glycoprotein expression and function in various tissues (Fromm, 2004).

4) P-gp substrates and modulators

The substances that bind to P-gp can be divided into two classes: the substrates and the modulators. The P-gp substrates can be defied as the compounds which are transported out of the cells via P-gp action. The P-gp modulators are the substances with capability to reverse MDR by blocking P-gp efflux pump.

The P-gp substrates include a broad spectrum of chemical structures with inconclusive structure-activity relationship. A great number of variety of pharmacological agents used in cancer chemotherapy, hypertension, infection, immunosuppression and inflammation are known as either P-gp substrates or modulators with the potential to cause drug interaction upon multidrug usage. Mostly, the P-gp substrates feature hydrophobic, positively charged or neutral molecules with a planar structure. However, the negatively charged compounds such as methotrexate and phenytoin have also been reported as P-gp substrates (Morris and Zhang, 2006). The lipophilicity and the number of hydrogen bonds appear to be relevant parameters for the binding affinity to P-gp (Marzolini et al., 2004). Examples of P-gp substrates are listed in Table 1.

Table 1: List of P-gp substrates (Stouch and Gudmundsson, 2002)

Anticancer drugs	**Hormones**
Vinca alkaloids (vincristine, vinblastine)	Hydrocortisone
Anthracyclines (doxarubicin, daunorubicin)	Progesterone
Epipodophyllotoxins (etioposide, teniposide)	Testosterone
Taxol (paclitaxel, docetaxel)	Dexamethasone
Actinomycin D	Estradiol
Mitomycin C	**Immunosuppressants**
Topotecan	Cyclosporine
Mithramycin	FK506
	Rapamycin

Other cytotoxic agents	
Colchicine	**Detergents**
Emitine	Triton X-100
Puromycin	Tween 80
	Solutol HS-15
Calcium channel blocker	
Diltiazem	**Hydrophobic peptides**
Felopdipine	Gramicidin D
Verapamil	Valinomycin
Nicarpedine	*N*-acetyl-leucyl-leucyl-norleucine
HIV protease inhibitors	**Other compounds**
Ritinovir	Digoxin
Indinavir	Tamoxifen
Saquinavir	Terfenadine
	Rhodamine 123
Antifungals	Calcein-AM
Itraconazole	Morphine
Ketacopnazole	

The inhibition of P-gp activity can result in an increased accumulation of P-gp substrates in the cells. The consequence could be either beneficial outcome in enhancing drug efficacy or unfavorable outcomes in adverse drug reaction problems. A number of chemosensitizers are P-gp modulators with ability to increase the sensitivity of cancer cells to anticancer drugs and to improve the efficacy of chemotherapy. Verapamil (Figure 7) was the first P-gp inhibitor (modulator) that was shown to restore the in vitro sensitivity to vincristine in multidrug resistance cell lines through the competitive inhibition. Recently, a number of natural substances have been reported to possess inhibitory action against P-gp transporter. These substances include capsaicin in chilli peppers, [6]-gingerol in ginger, resveratrol in grapes (Nabekura et al., 2005), curcumin in turmeric (Anuchapreeda et al., 2002; Nabekura et al., 2005; Ampasavate et al., 2010) and flavonoids (Khantamat et al., 2004; Kitagawa et al., 2005; Morris and Zhang, 2006) (Figure 8).

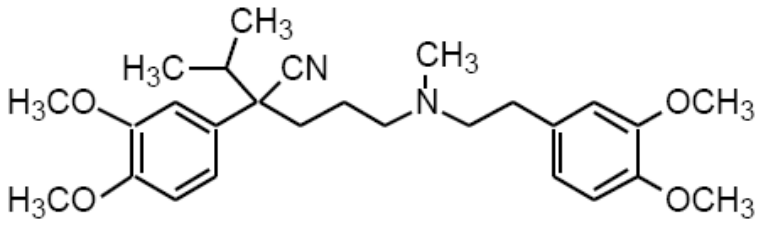


Figure 7. Chemical structures of verapamil (Avendano and Menendez, 2002).

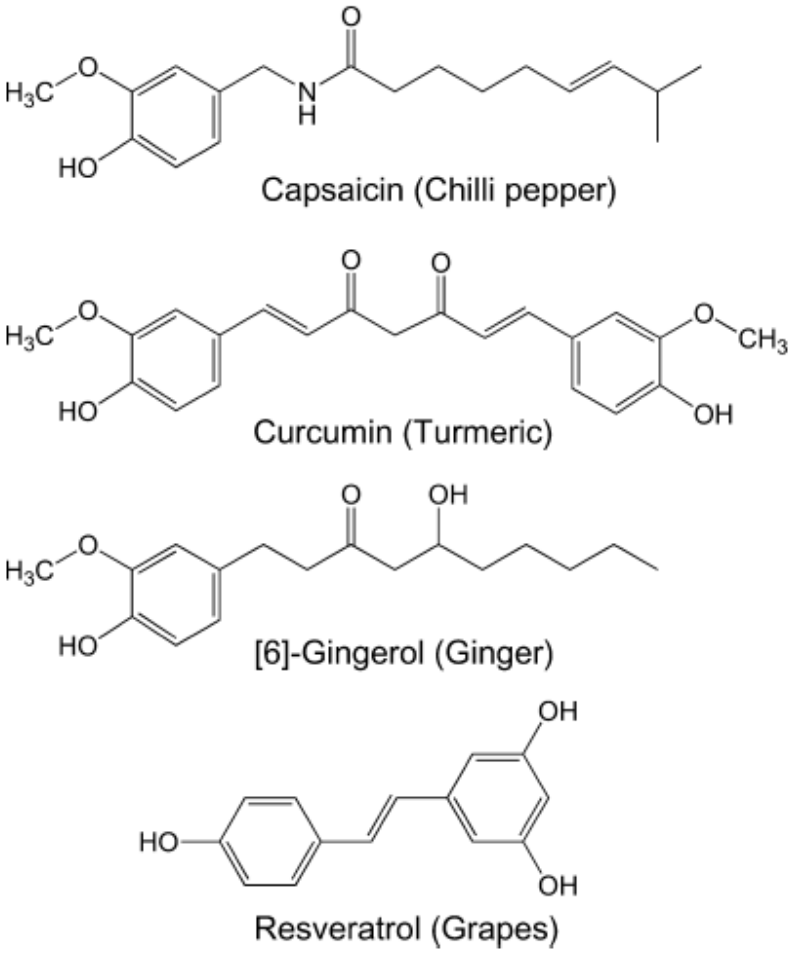


Figure 8. Chemical structures of phytochemicals act as P-gp modulators (Nabekura et al., 2005).

The P-gp modulators might exert its inhibitory actions via several mechanisms (Figure 9). It is possible that the modulators bind directly to substrate binding sites of P-gp thus blocking transport by acting as competitive or non-competitive inhibitors. Another mechanism involves with the inhibition of ATP binding, ATP hydrolysis or coupling of ATP hydrolysis to the translocation of the substrate. Furthermore, the modulators may affect the expression of P-gp through interfering transcription or translation processes. It is also possible that the interaction between modulators and lipid membrane allosterically modifies P-gp conformation and activities (Stouch and Gudmundsson, 2002; Zrieki et al., 2008).

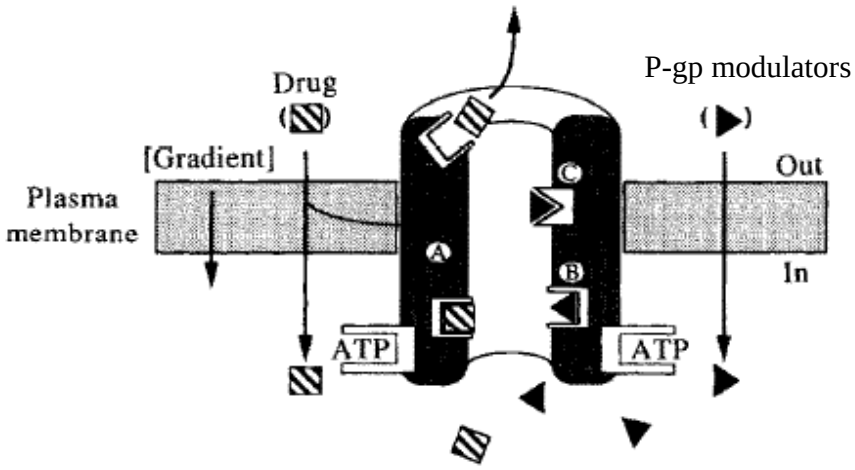


Figure 9. Mechanism of P-gp modulators. The model depicts a membrane efflux pump, which utilizes ATP energy to actively transport drug substrate across the plasma membrane (A). P-gp modulators may serve as a competitive inhibitor by occupying drug-binding sites (B) or a non-competitive inhibitor at P-gp modulators binding sites (C) (Ford, 1996).

5) Example of P-gp-mediated drug interaction

The expression of P-gp at the brush-border of intestinal epithelial cell membrane can limit the drug absorption. Thus, the substrates of P-gp may not be able to enter the circulation via transcellular pathway. Any substances with the capability to modulate P-gp function can cause problems regarding drug interactions.

A number of P-gp mediated-drug interactions were reported in the literature, as shown in Table 2. For example, the drug interaction between digoxin and other drugs such as verapamil, talinolol, clarithromycin and ritonavir (Marchetti et al., 2007). These interactions can lead to an alteration in pharmacokinetics, efficacy and drug safety. In addition to the drugs, excipients in pharmaceutical formulations were reported affect P-gp function. Consequently, these excipients might be able to affect drug absorption. For example, polysorbate 80 was able to inhibit P-gp activity, leading to an increased intracellular levels of daunorubicin in cell cultures (Woodcock et al., 1992). Polyoxyl castor oil and polysorbate 80, which are solubilizers for certain lipophylic and/or poorly soluble drugs were reported to enhance the oral absorption of saquinavir and digoxin, through interaction with P-gp activity (Martin-Facklam et al., 2002; Tayrouz et al., 2003).

In addition, a variety of food and dietary constituents, such as grapefruit, orange, apple, and pummelo juice has been reported its modulating action on P-gp function (Marchetti et al., 2007). For example, flavonoids and furanocoumarins of grapefruit juice were able to inhibit P-gp and CYP3A4 activities in P-gp–overexpressing cell lines (Ohnishi et al., 2000; Spahn-Langguth and Langguth, 2001). Consequently, the intracellular accumulation of anticancer drugs that are P-gp substrates increased in these cells, resulting in an increased chemotherapeutic efficacy (Ohnishi et al., 2000; Spahn-Langguth and Langguth, 2001). In contrast, the apparent bioavailability after oral administration and the plasma concentrations of etoposide were significantly lower in subjects taking grapefruit juice (Reif et al., 2002). The reasons for these discrepancies may relate to the concentrations of drug interacting compounds in the juices (such as fouranocoumarins and flavonoids) (De Castro et al., 2006), as well as the presence of other transporters (such as

OATPs, multidrug resistance–associated proteins [MRPs]) and metabolizing enzymes in the cell.

Table 2: Clinically relevant drug-drug interactions mediated by P-gp

Drug	Inhibitor/inducer	Measured effect/toxicity	References
Digoxin	Verapamil	Greater plasma levels, lower renal clearance	Klein et al., 1982; Verschraagen et al., 1999
Digoxin	Talinolol	Greater plasma levels and AUC, lower renal clearance	Westphal et al., 2000
Digoxin	Clarithromycin	Greater plasma levels, lower renal clearance	Wakasugi et al., 1998
Digoxin	Ritonavir	Greater plasma AUC and terminal half-life and toxicity of digoxin	Phillips et al., 2003; Ding et al., 2004
Loperamide	Quinidine	Greater CNS adverse effects	Sadeque et al., 2000
Talinolol	Erythromycin	Greater AUC	Schwarz et al., 2000

Multidrug resistance-associated protein 2 (MRP2)

The human multidrug resistance-associated protein (MRP, Figure 10) subfamily contains 9 members: MRP1 (*ABCC1*), MRP2 (*ABCC2*), MRP3 (*ABCC3*), MRP4 (*ABCC4*), MRP5 (*ABCC5*), MRP6 (*ABCC6*), MRP7 (*ABCC10*), MRP8 (*ABCC11*), and MRP9 (*ABCC12*). MRP2 is an efflux transporter of the ATP binding-cassette family which was encoded by *ABCC2* gene. It was first functionally characterized as a canalicular multispecific organic anion transporter in the apical membrane of hepatocytes (Jansen et al., 1993). This transporter was firstly cloned from rat liver by Buchler et al. (1996).

