CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Test compound

Phyllanthin was kindly offered by Associate Professor Pornpen Pramyothin, Ph.D., Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Phyllanthin was isolated from the aerial part of *Phyllanthus amarus* Schum. et. Thonn. (family Euphorbiaceae), as described in Pramyothin et al., 2007. In brief, the fresh plant was pulverized and extracted with hexane in a soxhlet apparatus. Then the extract was isolated and characterized in each fraction with thin-layer chromatography using n-hexane:ethyl acetate (3:2) as mobile phase. Phyllanthin was identified by IR spectrum and HPLC comparing with standard reference (Chromadex Inc., USA). The purity of phyllanthin was at least 98%.

Phyllanthin was dissolved in dimethyl sulfoxide (DMSO) 99.9% and kept at -20 °C until used. On the day of experiments, phyllanthin was diluted with Hanks' Balanced Salt solution (HBSS) to the desired concentration. The final concentration of DMSO in the experiment was less than 0.5% (v/v).

1.2 Chemicals

Other chemicals included antipyrine, furosemide and theophylline from Fluka (Buchs, Switzerland). Hanks' Balanced Salt solution (HBSS), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic

acid) (HEPES), non-essential amino acids solution (NEAA), 2-(N-morpholino) ethanesulfonic acid hydrate, 4-morpholineethanesulfonic acid (MES hydrate), penicillin G sodium, streptomycin sulfate, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Triton X-100, rhodamine 123, verapamil hydrochloride, and vinblastine sulfate (VBL) were purchased from Sigma Chemical Company (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) and L-glutamine were from Gibco Life Technologies (Grand Island, NY, USA). Fetal Bovine Serum (FBS) was from Biochrom AG (Berlin, Germany). Acetonitrile and methanol of HPLC grade were purchased from Burdick and Jackson (Gyeonggi-do, Korea).

1.3 Experimental instruments

1. Autoclave: Hirayama, Saitama, Japan
2. Digital ultrasonic cleaning unit: T900H, Elma, Singen, Germany
3. Flask-trap aspirator: FTA-1, Biosan, Riga, Latvia
4. Hot air oven: MEMMERT, Buchenbach, Germany
5. HPLC system: Shimadzu, Kyoto, Japan
6. Humidified carbon dioxide incubator: Forma Scientific, Ohio, USA
7. Inverted microscope: Axiovert 135, Zeiss, Konstanz, Germany
8. Laminar air flow hood
9. Microplate reader: Wallac 1420 Perkin-Elmer Victor 3, Perkin Elmer Inc., Massachusetts, USA
10. Millicell®-ERS potentiometer: Millipore, USA
11. Multiwell plates: Corning, New York, USA
12. Orbital shaker: OS-20, Biosan, Riga, Latvia
13. pH meter: CG 842, Schott, Hofheim, Germany
14. Polycarbonate membranes, Transwell® inserts (0.4 µm pore size; 24 mm diameter): Corning, New York, USA
15. Refrigerated centrifuge: Z 383K, Hermle Labortechink, Burladingen, Germany
16. Tissue culture flasks : Corning, New York, USA
17. Vortex mixer: mode K550-GE, Scientific Industries, New York, USA
18. Water bath: WB22, Memmert, Germany

1.4 Cell culture

In this study, there were two cell culture models including the human colon adenocarcinoma (Caco-2) and the vinblastine-treated Caco-2 cells (VBL-Caco-2 cells). The Caco-2 cells (ATCC® HTB37™, Rockville, MD, USA) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) (Figure 8). The cells were cultured in DMEM supplemented with 10% FBS, 1% NEAA, 1% L-glutamine and 1% penicillin-streptomycin mixture at 37°C in a humidified atmosphere of 5% CO_2. The cells were sub-cultured using 0.25% trypsin in 1 mM EDTA solution every 3–4 days (at approximately 70 % confluent). The VBL-Caco-2 cells were obtained by culturing the cells (passage 96 to 112) in DMEM medium containing 10 nM vinblastine (VBL) (Shirasaka Y., Kawasaki M. et al. 2006, Siissalo S., Laitinen L. et al. 2007, Hellinger E., Bakk M.L. et al. 2012).

