CHAPTER IV

RESULTS

1. HPLC profiles

1.1 System specificity

Theophylline, antipyrine, furosemide, and phyllanthin were quantified by HPLC analysis at the wavelength of 220 nm. The HPLC system was able to separate each compound in the cocktail mixture with the retention times of 8.4 min (theophylline), 10.7 min (antipyrine), 15.7 min (furosemide), and 32.2 min (phyllanthin) (Figure 9). Chromatogram of 0.5% DMSO in transport buffer did not show any interfering peaks during the HPLC run time of 50 min (Figure 10). Hence, the HPLC system in this study was suitable for separation and detection of all test compounds in the cocktail mixture.

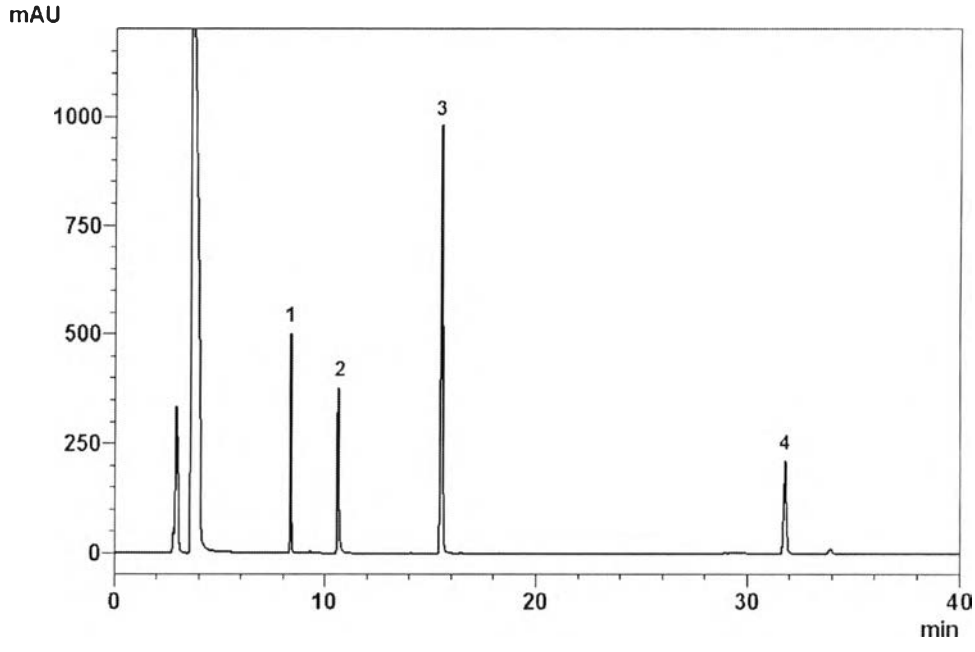

Figure 9. Representative of HPLC chromatograms of test compounds in the cocktail mixture: theophylline (1), antipyrine (2), furosemide (3), and phyllanthin (4).

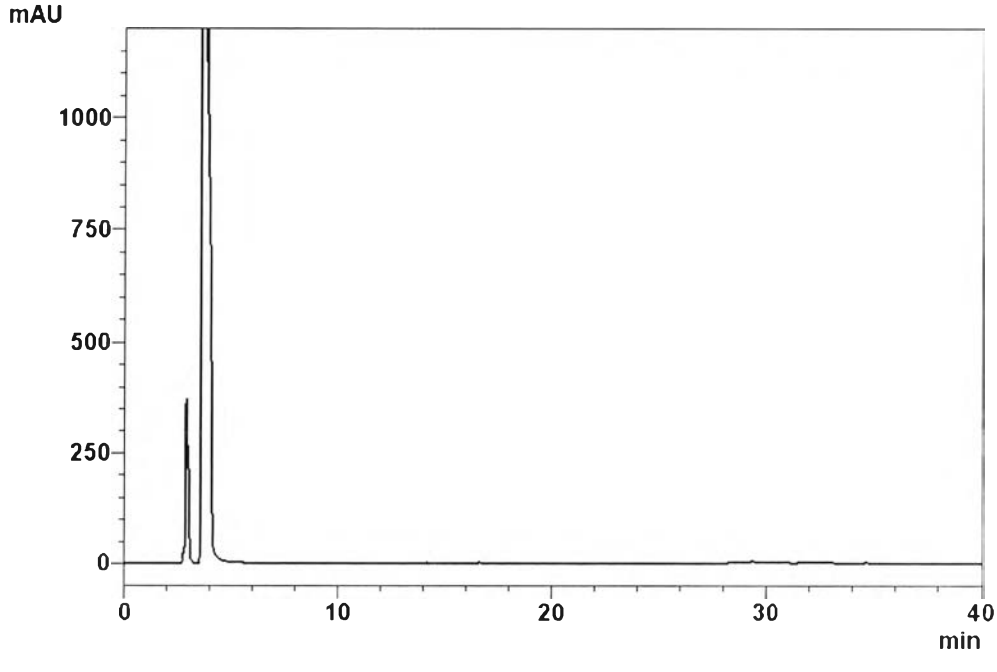

Figure 10. Representative of HPLC chromatograms of 0.5% DMSO in transport buffer.