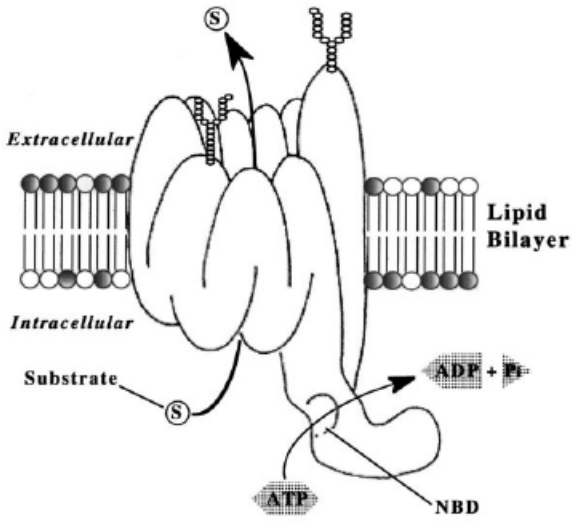


Figure 10: Multidrug resistance-associated protein (MRP) (Quan et al.,2000).

1) Structure of MRP2

MRP2 is a 190-kDa protein with 1545 amino acids. The molecular structure of MRP1, MRP2, MRP3, MRP6, and MRP7 is similar, having 2 ATP-binding domains and 17 transmembrane regions (Figure 11A). The structure of MRP4, MRP5, and MRP8 contain 2 ATP-binding domains and 12 transmembrane regions (Figure 11B) (Takano et al., 2006).

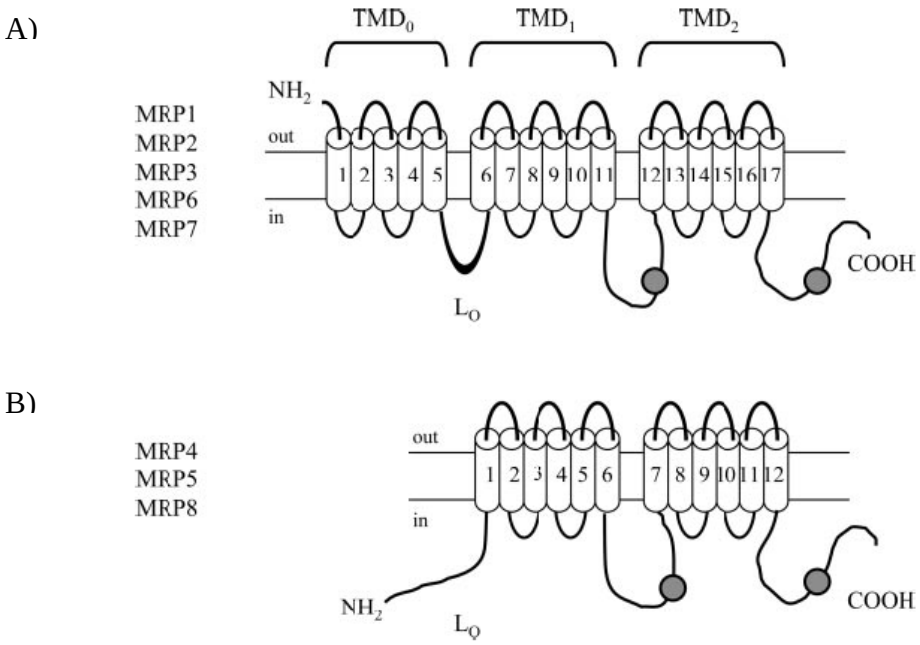


Figure 11: Topology of MRP. (A) Membrane topology of MRP 1,2,3,6,7 transporters; (B) Membrane topology of MRP 4,5,8 transporters (Dallas et al., 2006).

2) Physiological function and localization of MRP2

The tissue distribution and cellular localization of MRP2 influences the efficacy and the toxicity of drugs and environmental toxins. MRP2 is predominantly expressed at the hepatocyte canalicular membrane with the function to excrete conjugated compounds into the bile (Jansen et al., 1993; Koenig et al., 2003). It is suggested that MRP2 plays an important role in detoxification of xenobiotics (Takikawa, 2002). In addition, MRP2 is also expressed in the apical membranes of the polarized cells such as kidney, intestine, gallbladder, bronchi and placenta (Nies and Keppler, 2007). In the intestine, MRP2 is concentrated in the proximal duodenum, jejunum, and little in the distal ileum (Mottino et al., 2000; Dietrich et al., 2003). Moreover, a number of cancerous cells and tumors are also found to express this transporter, which may contribute to multidrug resistance in chemotherapeutic failure.

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3) MRP2 substrates and modulators

Like those of P-gp, the substrates of MRP2 are structurally diverse. MRP2 transports
a variety endogenous and xenobiotic compounds. MRP2 can transport a number of substances including organic anions, glucuronate and GSH conjugates of lipophilic substances (Schinkel and Jonker, 2003). Besides cysteinyl leukotrienes and bilirubin glucuronides, glucuronosyl conjugates of estradiol, tri-iodo-L-thyronine, as well as sulfate conjugates of several bile salts, are important endogenous substrates of MRP2 (Table 3). In addition to amphiphilic anionic substances as these conjugates, MRP2 can transport certain neutral or cationic compounds in co-transport together with reduced GSH such as vincristine and vinblastine (Jedlitschky et al., 2006)

Apart from conjugates, MRP2 also can transport GSH-dependent uncharged or cationic substrates or other amphiphilic anions that do not represent conjugates, including benzylpenicillin (Ito et al., 2004), fusidate (Bode et al., 2002), methotrexate and bromosulfophthalein (Hooijberg et al., 1999; Cui et al., 2001). In addition, it was reported

that natural substances and dietary components such as flavonoids, catechin, chrysin were also able to modulate MRP2 function (Takano et al., 2006).

In addition, among these MRP2 substrates, there are some substrates that overlap with P-gp such as vinblastine, vicristine, doxorubicin, tetramethylpyrazine, ritonavir, grepafloxacin, octreotide, saquinavir (Guttman et al., 1999; Flanagan et al., 2002; Dietrich et al., 2003; Wang et al., 2010).

Table 3: Substrates of MRP2 (Jedlitschky et al., 2006)

Physiological compounds	Exogenous compounds
Glutathione Leukotrienes C4, D4, E4 Conjugated bile salts Bilirubin glucuronides Steroids (17ß-glucuronosyl estradiol)	Anticancer substrates: doxorubicin, etoposide, methotrexate, mitoxantrone, cisplatin, vincristine, vinblastine, camptothecin HIV drugs: indinavir, ritonavir, saquinavir, adevovir, cidofovir, nelfinavir Antibiotics: ampicillin, cefodizime, ceftriaxone, grepafloxacine, irinotecan, azithromycin Further drugs: pravastatine, temocaprilate, conjugates of a variety of drugs (acetaminophen, indomethacin, phenobarbital, sulfinpyrazone) Toxicants: *S*-glutathionyl-2,4-dinitrobenzene, *S*-glutathionyl ethacrynic acid, ochratoxin A, 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridin, 4-(methylnitrosamino)-1-(3-pyridyl)-1-buta-nol, heavy metal complexes (arsenic glutathione, Sb, Zn, Cu, Mn, Cd) Dyes: fluo-3, carboxydichloro fluorescein, sulfobromophthalein

4) Role of MRP2 in drug interactions

Alterations in MRP2 function can change the absorption, organ distribution and clearance of many clinically important drugs, including cancer chemotherapeutics (irinotecan, methotrexate and vinblastine), antibiotics (ampicillin, ceftriaxone and rifampin), antihyperlipidaemics and angiotensin-converting enzyme inhibitors, as well as many toxins and their conjugates (Jedlitschky et al., 2006). Clinically important pharmacokinetic drug–drug interactions between MRP2 substrate and modulators were reported (Jedlitschky et al., 2006). For example, the drug interaction between MTX and certain compounds including NSAIDs, salicylic acid, diuretics and probenecid led to bone marrow suppression and renal failure (Jedlitschky et al., 2006). In addition, it had been reported that concomitant intake of indomethacin and sulfasalazine led to an increased permeability of sulfasalazine in the small intestine via inhibition of MRP2 (Dahan and Amidon, 2010). Consequently, the colonic concentrations of the drug and its therapeutic action were changed (Dahan and Amidon, 2010).

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Assessment of the function of efflux transporters

Accumulation assays are easy and fast method for functional screening of drug efflux pump. The principle of this assay is to determine the intracellular accumulation of selective fluorescent substrate (Kessel et al., 1991). Although this method is quite useful, it has certain limitations such as its incapability to distinguish the differences between substrate and inhibitor. In addition, this method may underestimate pump activity in the presence of the low permeability compounds (Zhang et al., 2003).

Fluorescent substrates for P-gp and MRP2

1. Calcein-AM (Calcein Acetoxymethyl Ester)

Calcein-AM is a good substrate for the P-gp and MRP1 (Hollo et al., 1998). Calcein-AM is a non-fluorescent and highly lipid-soluble dye. Thus, it rapidly diffuses through the plasma cell membrane into the cell and is hydrolyzed by cellular esterases into the fluorescent calcein (Essodaigui et al. 1998). Calcein has favorable properties such as bright fluorescence, and insensitive to Ca^{2+} and Mg^{2+} concentrations or pH (Hollo et al., 1994). Being hydrophilic with organic anion charge, calcein diffuses across lipid bilayers slowly. However, except for P-gp, this fluorescent calcein is a substrate of both MRP1 and MRP2 efflux pump (Essodaigui et al. 1998; Wortelboer et al., 2003; Prime-Chapman et al. 2004) (Figure 12). It has been shown that MRP2 is primarily responsible for the efflux of calcein in the Caco-2 cells (Prime-Chapman et al., 2004).

In the presence of P-gp activity, non fluorescent calcein-AM is pumped out of the cells via P-gp action. Consequently, there is small amount of this compound to be converted into fluorescent calcein within the cell (Homolya et al., 1993). Inhibition of P-gp activity leads to an increased accumulation of calcein-AM which can be reflected by an increased calcein level.

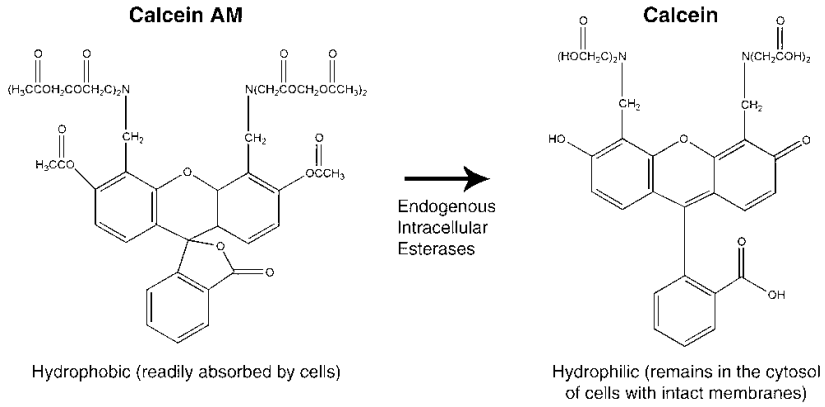


Figure 12: Molecular structure of calcein-AM and its fluorescent derivative, calcein.

2. CDCFDA (5(6)-carboxy-2',7'-dichlorofluorescein diacetate)

CDCF (5(6)-carboxy-2',7'-dichlorofluorescein) is a specific substrate for MRP2 activity (Haimeur et al.,2004). At pH 7.4, the fluorescent CDCF is in highly ionized form with low permeability. Therefore, it accmulates within the cells unless it is pumped out via MRP2. In the assay, the cells are incubated with the non-fluorescent diacetate derivative of CDCF (or CDCFDA) which can passively diffuse across the cell membrane. After passing through the membrane, the intracellular CDCFDA can be readily hydrolyzed into the fluorescent CDCF by the intracellular esterases (Breeuwer et al., 1995) (Figure 13). Thus, inhibition of MRP2 activity can lead to an increased accumulation of CDCF within the cells.