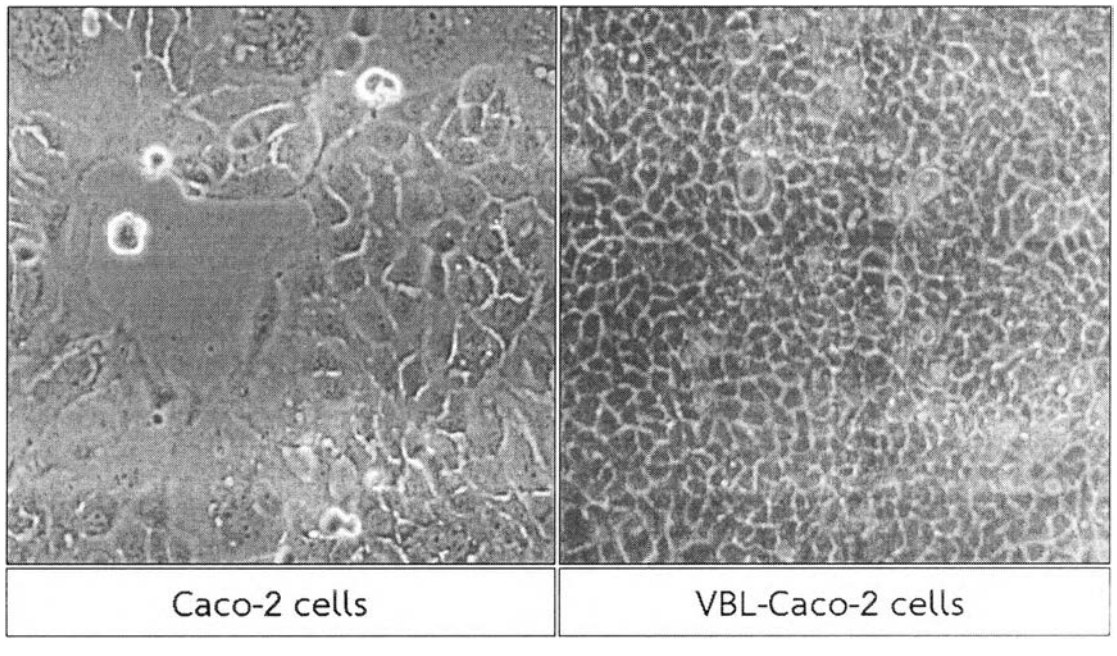

Figure 8. The morphology of the Caco-2 cells (left) and VBL-Caco-2 cells (right).

2. Method

2.1 Cell viability

Cell viability was determined by an MTT assay. MTT is a yellow water-soluble tetrazolium dye that is reduced by mitochondria enzymes to a purple insoluble formazan.

The Caco-2 cells were cultured in 96-well plates for 24 hour at seeding density of 1×10^4 cells/well. The cells were incubated with phyllanthin (5-200 μM) for 4 hour. At the end of incubation period, the medium was removed, and replaced with MTT solution (0.4 mg/ml in DMEM). After 4-hour of incubation period in the dark, the MTT solution was discarded and the formazan crystals were dissolved in DMSO. The amount of formazan was determined by a microplate reader at 570 nm. Cell viability was calculated as the percentage of the control.

2.2 Uptake study

The cells were seeded onto a 24 well-plate and were fed with fresh medium every 2 days until they were used in the assay at 21 days after seeding. In the experiment using VBL-Caco-2 cells, the medium was switched from VBL-containing medium to VBL-free medium on the day before an uptake assay.