1.2 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 (Figure 18.1-18.3 in Appendix). The six point calibration curves of phyllanthin were constructed in the concentration range of 1.5-60 μM (Figure 18.1-18.3 in Appendix). The linearity of the curves was determined by linear regression analysis. As shown in Table 3, the calibration curves of each test compound exhibited the linearity with the correlation coefficient (r) equal or close to 1.0000.

Table 3. Regression parameters of the calibration curves of theophylline, antipyrine, furosemide, and phyllanthin.

Compound	Regression analysis[a]			
	Range (μM)	Slope[b]	Intercept[b]	r[c]
Theophylline	0.1 – 80	1.0000	-2744.19 ± 4019.53	22353.48 ± 123.43
Antipyrine	0.1 – 80	1.0000	-6735.07 ± 4920.88	29446.26 ± 151.11
Furosemide	0.1 – 80	1.0000	-21661.99 ± 13355.99	87077.82 ± 410.12
Phyllanthin	1.5 – 60	0.9983	-85356.07 ± 15156.53	36565.20 ± 537.42

[a] Linear regression analysis with a regression equation of $y = mx + c$, in which x is the concentration in μM and, y is the peak area.

[b] Data represent mean ± S.E. of six independent experiments.

[c] r is the correlation coefficient in calibration curve of six independent experiments.

1.3 Accuracy and precision

The accuracy and precision of HPLC analysis was determined to ensure validity of the set-up analytical system. Accuracy was evaluated by the percentage of analytical recovery in triplicate samples run within a day (intra-run). This procedure was repetitively carried out on three consecutive days (inter-run). As shown in Table 4, all the samples, except antipyrine and phyllanthin at the 5 μM, had the percentage of analytical recovery within the range of 100 ± 10%. The intra-run and inter-run precision were assessed by determining the coefficient of variation (C.V.) values. As shown in table 4, the C.V. value of each test compound did not exceed 10%. Therefore, the HPLC analytical system had accuracy and precision for quantifying the amount of each compound in this study.

Table 4. Accuracy and precision data (intra-run and inter-run) of chromatographic method for analysis of theophylline, antipyrine, furosemide, and phyllanthin in transport buffer (pH 7.4).

Compound	Theoretical conc. (μM)	Intra-run			Inter-run		
		Measured conc. (μM)	% Analytical recovery	C.V.	Measured conc. (μM)	% Analytical recovery	C.V.
Theophylline	75	72.19	96.26	9.42	75.46	100.61	0.69
	25	24.10	96.42	6.47	25.04	100.17	1.28
	5	5.02	100.49	0.93	5.06	101.11	11.1
Antipyrine	75	75.83	101.10	9.84	77.35	103.13	2.46
	25	25.36	101.44	6.83	25.99	103.96	0.62
	5	5.56	111.21	8.15	5.14	102.75	5.50
Furosemide	75	72.72	96.96	9.20	75.31	100.41	1.79
	25	24.60	98.40	5.73	25.16	100.63	0.91
	5	5.46	109.22	3.85	5.29	105.81	1.20
Phyllanthin	50	49.21	98.42	8.78	49.86	99.71	3.40
	25	24.59	98.36	6.98	24.46	97.83	4.34
	5	6.15	123.10	2.97	5.97	119.39	9.52

Intra-run and inter-run accuracy and precision was determined with triplicates on three consecutive days for each concentration.

1.4 Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (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. In this study, the LOD and LOQ for measurement of theophylline, antipyrine, furosemide, and phyllanthin were shown in Table 5.

Table 5. Limit of detection (LOD) and limit of quantification (LOQ) of chromatographic method for analysis of theophylline, antipyrine, furosemide, and phyllanthin in transport buffer (pH 7.4).

Compound	LOD[a] (μM)	LOQ[b] (μM)
Theophylline	0.016 ± 0.002	0.055 ± 0.007
Antipyrine	0.012 ± 0.001	0.042 ± 0.005
Furosemide	0.004 ± 0.004	0.014 ± 0.002
Phyllanthin	0.011 ± 0.002	0.037 ± 0.006

Data represent mean ± S.E. of six independent experiments.