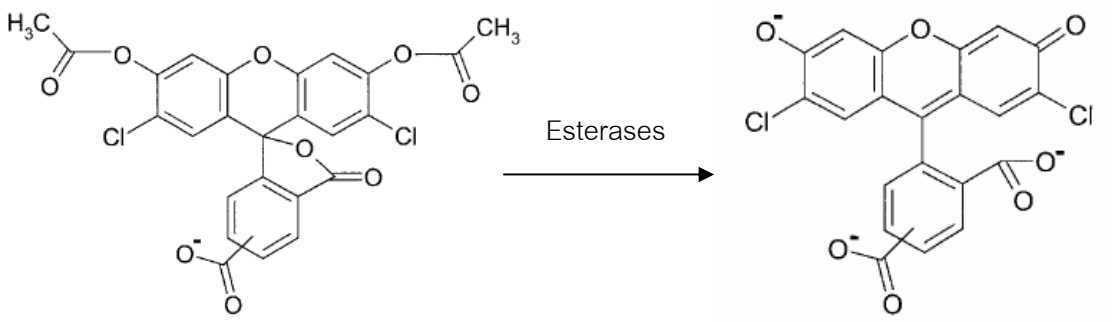


Figure 13: Structure of non-fluorescent 5(6)-carboxy-2',7' dichlorofluorescein diacetate (CDCFDA) and fluorescent 5(6)-carboxy-2',7'dichlorofluorescein (CDCF) at pH 7.4 (Zamek-Gliszczynski et al., 2003).

Caco-2 cells

In order to study the function of P-gp, the *in vitro* cell culture model of the Caco-2 monolayers is well established and widely used for the transporter studies (Wahlang et al., 2009). The Caco-2 cell is an epithelial human colon adenocarcinoma cell line which has been widely accepted as a model of the intestinal barrier to predict absorption of drug molecules (Artursson et al., 2001). This cell line is derived from epithelial human colorectal adenocarcinoma (Ferrec et al., 2001). Upon cultured on microporous filter, the Caco-2 cells form monolayer and differentiate spontaneously into enterocytes in 14-21 days after confluency in standard culture medium. At this age, the cells monolayer expresses of the morphological and functional characteristics of small intestinal absorptive cells, including brush border on the apical surface and tight junction complexes between adjacent cells. In addition, the Caco-2 cell expresses several active transporter along with phase I- and Phase II- metabolizing enzymes including cytochrome P450 (CYP 450) and UDP-glucuronosyl transferases. However, CYP3A4, which can be found in almost all intestinal cells, expresses in the Caco-2 cell at very low levels (Prueksaritanont et al. 1996; Nakamura et al. 2002; Sambuy et al., 2005). It has been known that the Caco-2 cell also expresses various efflux transporters in the ATP binding cassette (ABC) superfamily such as P-glycoprotein (P-gp), multidrug resistant associated protein (MRP) and lung cancer associated resistance protein (BCRP) (Figure 14) (Taipalensuu et al., 2001; Ferrec et al., 2001).

Upon being cultured in optimum condition, the Caco-2 cell monolayers can be a suitable model to study the function and activity of the efflux pumps. Examples of experimental Caco-2 model for the study of P-gp modulators were listed in Table 4. There are several factors influencing the differentiation of the Caco-2 cells into absorptive barriers as well as the functional expression of transporters. These factors include the experimental conditions, number of passages, seeding density, and cultured period (Sambuy et al., 2005). In order to obtain an acceptable level of P-gp expression, Anderle et al. suggested

that the subculture process with trypsinization should be performed at 70% confluency (Anderle et al., 1998).

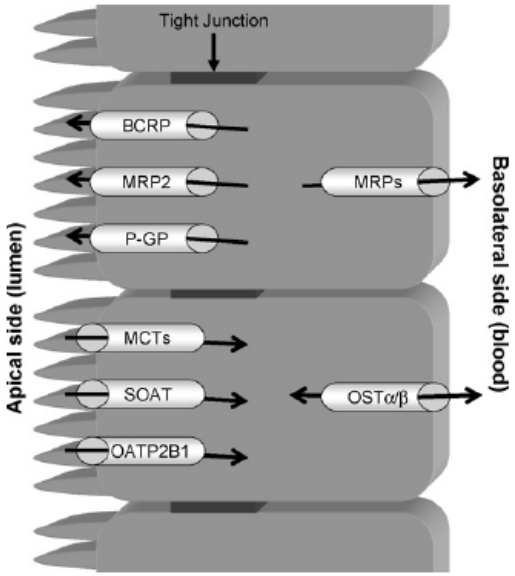


Figure 14: The Caco-2 cell model (Gram et al., 2009).

Table 4: Examples of studies related P-gp using the Caco-2 cells culture model.

Method	P-gp substrate	Test compound	Reference
1. Cellular accumulation	Rhodamine 123	Bitter melon extract	Konishi et al., 2004
2. Cellular accumulation	Calcein-AM	Cadmium	Huynh-Delerme et al., 2005
3. Cellular accumulation and bidirectional transport	[^{3}H]-digoxin and [^{3}H]-vinblastine	Flavonoids	Zhang and Morris., 2003
4. Bidirectional transport	[^{3}H]-digoxin	Capsaicin	Han et al, 2006
5. Bidirectional transport	[^{3}H]-digoxin	Piperine	Han et al., 2008
6. Cellular accumulation and bidirectional transport	Rhodamine 123 and [^{3}H]-digoxin	Curcumas and curcumin	Hou et al., 2008
7. Cellular accumulation	Rhodamine 123 and [^{3}H]-digoxin	Cyclooxygenase Inhibitors	Zrieki et al., 2008
8. Cellular accumulation and bidirectional transport	Rhodamine 123 and digoxin	Diosmin	Yoo et al., 2007
9. Bidirectional transport	Digoxin and vinblastine	Organophosphate pesticide diazinon	Lecoeur et al., 2006
10. Cellular accumulation and bidirectional transport	Rhodamine 123 and calcein-AM	Curcuma longa and Curcuma sp.	Ampasavate et al., 2010
11. Bidirectional transport	Rhodamine 123	Astilbin and taxifolin	Wang et al., 2009
12. Bidirectional transport	Rhodamine 123	Benzo[a]pyrene	Sugihara et al., 2006

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Test compounds

Phyllanthin and hypophyllanthin were kindly provided by Assoc. Prof. Pornpen Pramyothin, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Phyllanthin and hypophyllanthin were kept at -20 ºC until used. Throughout the study, the two test compounds were dissolved in dimethyl sulfoxide (DMSO) 99.9% and further diluted to the desired concentration with the final concentration of DMSO in each experiment at no more than 0.5% (v/v).

1.2 Chemicals

The chemicals in this study included verapamil, probenecid, non essential amino acid (NEAA), bovine serum albumin (BSA), Bradford reagent, ethylenediamine tetraacetic acid (EDTA), penicillin G sodium, Triton X-100, streptomycin sulfate, trypsin, Hanks' balanced salts (HBSS), 0.04% Trypan blue. These chemicals were purchased from Sigma Chemical Company (St.Louis, MO ,USA). In addition, other chemicals included DMSO 99.9% (Labscan; Gliwice, Ireland); potassium dihydrogen phosphate, potassium hydroxide, sodium bicarbonate and sodium chloride (Merck, Darmstadt, Germany). Calcein acetoxymethyl ester (Calcein-AM) and 5(6)-carboxy-2´,7´-dichlorofluorescein diacetate (CDCFDA) were purchased from Fluka (Buchs,Switzerland).

Materials for cell culture were Dulbecco's Modified Eagle's Medium (DMEM) and 200 mM L-glutamine obtained from Gibco Life Technologies (Grand Island, NY, USA) and fetal bovine serum (FBS) obtained from Biochrome AG, USA.

1.3 Cell cultures

The Caco-2 cell line was purchased from American Type Culture Collection (ATCC N°HTB37). In this study, the Caco-2 cells between passage 50-78 were used. The morphology of cultured Caco-2 cells was shown in Figure 15, as observed by an inverted microscope with phase contrast optics in combination (Axiovert 135, Zeiss, Konstanz, Germany) with microcomputer-assisted image capture system (Pinnacle 8, Pinnacel system, Germany).

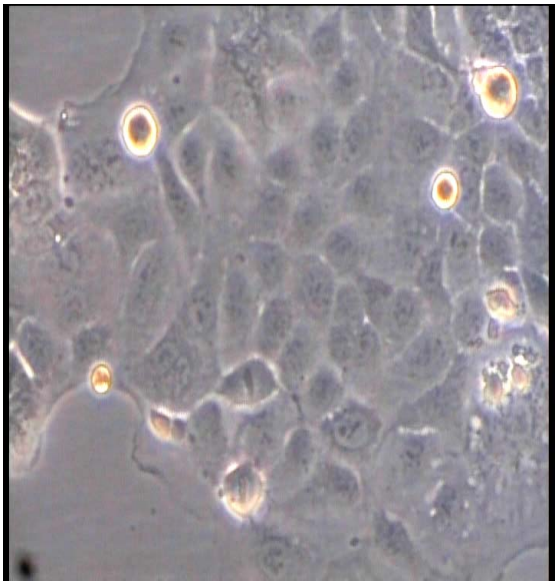


Figure 15: The morphology of Caco-2 cells in the passage number 66th cultured in DMEM with normal culture condition for 3 days. Magnification: x10.

Maintenance of the Caco-2 cells

The Caco-2 cells were cultured in DMEM containing 4.5 g/L glucose and sodium pyruvate, and supplemented with 10% heat-inactivated fetal bovine serum (HIFBS, inactivation at +56 ºC for 30 min), 1% non-essential amino acids (NEAA), 1% L-glutamine, 1% penicillin–streptomycin mixture. The cells were maintained in a 37°C incubator with 5% carbon dioxide and 95% atmospheric air (Forma Scientific, OH, USA).

The Caco-2 cells were seeded at initial density of 5-6 x 10^5 cells per 15 ml in 75 cm^2 tissue culture flasks. The cells were subcultured when the confluency was about 70-80% (approximately 3-4 days after seeding). In the subculture process, the cell monolayer was washed with phosphate buffered saline (PBS) 5 ml, and incubated with 0.25% trypsin in 1 mM EDTA solution (2 ml/ 75 cm^2) for 2-3 minutes at 37°C to detach the cell from the culture flask. At the end of the incubation period, the medium (8 ml) was added to stop the action of trypsin. Thereafter, the cell suspension was dispersed and centrifuged at 1,500 rpm for 4 min. The supernatant was discarded. The pellet was collected and resuspended in the fresh DMEM medium containing 10% FBS, 1% NEAA, 1% L-glutamine and 1% penicillin-streptomycin and transfer to new culture flask. The split ratio was 1:3.

2. Methods

2.1 Confirmation of P-gp and MRP2 activities in the Caco-2 cell culture model

In order to determine the function of each transporter, the accumulation of specific substrate in the presence and absence of specific inhibitor was applied in this study. The specific substrates and inhibitors were listed in Table 5.

Table 5: Specific substrates and inhibitors of efflux transporters.

efflux transporter	substrate	inhibitor	reference
P-gp	Calcein-AM	Verapamil (100 μM)	Ampasavate et al., 2009
MRP2	CDCFDA	Indomethacin, Probenecid (500 μM)	Siissalo et al., 2009; Prime-Chapman et al., 2004

Substrate accumulation assays

For the experiments, the Caco-2 cells were seeded onto 24- well plates at the density of $1.3x10^4$ cells/cm². The medium was replaced every 2 days. After 21 days post seeding, the cells were washed twice with HBSS (pH 7.4). Then the cells were pre-incubated for 30 min in HBSS in either the absence or the presence of each inhibitor. After 30 min, a specific substrate of each transporter (either calcein-AM or CDCFDA) was added to the assay medium and further incubated for another 30 min. The concentrations of calcein-AM and CDCFDA in this study were 0.4 μM and 5 μM, respectively. At the end of this co-incubation period, the test solutions were removed and the cells were washed with ice-cold PBS three times. Then the cells were lyzed with 500 μl of 0.1% Triton X-100 in PBS for 30 min. The cell lysates (200 μl) were transferred to a 96 well black plate for determination of fluorescent density with the microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., USA) at an excitation wavelength and an emission wavelength of 485 nm and 535 nm, respectively.