The Caco-2 monolayers were pretreated with the test extract for 30 minutes, followed by addition of calcein-AM (0.4 μM). After the incubation period of 30 minutes, the cells were washed with ice cold HBSS and lysed with 0.3N NaOH in 1% Triton X-100. The fluorescent intensity of calcein was determined with the microplate reader at an excitation wavelength and an emission wavelength of 485 nm and 535 nm, respectively. The amounts of proteins in each sample were

determined with Bradford reagent in order to normalize the total protein in each experiment.

2.3 Validation of the Caco-2 monolayers for transport study

The cells were grown onto Transwell® inserts (polycarbonate, 0.4 μm pore size, 24 mm diameter; Corning Incorporated, Corning, NY, USA) at a seeding density of 6.0×10^4 cells per cm^2. The cells were fed with fresh medium every 2 days until they were used in the assay at 21 days after seeding. The Caco-2 cells between passages 60-70 were used in this study.

2.3.1 Caco-2 cell monolayer integrity tests

The cell monolayer integrity was evaluated by measuring the transepithelial electrical resistance (TEER) and the paracellular transport of lucifer yellow (LY).

Before and after the transport studies, TEER values of the Caco-2 cell monolayer were measured with the use of a chopstick-like electrode connected to a the Millicell®-ERS (electrical resistance system) (Millipore Corporation, Bedford, MA, USA). Only the monolayer having TEER values above 300 Ω cm^2 were used for the transport experiments.

The TEER value was determined from the following equation:

$$TEER_{monolayer} = (R_{monolayer} - R_{blank}) \times A$$

Where $R_{monolayer}$ is the resistance of the cell monolayer on the insert, R_{blank} is the resistance of insert and A is the surface area of the monolayer (4.67 cm^2 in 6-well plates).

In addition to TEER measurement, the paracellular flux of LY was determined at the end of each transport study. LY (100 μM) was added to HBSS-HEPES in the apical compartment. After incubation period of 30 min at 37°C, the sample was taken from the basolateral compartment for fluorescence analysis. The fluorescence intensity of LY was determined with a microplate reader (Wallac 1420 VICTOR 3, PerkinElmer Inc., Massachusetts, USA) at 485/535 nm excitation/emission wavelengths.

2.3.2 P-gp function test

The function of P-gp in the Caco-2 monolayers was assessed by the extent of transported rhodamine 123, a known P-gp substrate across the monolayers in the presence and absence of verapamil, a known P-gp inhibitor. For the determination of the AP to BL transport, 1.5 ml of HBSS (pH 6.5) consisting of rhodamine123 (20 μM) in the presence and absence of verapamil (100 μM) was added to the AP side (i.e., the donor chamber) whereas 2.6 ml of HBSS (pH 7.4) without the test compound was added to the BL side (i.e., the receiver chamber). For the determination of the BL to AP transport, 2.6 ml of HBSS (pH 7.4) consisting of rhodamine123 (20 μM) in the presence and absence of verapamil (100 μM) was added to the BL side (i.e., the donor chamber) whereas 1.5 ml of HBSS (pH 6.5) without the test compound was added to the AP side (i.e., the receiver chamber). Samples were withdrawn from the receiver chamber every 10 min for 90 min and immediately replaced them with the equal volume of fresh HBSS.

The amount of Rhodamine 123 in the samples was detected by using a fluorescence microplate reader (Wallac 1420 Perkin-Elmer Victor 3; Perkin Elmer Inc., Massachusetts, USA) at 485/535 nm excitation/emission wavelength.

2.4 Transport studies

2.4.1 Unidirectional transport

Transport of phyllanthin in cocktail mixture of three permeability markers (theophylline, antipyrine, and furosemide) was conducted in the apical (AP) to basolateral (BL) direction on an orbital shaker (60 rpm) at 37°C (Hubatsch I., Ragnarsson E.G. et al. 2007). After TEER measurement, the monolayers were rinsed and equilibrated for 15 min with 1.5 ml of HBSS containing 25 mM HEPES (pH 6.5) (for the apical side) and 2.6 ml of HBSS containing 10 mM MES (pH 7.4) (for the basolateral side). Then further, the apical buffer was changed to HBSS containing theophylline (100 µM), antipyrine (100 µM) and furosemide (100 µM). The test lignan phyllanthin (75 µM) was also introduced to the apical transport buffer. After that, 1 ml of basolateral buffer was withdrawn every 10 min for 90 min. The equal amount of fresh HBSS (1ml) was immediately added to the basolateral chamber when the sample was taken. Samples were analyzed by HPLC with ultraviolet (UV) detector (Shimadzu LC-20AD Prominence series; Kyoto, Japan).