[a] LOD was assigned when the signal to noise ratio of 3:1

[b] LOQ was assigned when the signal to noise ratio of 10:1

2. Cell viability

The effect of phyllanthin on cell viability was evaluated with the use of MTT assay. As shown in Figure 11, phyllanthin at the concentrations in the range of 5-100 μM had no effect on cell viability. However, when the concentration increased to 150 and 200 μM, the viability slightly decreased to 87.02 ± 7.26% and 80.56 ± 6.35%, respectively.

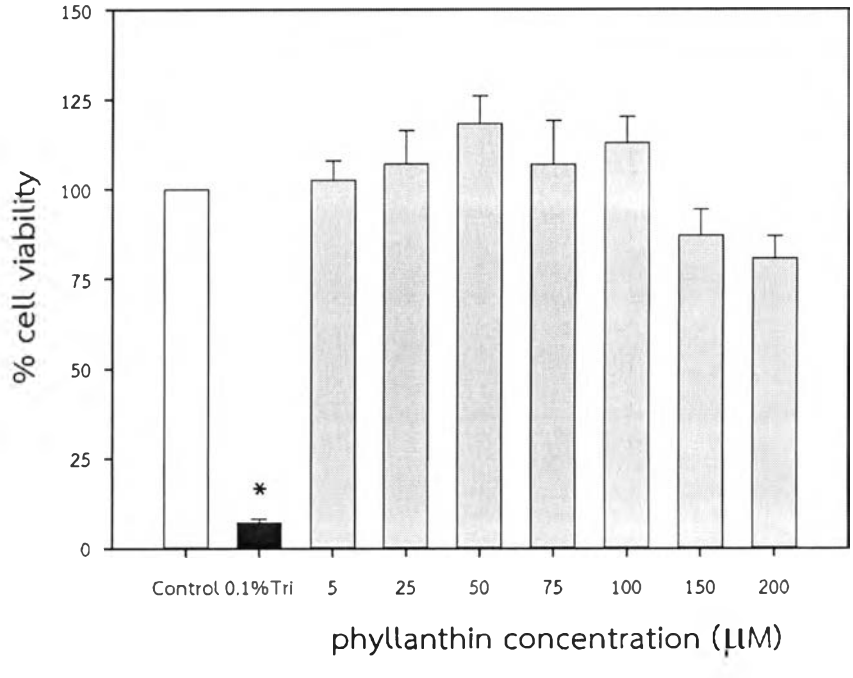

Figure 11. Effect of phyllanthin on cell viability. The Caco-2 monolayers were treated with phyllanthin at various concentrations in the range of 5 to 200 μM for 4 hours prior to MTT assay. Triton X-100 (Tri) at the concentration of 0.1% was used as a positive control group. Data represent mean ± S.E. of three to four independent experiments.

*$p<0.05$, significant difference from the control (untreated group).

3. Uptake study

The expression of active P-gp in VBL-resistant Caco-2 cells after cultured for 21 days in VBL-containing medium was at the appreciable level. The presence of verapamil (100 μM) and cyclosporine A (10 μM) in VBL-resistant Caco-2 cells, which are known inhibitors of P-gp, increased intracellular accumulation of calcein by 5 and 6.8 folds, respectively (Figure 12). The presence of verapamil (100 μM) in the VBL-resistant Caco-2 cells at the high passage number (96-112) and the native Caco-2 cells at the lower passage number (50-70) were increased intracellular accumulation of calcein by 5 and 3.4 folds, respectively (Figure 13).

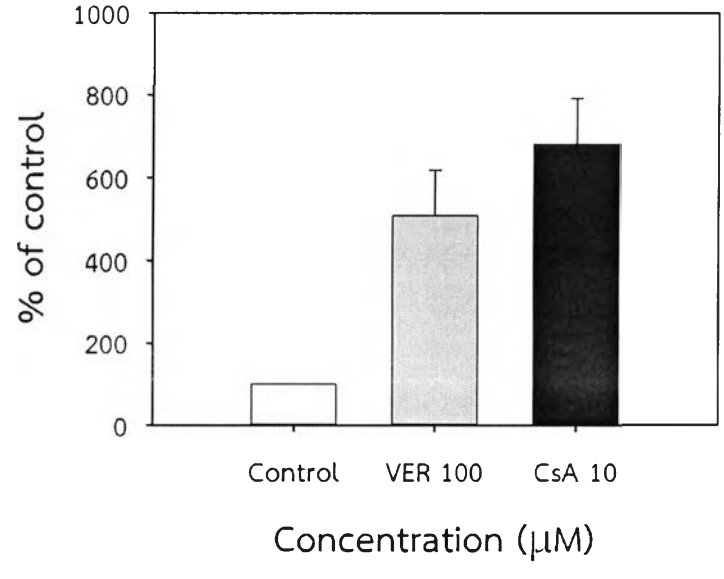

Figure 12. Intracellaular accumulation of calcein in the VBL-resistant Caco-2 monolayers at the high passage number (96-112). The effects of verapamil (Ver) and cyclosporine A (CsA), the known P-gp inhibitors, were determined. Data were normalized per mg of proteins and expressed as the percentage of control (untreated group). Values represented the mean ± SEM (n=5-6).