2.2 Effect of the test materials on P-gp and MRP2 function

2.2.1 Inhibitory effect

The effect of the test compounds (phyllanthin and hypophyllanthin (1, 10, 100 μM)) on the function of P-gp and MRP2 was determined in the Caco-2 cells at day 21 after seeding. The experiment was performed as described above in *Substrate accumulation assays*. The transporter that was inhibited by the test materials was investigated further in the section 2.2.2 and section 2.3.

2.2.2 Reversibility of inhibitory effect

The reversibility of inhibitory action of both phyllanthin and hypophyllanthin was tested by comparing the inhibitory effect observed in three experimental conditions as follows.

a. the pre-treatment condition:

In this condition, the test compound was pre-incubated with the cells in HBSS for 30 min at 37 ºC. Then, the solution was replaced with the HBSS containing the specific substrates, and incubated for another 30 min at 37 ºC prior to determination of fluorescent intensity with the cells.

b. the co-treatment condition:

In this condition, the cells were incubated with the test compound in HBSS containing the specific substrates for 30 min at 37 ºC. At the end of this incubation period, the fluorescent intensity was measured.

c. the pre-treatment followed by co-treatment condition:

In this condition, the cells were treated with HBSS containing the test compound for 30 min at 37 ºC (condition a). Then, the solution was replaced with the HBSS containing the specific substrates and the test compound (condition b). This assay solution was incubated with the cells for another 30 min prior to measurement of fluorescent intensity.

2.3 Effect of prolonged exposure to the test compounds on P-gp function

Effects of prolongs exposure to the test compounds on function of P-gp in the Caco-2 cells were also determined in this study with the calcein-AM accumulation assay. In this study, the Caco-2 cells were seeded on 24-well plates at a density of $1.3x10^4$ cells/cm². On day 14 post-seeding, the culture medium was replaced with the medium containing either with phyllanthin or hypophyllanthin. Then, the P-gp activity was determined periodically at 2 and 7 days later (equivalent to day 16 and day 21 after seeding). On the day of P-gp measurement, the culture medium was changed to a fresh medium in the absence of the test material for 3 h prior to the experimentation. Thereafter, the cells were washed twice with HBSS (pH 7.4) and then incubated in HBSS for 30 min at 37 ºC in either the absence or the presence of verapamil (100 µM). At the end of this incubation period, the solution was changed to HBSS containing either calcein-AM (0.4 µM) alone or calcein-AM with verapamil (100 µM). After the 30 min incubation period, the assay solution was removed and the cells were measured for fluorescent intensity with the microplate reader (Wallac 1420 VICTOR 3 PerkinElmer Inc., USA) at an excitation wavelength and an emission wavelength of 485 nm and 535 nm, respectively.

2.4 Protein determination:

The protein concentration was determined according to Bradford method (1976), using bovine serum albumin as the protein standard. Briefly, samples of homogenized cell lysates (4 µl) were transferred to a 96-well clear plate followed by addition of 200 µl of Bradford reagent®. The absorbance at 595 nm was measured with microplate reader (Wallac 1420 VICTOR 3 PerkinElmer Inc., USA).

2.5 Statistical analysis

Results were expressed as mean±SEM. Statistical analysis was performed by the one-way analysis of variance (ANOVA) followed by the Scheffe's test. Differences were considered statistically significant when $P < 0.05$.

CHAPTER IV

RESULTS

1. The function of P-gp and MRP2 in Caco-2 cells

In this study, the specific substrate and known inhibitor of either P-gp or MRP2 were used to demonstrate their transport function. As shown in Figure 16, verapamil (100 μM), which is a known inhibitor of P-gp, increased intracellular accumulation of calcein by approximately 3.0-folds. In addition, indomethacin (500 μM) and probenecid (500 μM), which are known inhibitors of MRP2, increased intracellular accumulation of calcein by approximately 3.2- and 2.1-folds, respectively. Upon changing the substrate from calcein-AM to CDCFDA, verapamil (100 μM) had no effect on CDCF accumulation implying that verapamil inhibited only on the P-gp activity. In addition, indomethacin and probenecid (500 μM) significantly increased accumulation of CDCF by 4.6- and 3.0-folds, respectively (Figure 17). Taken together, these results suggested that the Caco-2 monolayers at 21 days post-seeding expressed appreciable levels of P-gp and MRP2 activities.

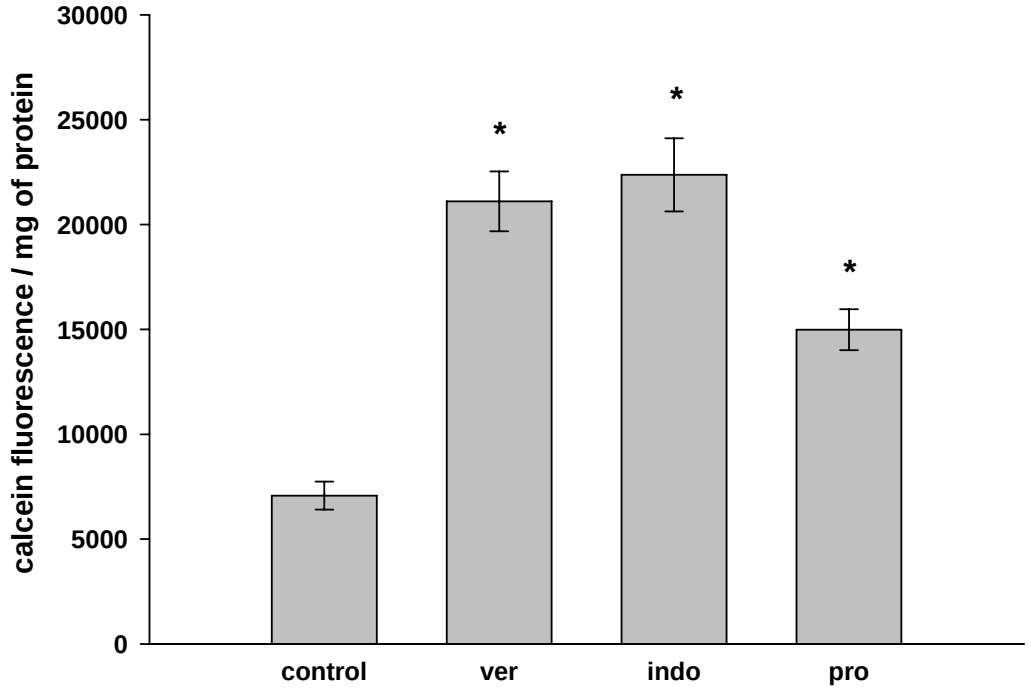


Figure 16: Effect of known inhibitors on accumulation of calcein in the Caco-2 cells. The Caco-2 cells were pre-incubated with known inhibitors; verapamil (ver; 100 μM) or indomethacin (indo; 500 μM) or probenecid (pro; 500 μM) for 30 min at 37ºC suited with 30 min of incubation with calcein-AM (0.4 μM) in the presence of inhibitors at 37ºC, as described in *Materials and Methods*. Each bar represents the mean ± SEM (n=4-6). * $P < 0.05$ vs control.

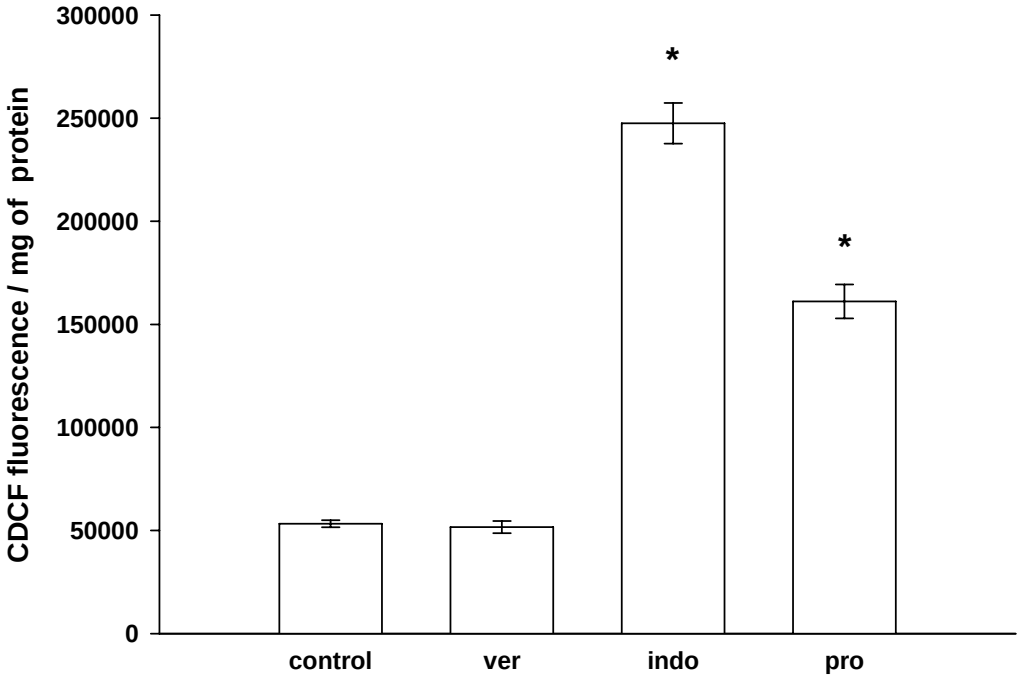


Figure 17: Effect of known inhibitors on accumulation of CDCF in the Caco-2 cells. The Caco-2 cells were pre-incubated with known inhibitors; verapamil (ver; 100 μM) or indomethacin (indo; 500 μM) or probenecid (pro; 500 μM) for 30 min at 37ºC suited with 30 min of incubation with CDCFDA (5 μM) in the presence of inhibitors at 37ºC, as described in *Materials and Methods*. Each bar represents the mean ± SEM (n=5-6). * $P < 0.05$ vs control.

2. Effect of the test materials on function of P-gp and MRP2

Neither phyllanthin nor hypophyllanthin was cytotoxic to the Caco-2 cells in this experimental condition, as determined by trypan blue extrusion method. After treatment with the test compounds, the cells had no change in morphology, no detachment and unstained with trypan blue.

2.1 Inhibitory effects

2.1.1 P-gp function

Both phyllanthin and hypophyllanthin (at the concentration ranging from 1-100 μM) increased the amount of intracellular calcein in concentration related manner (Figure 18). In addition, the effects of these two compounds at the same concentration on accumulation of calcein were statistically indifferent. At the concentration of 100 μM, phyllanthin and hypophyllanthin inhibited P-gp function by 3.7- and 3.2-folds, which were quite comparable to the effect of verapamil (100 μM). These findings suggested that hypophyllanthin was able to inhibit P-gp activities with the comparable potency to phyllanthin.