2.4.2 Bidirectional transport

The bidirectional transport (AP to BL and BL to AP direction) of phyllanthin as a single compound was also determined in a similar procedure described above. For the determination of the AP to BL transport, 1.5 ml of HBSS (pH 6.5) consisting either

phyllanthin (75 μM) or rhodamine123 (20 μM) was added to the AP side (i.e., the donor chamber) whereas 2.6 ml of HBSS (pH 7.4) without the test compound was added to the BL side (i.e., the receiver chamber). For the determination of the BL to AP transport, 2.6 ml of HBSS (pH 7.4) consisting either phyllanthin (75 μM) or rhodamine123 (20 μM) was added to the BL side (i.e., the donor chamber) whereas 1.5 ml of HBSS (pH 6.5) without the test compound was added to the AP side (i.e., the receiver chamber). Samples were withdrawn from the receiver chamber every 10 min for 90 min and immediately replaced them with the equal volume of fresh HBSS.

The amount of Rhodamine 123 in the samples was detected by using a fluorescence microplate reader (Wallac 1420 Perkin-Elmer Victor 3; Perkin Elmer Inc., Massachusetts, USA) at 485/535 nm excitation/emission wavelength. The amount of phyllanthin was determined by HPLC with ultraviolet (UV) detector (Shimadzu LC-20AD Prominence series; Kyoto, Japan).

2.4.3 Calculation of the permeability coefficients

The apparent permeability coefficients, Papp (cms^{-1}), for both AP to BL and BL to AP directions of each test compounds were calculated from the following equation:

$$Papp = (dQ/dt) \times (1/C_0A)$$

Where dQ/dt is the cumulative transport rate (nmol/min), C_0 is the initial drug concentration on the donor side (μM), and A is the surface area of the inserts (4.67 cm^2 in 6-wells).

The efflux ratio (ER) was calculated from the following equation:

$$ER = Papp_{BL-AP} / Papp_{AP-BL}$$

2.5 HPLC analysis

2.5.1 HPLC system

HPLC was performed with the use of a Shimadzu LC-20AD Prominence controller, SIL-20A Prominence autosampler, SPD-M20A Prominence diode array detector and CTO-20A Prominence column oven (Shimadzu, Kyoto, Japan). Samples (50 µl) were injected into a BDS Hypersil C18 column (5 µm, 4.6 mm × 250 mm, Thermo Scientific, PA, USA) which was maintained at 35°C. The mobile phase system consisted of Milli-Q water, adjusted to pH 3 with 20% ortho-phosphoric acid (solvent A) and 100% acetonitrile (solvent B). The column was equilibrated with 2 % solvent B at a flow rate of 1 ml/min. After injection, the concentration of solvent B increased to 30% over 4 min, then further increased to 40% in 8 min, and to 45% in the next 10 min, and finally to 60% in 2 min. The isocratic flow was maintained at 60% solvent B for 14 min before return to original conditions (2% solvent B).

All the parameters of HPLC were controlled by LC solution software version 1.21 SP1. Separation was achieved using BDS Hypersil C18 column, 5 µm, 4.6 mm × 250 mm (Thermo scientific, PA, USA) and an ultraviolet (UV) detector with 220 nm, which was maintained at 35°C.

2.5.2 HPLC method validation

2.5.2.1 Sample preparation

Stock solution of each test compound (including theophylline, antipyrine, furosemide, and phyllanthin) was freshly prepared in 99.9% DMSO on the day of experiment. These stock solutions were further diluted in transport buffer to the desired concentration as need. The mixture of theophylline, antipyrine, furosemide, and phyllanthin was freshly prepared from these stock solutions before the validation process.

2.5.2.2 System suitability

The HPLC system suitability for analyzing the mixture of theophylline (100 μM), antipyrine (100 μM), furosemide (100 μM), and phyllanthin (75 μM) was determined. The peak area and retention time of each test compound were measured and compared to its standard reference. All sample runs were performed in triplicate.

2.5.2.3 System specificity

System specificity of the HPLC method was evaluated for analyzing the mixture of theophylline, antipyrine, furosemide, and phyllanthin. The HPLC chromatograms of each test compound were measured and compared to the solvent control samples (0.5% DMSO in transport buffer). Each sample from 6-separated experiments was run in triplicate.

2.5.2.4 Linearity and range

The eight point calibration curves of each permeability marker (theophylline, antipyrine, and furosemide) in the cocktail mixture were constructed in the concentration range of 0.1-80 μM. The six point calibration curves of phyllanthin

were constructed in the concentration range of 1.5-60 μM. Each sample from 6-separated experiments was run in triplicate. The linearity of the calibration curves was determined by linear regression analysis.

2.5.2.5 Accuracy and precision

The accuracy and precision of HPLC analysis were determined to ensure validity of the set-up analytical system. The accuracy and precision of each test compounds in the cocktail mixture were measured in three concentrations (low, medium, high) within the calibration curve. Accuracy was evaluated by determining the percentage of analytical recovery in triplicate samples that were run within a day (intra-run). This procedure was repetitively carried out on three consecutive days (inter-run). The intra-run and inter-run precision were assessed by determining the coefficient of variation (C.V.) values.

The coefficient of variation (C.V.) value was calculated from the following equation:

$$\text{C.V.} = (\text{standard deviation} / \text{mean}) \times 100$$

2.5.2.6 Limit of detection and limit of quantification

The limit of detection (LOD) is the lowest concentration of test compound in sample which can be detected. The limit of quantification (LOQ) is the lowest concentration of the test compound in sample which can be detected with acceptable accuracy and precision. The LOD and LOQ were determined by measurement of the signal-to-noise ratio. LOD and LOQ were assigned at the ratios of 3:1 and 10:1, respectively. Each sample from 6-separated experiments was run in triplicate.

2.6 Stability of phyllanthin in HBSS assay

Phyllanthin (75 µM) was dissolved in HBSS buffer solution pH 4.5, 6.5, and 7.4. The solution was kept in a shaking incubator (60 rpm) at 37°C. Samples were taken for HPLC analysis at 0, 30, 60, 90, 120, 150, and 180 min. Stability of phyllanthin was calculated as percentage of phyllanthin remaining.

2.7 Solubility studies

Solubility of phyllanthin was determined at 37 ± 1°C in aqueous media with a pH in the range of 1.0-7.5 (including: pH 1.0, 4.5, 6.5, and 7.5). Aqueous media was adjusted with 10 mM sodium acetate trihydrate for pH 1.0 and 4.5; with 10 mM MES for pH 6.5; and with 25 mM HEPES for pH 7.4. The aqueous solubility of phyllanthin was estimated by visual observation following the procedure described by Steele and Austin 2009. Initially, 0.2 mg of phyllanthin was dissolved in 10 µl of aqueous media at the designated pH (pH 1.0 or 4.5 or 6.5 or 7.5). If the compound did not dissolve, a further 40 µl of aqueous media was added. Each of 40 µl of aqueous media would be added repeatedly until the compound dissolved. The total amount of aqueous media was combined to give an approximate value of phyllanthin solubility (Steele G. and Austin T. 2009).

2.8 Data analysis

Data were presented as mean ± SEM (standard error of mean). Data were obtained from three or four separated experiments. Statistical analysis was performed by either the Student's *t* test or the one-way analysis of variance

(ANOVA), followed by the post-hoc Tamhane's T2 test. Statistical significance was considered at $p < 0.05$.