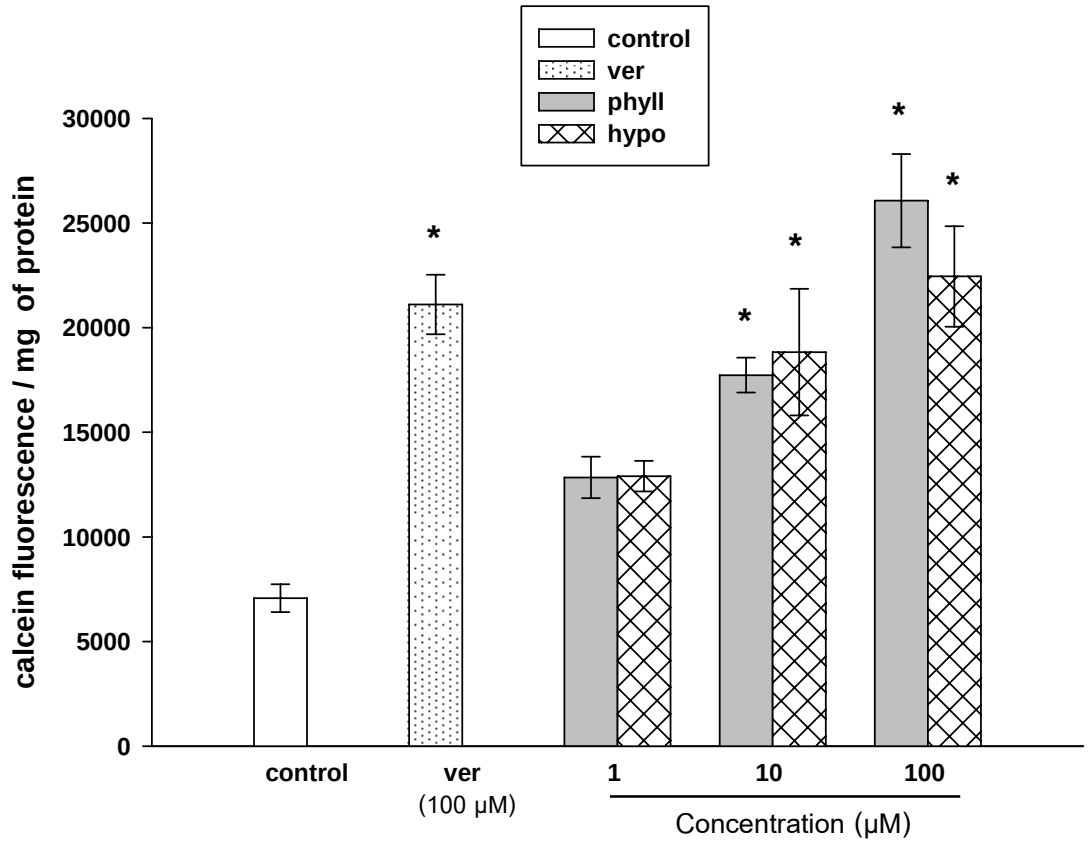


Figure 18: Effects of Phyllanthin (phyll) and hypophyllanthin (hypo) on calcein accumulation in the Caco-2 cells. The Caco-2 cells were pre-incubated with various concentrations of phyllanthin or hypophyllanthin (1, 10, 100 μM) for 30 min at 37ºC suited with 30 min of incubation with calcein-AM (0.4 μM) in the presence of phyllanthin or hypophyllanthin at 37ºC, as described in *Materials and Methods*. Verapamil (ver; 100 μM) was use as a positive control. Data were presented as mean ± SEM (n=6). * $P < 0.05$ vs control.

2.1.2 MRP2 function

As shown in Figure 19, indomethacin and probenecid (500 μM), the known positive inhibitors of MRP2, increased the levels of intracellular CDCF by 4.7- and 2.7-folds, respectively. Neither phyllanthin nor hypophyllanthin at the concentration up to 100 μM enhanced the accumulation of CDCF within the Caco-2 cells. The data suggested that none of the test materials in this study was able to inhibit MRP2 efflux pump.

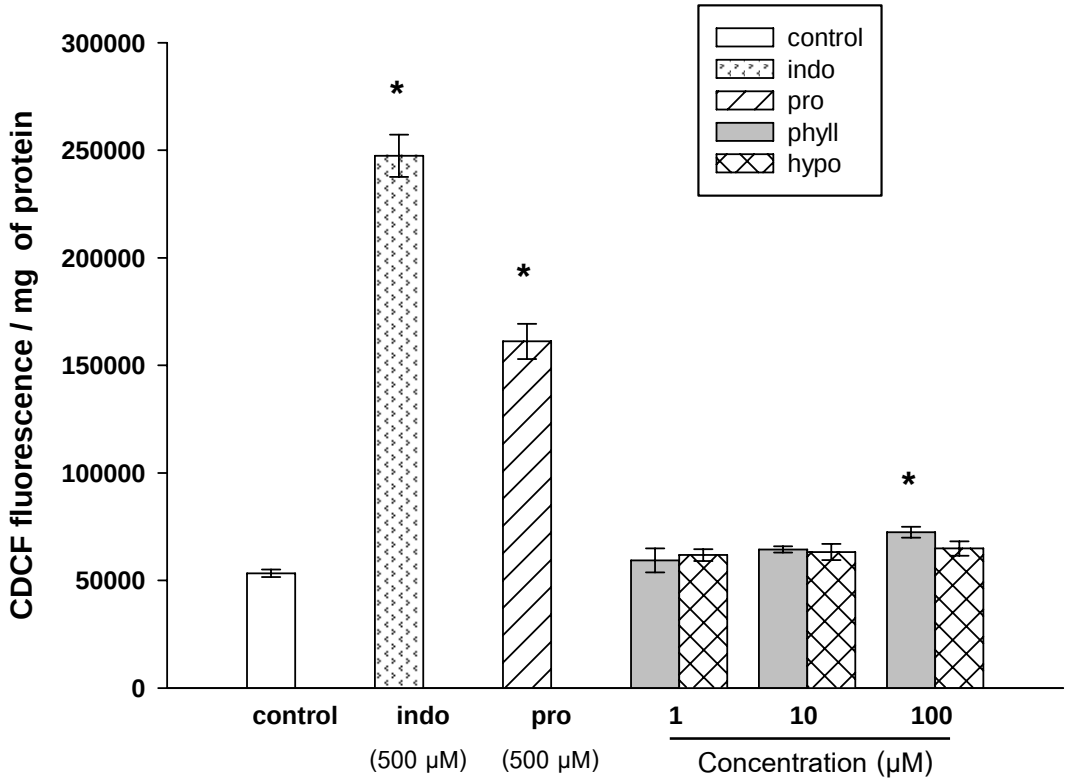


Figure 19: Effect of phyllanthin (phyll) and hypophyllanthin (hypo) on intracellular accumulation of CDCF in the Caco-2 cells. The Caco-2 cells were pre-incubated with various concentrations of phyllanthin or hypophyllanthin (1, 10, 100 μM) for 30 min at 37ºC suited with 30 min of incubation with CDCFDA (5 μM) in the presence of phyllanthin or hypophyllanthin at 37ºC, as described in *Materials and Methods*. Indomethacin (indo; 500 μM) and probenecid (pro; 500 μM) were use as a positive control. Values represented the mean ± SEM (n=5-6). * $P < 0.05$ vs control.

Reversibility of inhibitory effect

As shown in the previous section, both phyllanthin and hypophyllanthin were able to inhibit the activity of P-gp, but not the activity of MRP2. Hence, the reversibility of inhibitory action of the test compounds on P-gp function was further examined. In this experiment, the intracellular accumulation of calcein was measured in two other experimental conditions in addition to the pre-treatment with the test compound followed by the coexistence of the test compound and substrate.

In the condition that the test compounds were pre-incubated for 30 min and washed out before calcein-AM incubation (the pre-treatment condition), neither phyllanthin nor hypophyllanthin could exert their inhibitory action against P-gp activity (Figure 20). The inhibitory actions of the known P-gp competitive inhibitor, verapamil was not observed under this experiment condition. In case that the test compounds and calcein-AM was incubated concurrently (the co-treatment condition), these test compounds appeared to increase intracellular calcein (as shown in Figure 21). However, the results were obtained with high variation, leading to statistical insignificance. The presence of the test compounds for 30 min prior to addition of substrate provided the similar data to those of the co-incubation condition, but with less variation and better inhibition (Figure 22). Taken together, the two lignans exerted their inhibitory action in these experimental conditions similar to verapamil. Hence, it was possible that both phyllanthin and hypophyllanthin might reversible interact with the substrate binding site of P-gp.

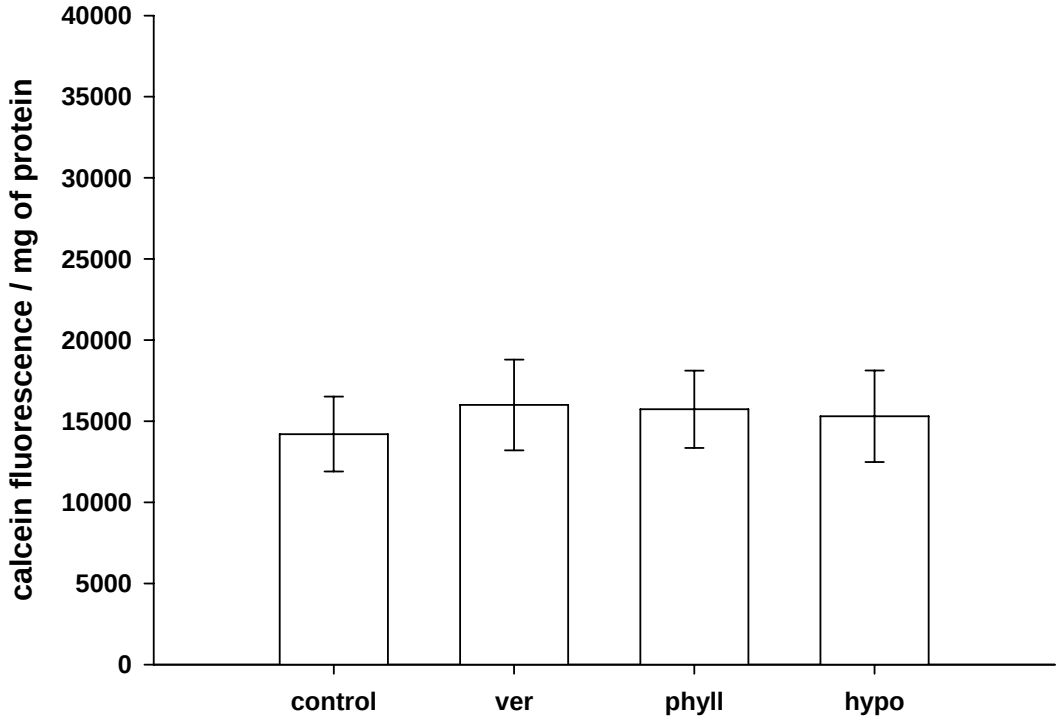


Figure 20: The pre-treatment condition. The Caco-2 cells were pre-incubated with the test materials; verapamil (ver; 100 μM) or phyllanthin (phyll; 100 μM) or hypophyllanthin (hypo; 100 μM) for 30 min at 37ºC followed by another 30 min incubation time with calcein-AM (0.4 μM) in the absence of test materials at 37 °C, as described in *Materials and Methods*. Data were shown as mean ± SEM (n = 4). * $P < 0.05$ vs control.

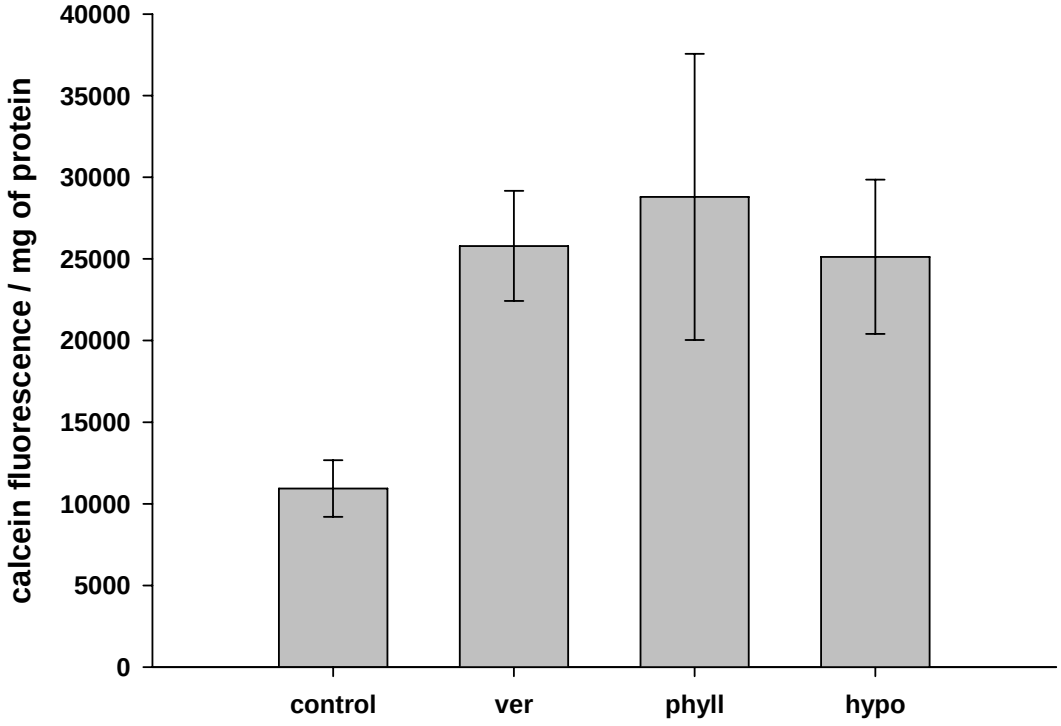


Figure 21: The co-treatment condition. The Caco-2 cells were incubated with calcein-AM (0.4 μM) in the presence of verapamil (ver; 100 μM) or phyllanthin (phyll; 100 μM) or hypophyllanthin (hypo; 100 μM) for 30 min at 37ºC, as described in *Materials and Methods*. Data were shown as mean ± SEM (n = 4). * $P < 0.05$ vs control.

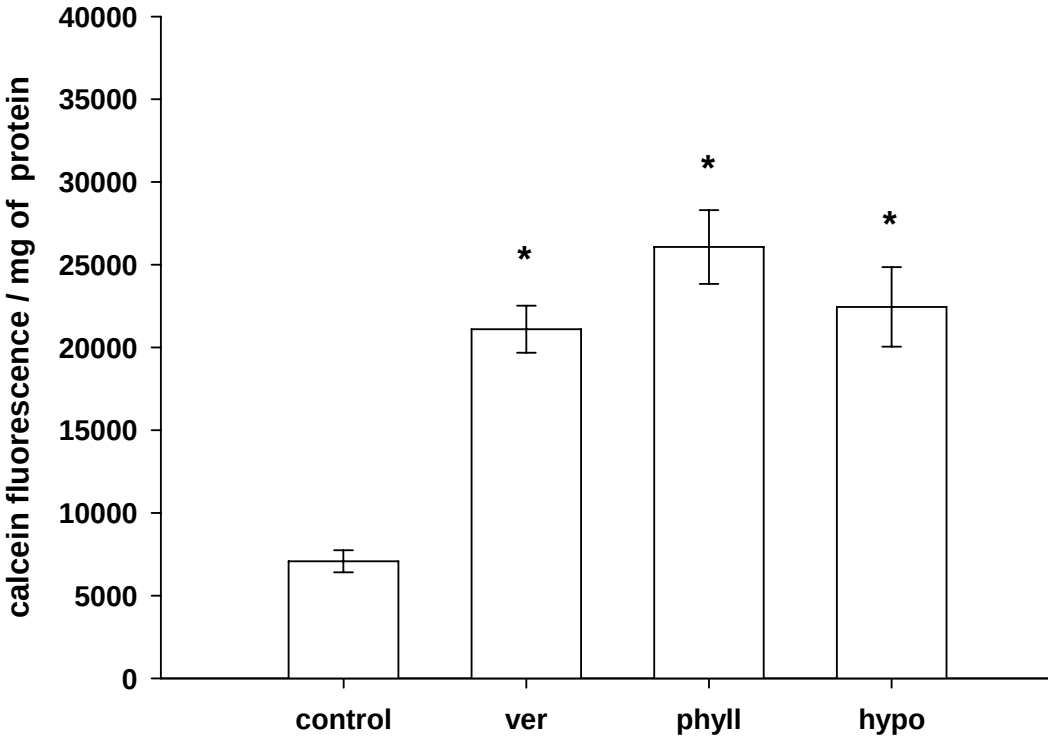


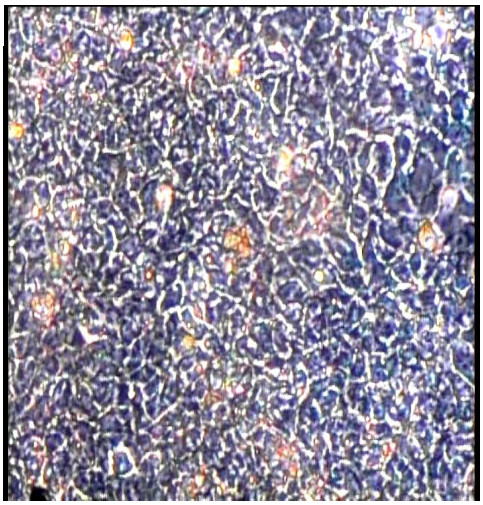
Figure 22: The pre-treatment followed by co-treatment condition. The Caco-2 cells were pre-incubated with the test materials; verapamil (ver; 100 μM) or phyllanthin (phyll; 100 μM) or hypophyllanthin (hypo; 100 μM) for 30 min at 37ºC followed by another 30 min incubation time with calcein-AM (0.4 μM) in the presence of test materials at 37 °C, as described in *Materials and Methods*. Data were shown as mean ± SEM (n = 6). * $P < 0.05$ vs control. (The graph was redrawn from the data set for Figure 18)

3. Effect of prolonged exposure to either phyllanthin or hypophyllanthin on P-gp function

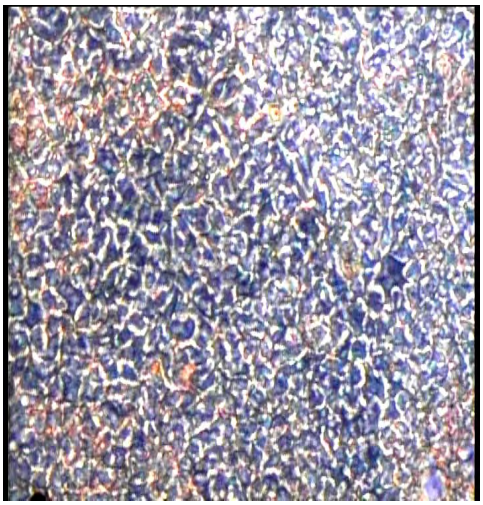
The effects of either phyllanthin or hypophyllanthin on P-gp function were also tested in Caco-2 cells upon 2- and 7- day exposure duration. In the preliminary screening, phyllanthin at the concentration of 100 μM was toxic to the cells after 3 days exposure, as evidenced by cells detachment (Figures 23, B&G). In addition, exposure the cells with hypophyllanthin at 100 μM for 3 days resulted in crystal formation in the medium although the morphology of the cells appeared normal (Figures 23, C&H). Hence, the maximal concentration of the test compounds used for this experiment was at 50 μM.

The P-gp activity in Caco-2 cells was assessed by measurement of the intracellular calcein accumulation. In this experiment, the Caco-2 cells were maintained for 14 days after seeding, and tested for P-gp activity on day 14, day 16 and day 21 after seeding. At 14-day post-seeding duration, the Caco-2 monolayers exhibited the 2.61-fold difference of calcein accumulation in the presence versus in the absence of verapamil (100 μM), suggesting the expression of P-gp activities. On days 16 and 21 after seeding, calcein accumulations were 2.86 and 2.90-fold, respectively. Exposure the cells with phyllanthin (10 and 50 μM) either for 2 days or 7 days had no influence on the fold increase of calcein accumulation in relative to those of the control group (Figure 24). In addition, exposure the cells with hypophyllanthin (10 and 50 μM) also had no influence on the fold increase of calcein accumulation (Figure 25). These findings suggested that the prolonged exposure with phyllanthin or hypophyllanthin did not influence the activity of P-gp.

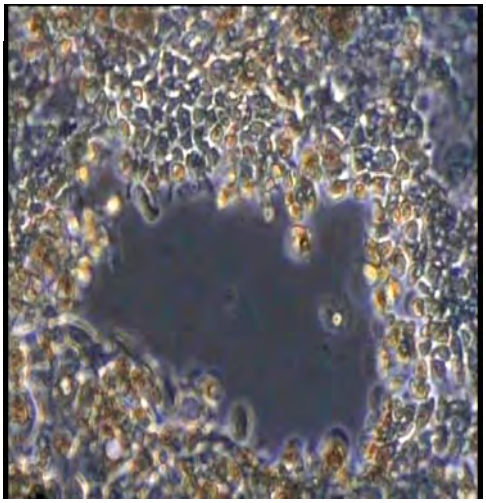
A)



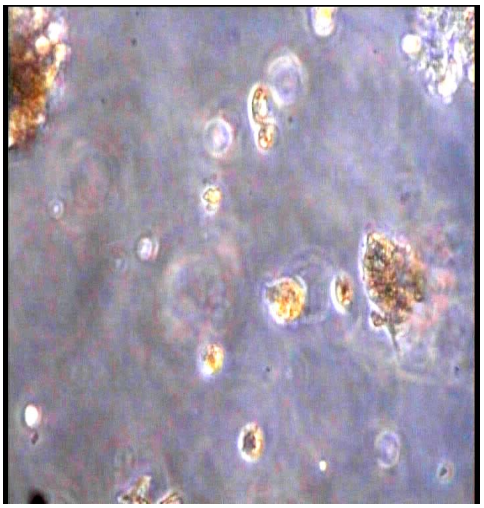
F)



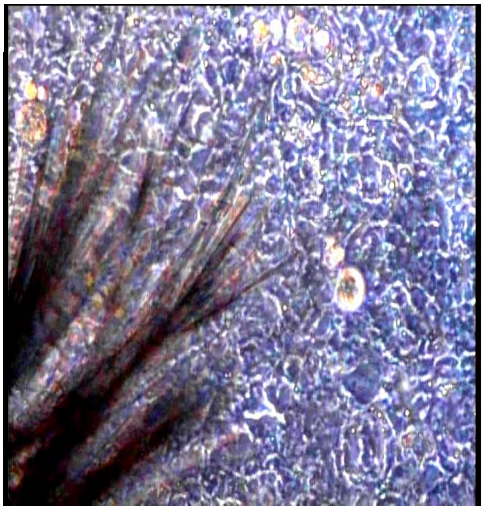
B)



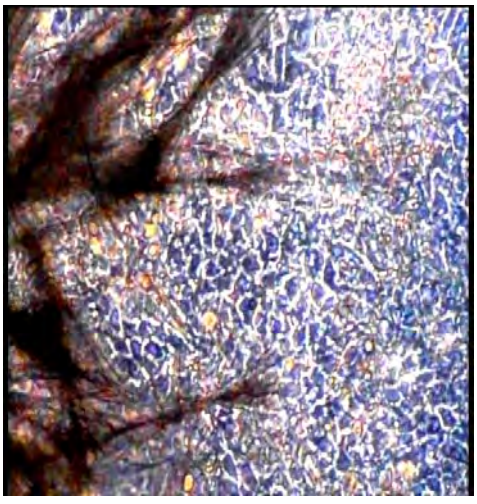
G)



C)



H)



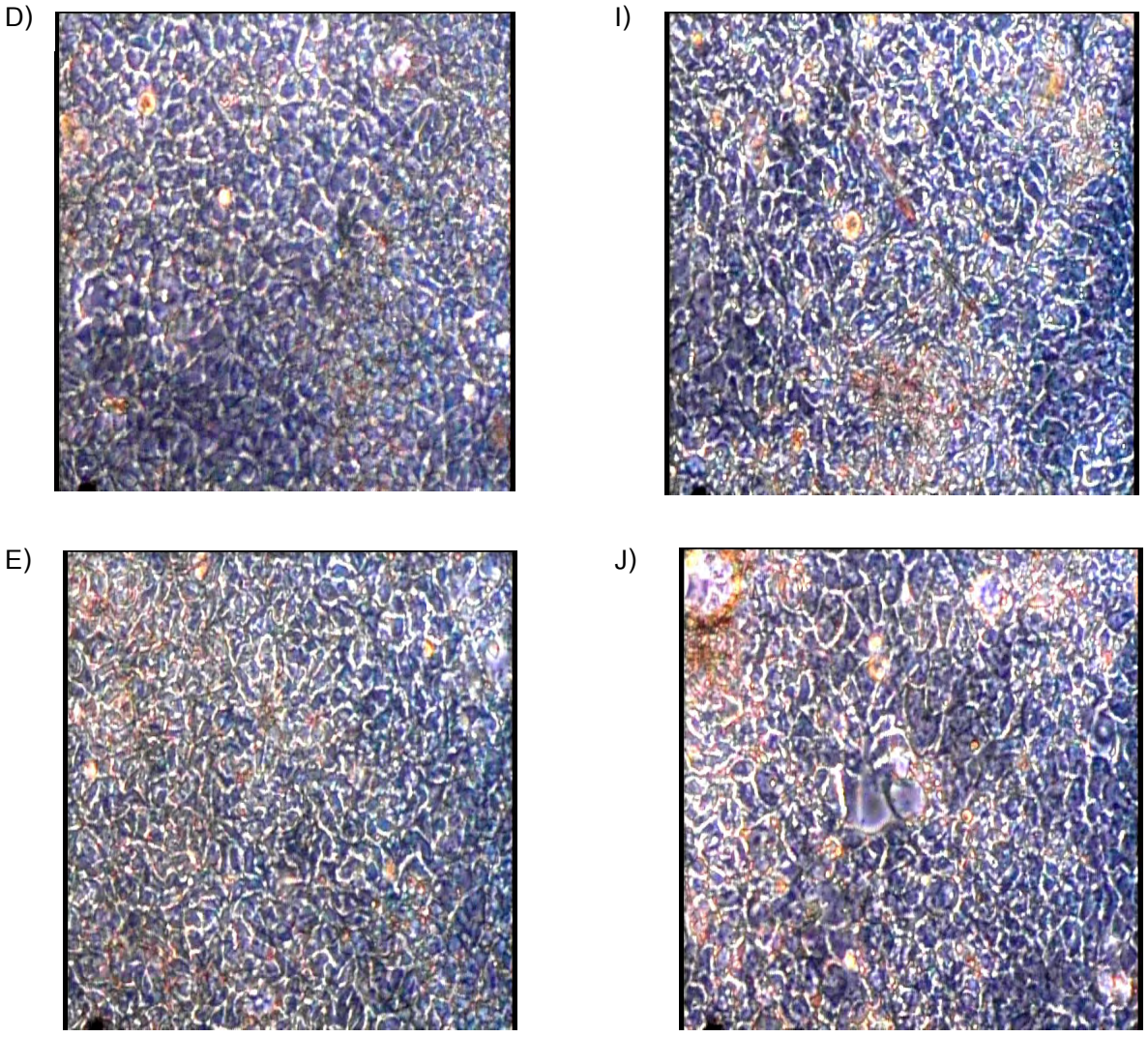


Figure 23: The morphology of Caco-2 cell treated with phyllanthin and hypophyllanthin for 3 (A-E) or 7 (F-J) days. A,F) Caco-2 cells no exposure; control. B,G) phyllanthin (100 μM). C,H) hypophyllanthin (100 μM). D,I) phyllanthin (50 μM). E,J) hypophyllanthin (50 μM).

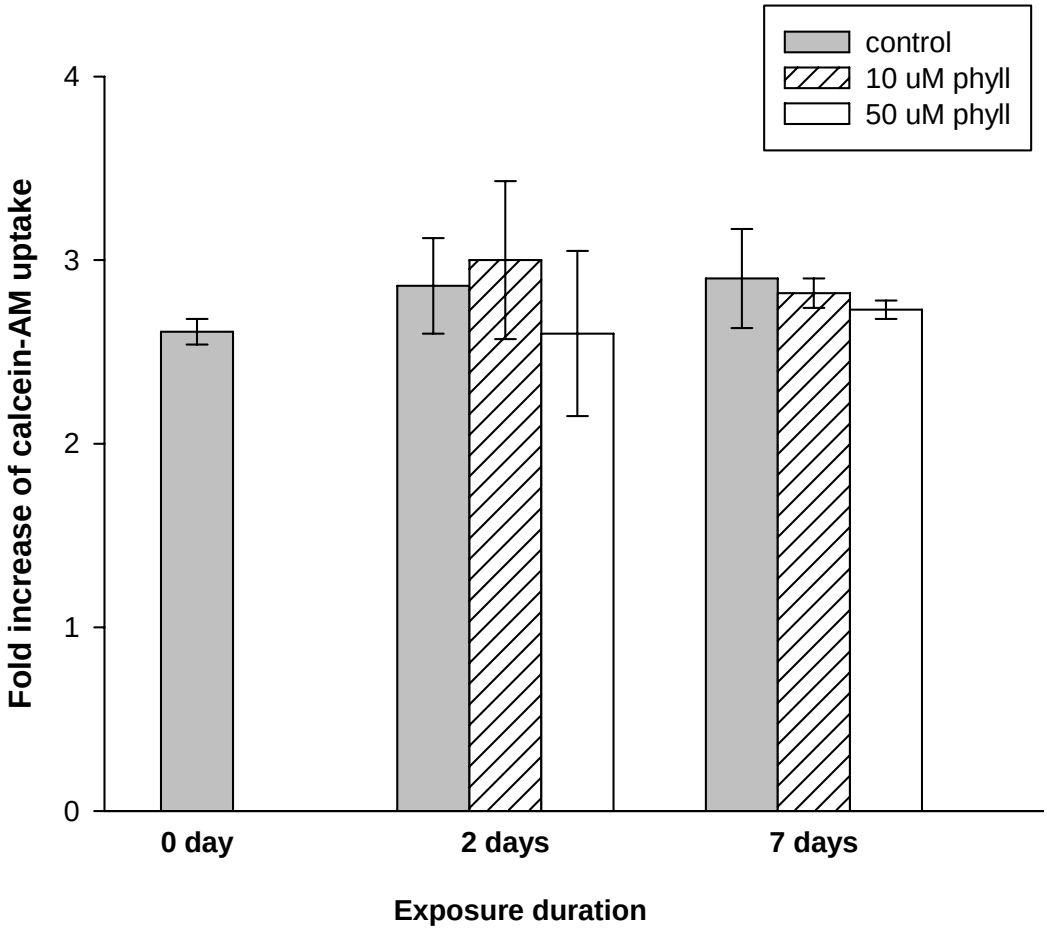


Figure 24: Effect of phyllanthin on the activity of P-gp in the Caco-2 cells. The Caco-2 cells were maintained in DMEM medium for 14 days prior to exposure with either phyllanthin (phyll; 10, 50 μM) for 2 or 7 days duration. The activity of P-gp was assessed by calcein-AM uptake at day 14, day 16 (at 2 day exposure duration) and day 21 (at 7 day exposure duration) after seeding. The bar graph shows the fold increase of calcein accumulation. Data were expressed as mean ± SEM (n=3).

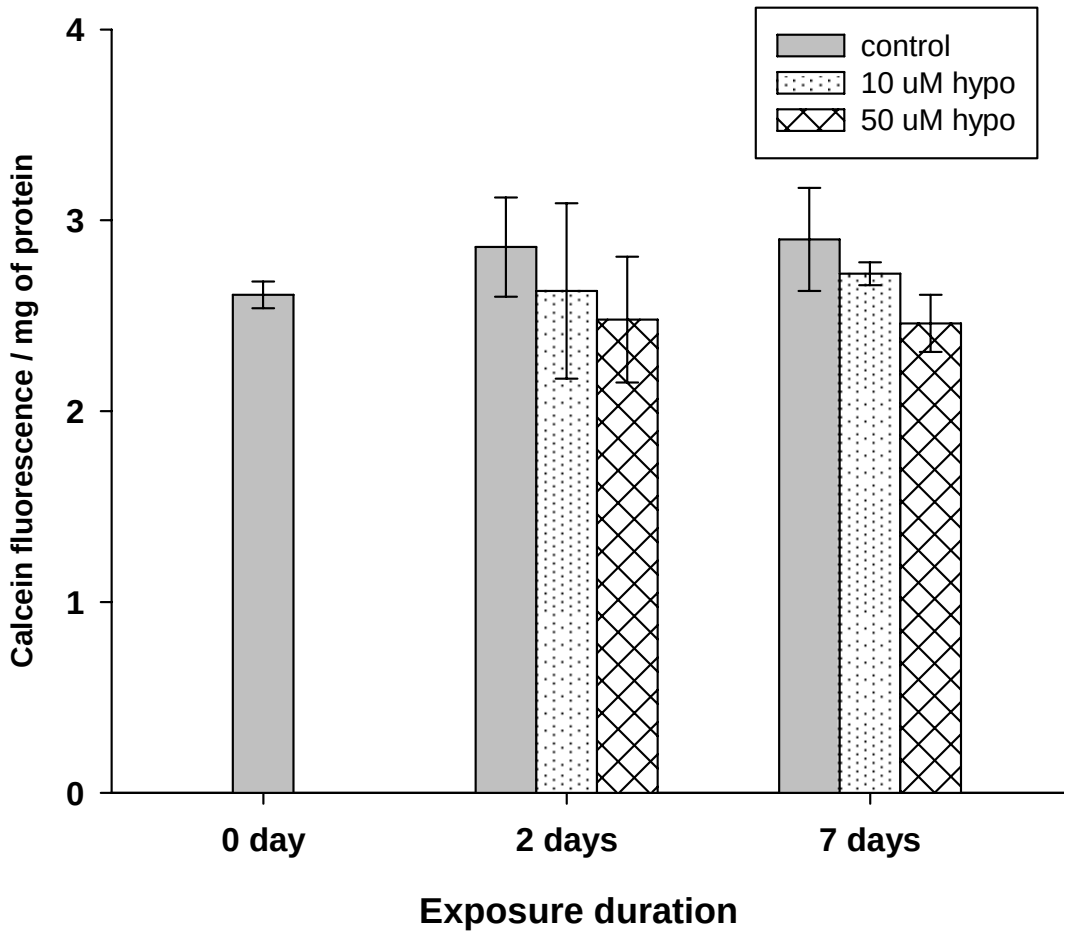


Figure 25: Effect of hypophyllanthin on the activity of P-gp in the Caco-2 cells. The Caco-2 cells were maintained in DMEM medium for 14 days prior to exposure with either hypophyllanthin (hypo; 10, 50 μM) for 2 or 7 days duration. The activity of P-gp was assessed by calcein-AM uptake at day 14, day 16 (at 2 day exposure duration) and day 21 (at 7 day exposure duration) after seeding. The bar graph shows the fold increase of calcein accumulation. Data were expressed as mean ± SEM (n=3).

CHAPTER V

DISCUSSION AND CONCLUSIONS

Interference on the action of efflux transporters including P-gp and MRP2 can cause problems regarding drug efficacy, toxicity and drug interactions. The expression of P-gp and MRP2 efflux pumps in the intestine plays a significant role in limiting the absorption of several drugs and xenobiotics. Any substances with capability to alter either function of intestinal P-gp and/or MRP2 may affect the oral bioavailability and/or pharmacokinetics of other drugs, leading to P-gp and/or MRP2-mediated herb-drug or drug-drug interactions. In addition, P-gp transporter is also found in certain cancerous cells, which has been related to multidrug resistance issue in chemotherapy (Fojo et al.,1987; Fromm, 2003; Thuerauf and Fromm, 2006). In this study, the inhibitory effects of phyllanthin and hypophyllanthin, the major hepatoprotective lignans of *P. amarus*, on the function of P-gp and MRP2 have been determined. Furthermore, the effect of prolonged exposure with either phyllanthin or hypophyllanthin on the function of P-gp was also evaluated, using the cultured human intestinal Caco-2 cell line as an *in vitro* model.

In this study, the function of efflux transporters was evaluated using the specific substrates and inhibitors for each transporter. Calcein-AM and CDCFDA were used as specific substrates for P-gp and MRP2, respectively. Calcein-AM and CDCFDA were converted into fluorescent substances after the cleavage by intracellular esterases. The positive inhibitors in this study included verapamil (a known P-gp inhibitor), indomethacin and probenecid (inhibitors of MRP2). In agreement with other reports, the results showed that verapamil selectively increased the accumulation of calcein, but not CDCF, in the Caco-2 cells cultured for 21 days. By contrast, indomethacin and probenecid increased the accumulation of both calcein and CDCF in the Caco-2 cells. As known, CDCF and calcein are substrates of MRP2. Regarding to calcein accumulation in the presence of MRP2 inhibitors, it could be explained that calcein was transported out of the cells by MPR2.

Therefore, an increase of CDCF and calcein in the presence of specific MRP2 inhibitors suggested the presence of MRP2 activity. The finding was in consistent with the previous study that indomethacin was more potent than probenecid in affecting the MRP2 function (Forster et al., 2008). Taken together, the Caco-2 cells cultured for 21 days in this experimental condition elicited the P-gp and MRP2 activities at appreciable levels for further studies.

In agreement with the previous study by Leite et al. (2006), the inhibitory action of phyllanthin against P-gp activity was demonstrated. In addition, this study demonstrated that hypophyllanthin was able to inhibit P-gp activitiy with the comparable potency to phyllanthin. The effects of hypophyllanthin on P-gp were similar to those of other lignans such as macelignan (Im et al., 2008), phyllanthin, nirtetralin, niranthin, and phyltetralin (Leite et al., 2006). Moreover, neither phyllanthin nor hypophyllanthin even at the highest concentration (100 μM) used in this study could influence on the action of MRP2. These findings suggested that these two compounds might cause less drug interaction problems, comparing with the compounds that could inhibit both P-gp and MRP2 such as vinblastine (Flanagan et al., 2002), ritonavir (Guttman et al., 1999) and tetramethylpyrazine, a bioactive constituent isolated from the root of *Ligusticum chuanxiong Hort* (Wang et al., 2010).

Phyllanthin contains molecular structure that was quite agreeable for P-gp modulators. This compound has steriogenic quaternary center with cationic protonable site linked to an aromatic lipophilic part. It appears that the structure of phyllanthin shares common features with those of verapamil (Seelig and Landwojtowicz, 2000). Considering the molecular structure of the two lignans in this study, hypophyllanthin is more planar-like than phyllanthin, but with similar reactivity. It was suggested that the planar structure of flavonoids might be one of the factors influencing its binding to P-gp (Kitagawa et al., 2005). In this study, the indifferent inhibitory effects of phyllanthin and hypophyllanthin on P-gp activities suggested that the planarity was not critical for the inhibitory actions of these two lignans. Moreover, structures of these lignan compounds were not suitable for interaction with MRP2.

The inhibitory actions of these two lignans against P-gp activity might result from the

direct interaction at the substrate binding sites. The experimental inhibitory conditions, where the appearance of substrate and the test materials in the assay system varied, could affect the inhibition outcomes. In condition that the test material was pre-treated with the cells for 30 min, followed by co-incubation with the substrate, both of the test compounds clearly elicited its inhibitory actions against P-gp. The variation of the data increased markedly in the condition that left out the pre-treatment period. These findings suggested that the membrane permeability of substrate and the test material might be different and could influence the cellular availability of each substance to compete at the P-gp binding sites. The pre-treatment period for the test material might provide its equilibrium appearance to readily compete with the substrate during the incubation period. This condition should be useful especially in case that the permeability of the test compounds was less than that of substrate. Furthermore, the inhibitory actions of the test compounds and verapamil were loss in the condition that the inhibitors were present only in the pre-treatment period. In this condition, it was likely that the binding of the test compounds to the P-gp biding sites could be reversible. Verapamil was a known P-gp inhibitor which exerted its effect through competitive inhibition at the substrate binding sites (Ford, 1996). Hence, it was possible that both phyllanthin and hypophyllanthin acted as competitive inhibitors of P-gp. In this regard, further study on the kinetics of inhibition might be in need to elucidate the type of P-gp inhibition by these two lignans. Furthermore, the inhibitory actions on ATPase activity of these test compounds could be excluded in this study. This presumption was based upon the fact that both P-gp and MRP2 needed ATP hydrolysis for its action. In this study, it was unlikely that ATPase was the inhibitory target when the MRP2 could still function normally in the presence of the test compounds.

In addition to the direct inhibition of P-gp activity, prolonged exposure could affect the activity of P-gp. Several compounds including natural substances were shown to influence the level of P-gp function after the prolonged exposure. The examples of these compounds were capsaicin (Han et al., 2006), piperine (Han et al., 2008), curcumin (Hou et al., 2008), Cox-inhibitors (Zrieki et al., 2008), astilbin and taxifolin (Wang et al., 2009). In this study, the potential effect of either phyllathin or hypophyllathin on the function of P-gp upon

prolonged exposure (up to 7 days) was investigated by determining the level of P-gp activity. In this study, treatment the Caco-2 cells cultured for 14 days with each compound at the concentration of 10 and 50 μM for either 2 or 7 days had no effects on P-gp activity.

In the Caco-2 cells, the activity of P-gp could be possibly altered through changing in protein expression level (Hunter et al., 1993). For example, the loss of P-gp function in the Caco-2 after 48 and 72 h exposure to indomethacin heptyl ester was due to the reduction of P-gp expression (Zrieki et al., 2008). In contrast, capsaicin increased P-gp activity through an up-regulation of cellular P-gp protein and MDR1 mRNA levels (Han et al, 2006). In this study, neither phyllanthin nor hypophyllanthin affected the function of P-gp in the Caco-2 cells after prolonged exposure up to 7 days. Though it was possible that the expression of P-gp in the Caco-2 cells was not affected by the two lignans under the experimental condition used in this study, the effect on P-gp expression could not be ruled out. It was possible that the expressed protein did not function actively as the membrane bound efflux pump. Further study on the protein expression may be needed for this presumption.

In conclusion, this study demonstrated that phyllanthin and hypophyllanthin, the major lignans of *P. amarus*, could directly inhibit P-gp activity without any interference on MRP2 activity. It was likely that both phyllanthin and hypophyllanthin could reversibly bind to the substrate binding sites on P-gp. These results suggested that phyllanthin and hypophyllanthin might be able to cause problems regarding P-gp-mediated drug interactions.

Further studies should be performed to investigate whether phyllanthin and hypophyllanthin are substrates or inhibitors of P-gp as well as their effects on expression of P-gp.

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APPENDICES

APPENDIX A

Phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin were kindly provided by Assoc. Prof. Pornpen Pramyothin, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The extraction and identification processes were described in Chirdchupunseree & Pramyothin (2010).

In brief, phyllanthin and hypophyllanthin were isolated from the aerial part of *Phyllanthus amarus* Schum. et. Thonn. The plant was collected in around Faculty of Pharmaceutical Sciences, Chulalongkorn University, and was authenticated by Assoc. Prof. Chaiyo Chaichantipyuth, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. In order to obtain phyllanthin and hypophyllanthin, the plant underwent the processes of pulverization and extraction with hexane. Then these two lignans were isolated and further purified by column chromatography, using n-hexane and ethyl acetate as mobile phase. Phyllanthin and hypophyllanthin were identified by IR spectrum and HPLC comparing with standard references (Chromadex, Inc., U.S.A.). The purities of these two compounds were at least 98%.

APPENDIX B

List of experimental instruments

1. Autoclave: Hirayama, Japan
2. Fluorescence microplate reader: Wallac 1420 VICTOR3, PerkinElmer Inc., USA
3. Hot air oven: MEMMERT, Germany
4. Humidified carbon dioxide incubator : Forma Scientific, OH, USA
5. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany
6. Laminar air flow hood
7. pH meter: CG 842 Schott, Sciencetific promotion Co. Ltd.
8. Refrigerated centrifuge: Z 383K, Hermle Labortechink, Burladingen, germany
9. Sonication
10. Sterilization filtration membranes (hydrophilic polyethersulfone membrane, 0.2 μm): Life Sciences AcroCap Filter Unit, Maxico
11. Tissue culture flasks, multiwell cell culture plates: Corning, NY, USA
12. Vortex mixer: mode K550-GE, Scientific Industries, NY, USA
13. Spectrofluorometer: Jusco Model FP-777

APPENDIX C

Data of experimental results

Table 6: Results of three inhibitors on calcein accumulation in the Caco-2 cells.

Groups	Calcein fluorescence per mg of protein							
	n1	n2	n3	n4	n5	n6	mean	S.E.
control	8614	4595	7042	9092	6738	6350	7071.83	664.01
100 μM verapamil	21729	19819	16241	18854	25002	24987	21105.33	1426.24
500 μM indomethacin	27349	22724	16488	23341	21960	-	22372.40	1741.04
500 μM probenecid	12141	15447	16615	15733	-	-	14984.00	979.72

Table 7: Results of three inhibitors on CDCF accumulation in the Caco-2 cells.

Groups	CDCF fluorescence per mg of protein							
	n1	n2	n3	n4	n5	n6	mean	S.E.
control	50053	47555	59819	54622	54660	53274	53330.50	1728.56
100 μM verapamil	37709	42650	42806	49567	37154	-	51690.00	2929.99
500 μM indomethacin	205994	190495	219807	192203	179410	181417	247478.00	9820.54
500 μM probenecid	150381	117119	117390	125674	156919	121896	161136.60	8209.34

Table 8: Results of Phyllanthin (phyll) and hypophyllanthin (hypo) on calcein accumulation in the Caco-2 cells.

Groups	Calcein fluorescence per mg of protein							
	n1	n2	n3	n4	n5	n6	mean	S.E.
control	8614	4595	7042	9092	6738	6350	7071.83	664.01
100 μM verapamil	21729	19819	16241	18854	25002	24987	21105.33	1426.24
1 μM phyll	12057	11097	11673	10913	14099	17214	12842.17	990.68
10 μM phyll	18787	17751	15168	20427	18726	15517	17729.33	833.56
100 μM phyll	28653	20413	20815	22598	31346	32585	26068.33	2225.79
1 μM hypo	12124	14412	12371	14987	13486	10045	12904.17	730.71
10 μM hypo	16853	18442	13775	17227	33398	13283	18829.67	3028.63
100 μM hypo	19738	19974	17272	17858	29831	30020	22448.83	2402.55

Table 9: Results of Phyllanthin (phyll) and hypophyllanthin (hypo) on CDCF accumulation in the Caco-2 cells.

Groups	CDCF fluorescence per mg of protein							
	n1	n2	n3	n4	n5	n6	mean	S.E.
control	50053	47555	59819	54622	54660	53274	53330.50	1728.56
500 µM indomethacin	205994	190495	219807	192203	179410	181417	247478.00	9820.54
500 µM probenecid	150381	117119	117390	125674	156919	121896	161136.60	8209.34
1 µM phyll	60974	75294	63925	41253	55198	-	59328.80	5579.06
10 µM phyll	62774	67227	68199	63193	60442	-	64367.00	1452.33
100 µM phyll	72311	65472	73080	65367	78924	79683	72472.83	2539.71
1 µM hypo	58615	68488	63662	65567	52809	-	61828.20	2769.16
10 µM hypo	56472	69116	60963	74783	54922	-	63251.20	3794.39
100 µM hypo	65295	55366	67007	61586	79320	60591	64860.83	3331.52

Table 10: Results of pre-treatment condition of calcein-AM uptake.

Groups	Calcein fluorescence per mg of protein					
	n1	n2	n3	n4	mean	S.E.
control	10917	17269	9632	18987	14201.25	2309.02
100 μM verapamil	12833	21997	9918	19247	15998.75	2791.77
500 μM indomethacin	12217	16721	9866	17103	13976.75	1763.03
500 μM probenecid	10485	12282	9308	17175	12312.50	1732.33
100 μM phyll	13126	20139	10359	19312	15734.00	2378.70
100 μM hypo	10443	19339	10460	20962	15301.00	2819.39

Table 11: Results of co-treatment condition of calcein-AM uptake.

Groups	Calcein fluorescence per mg of protein					
	n1	n2	n3	n4	mean	S.E.
control	9768	15991	8100	9875	10933.50	1734.11
100 μM verapamil	32664	30355	18807	21358	25796.00	3372.63
500 μM indomethacin	42552	34947	16714	15377	27397.50	6740.94
500 μM probenecid	34609	20823	8769	13523	19431.00	5633.92
100 μM phyll	54939	22138	20602	17514	28798.25	8766.46
100 μM hypo	36936	24294	25467	13823	25130.00	4726.19

Table 12: Results of prolonged exposure to phyllanthin or hypophyllanthin in the Caco-2 cells.

Groups	Fold increase of calcein-AM uptake					
	0 day		2 days		7 days	
	mean	S.E.	mean	S.E.	mean	S.E.
Control	2.61	0.07	2.86	0.26	2.90	0.27
10 μM phyll			3.00	0.43	2.82	0.08
50 μM phyll			2.60	0.45	2.73	0.05
10 μM hypo			2.63	0.46	2.72	0.06
50 μM hypo			2.48	0.33	2.46	0.15

VITA

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