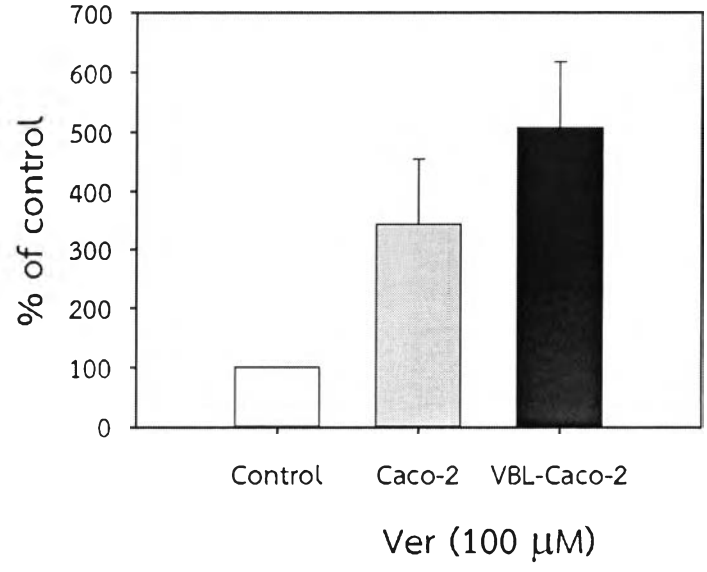

Figure 13. Intracellaular accumulation of calcein in the Caco-2 monolayers at the low passage number (51-56) and VBL-resistant Caco-2 monolayers at the high passage number (96-112). The effects of verapamil (Ver), the known P-gp inhibitors, were determined. Data were normalized per mg of proteins and expressed as the percentage of control (untreated group). Values represented the mean ± SEM (n = 3-6).

4. Validation of the Caco-2 monolayers for transport study

4.1 Monolayer integrity

The Caco-2 monolayer integrity was verified with the use of TEER values. The TEER values were measured every 2 days starting from day 5 to day 21 after seeding. As shown in Figure 14, the TEER values of Caco-2 monolayers increased in time dependent manner. On day 21, the TEER values before the transport experiments were 883.28 ± 18.11 Ω cm^2. These values were greater than 300 Ω cm^2 which was

generally accepted as an indicator of a good restrictive barrier for the transport study (Hunter et al., 1993; Troutman and Thakker, 2003).

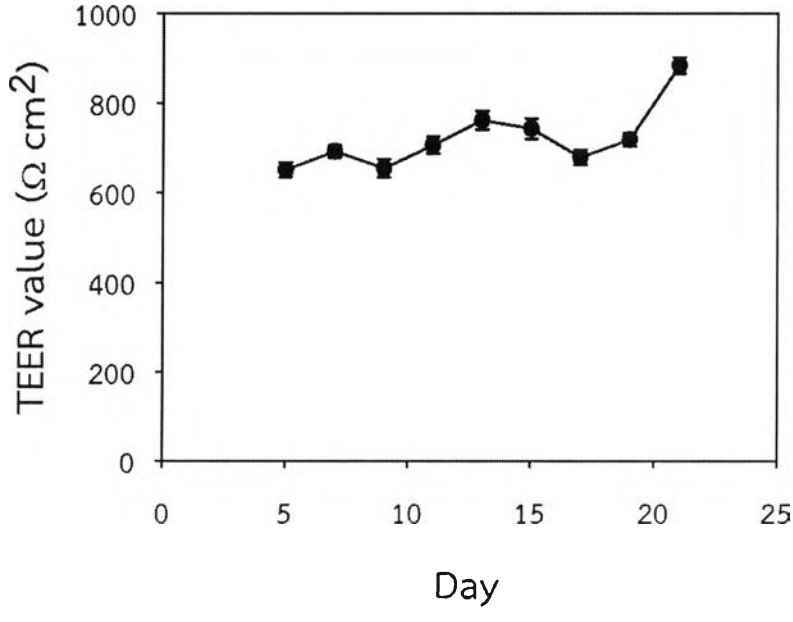

Figure 14. The TEER values of Caco-2 monolayers (passages number 60-64) during the period of day 5 to day 21 post seeding. Data are shown as mean ± S.E. of five independent experiments.

4.2 P-gp function

The function of P-gp in the Caco-2 monolayers was assessed by the extent of transported rhodamine 123 across the monolayers in the presence and absence of verapamil. The apparent permeability coefficients (Papp) of rhodamine 123 across the Caco-2 cell monolayers in both the apical-to-basolateral (AP-to-BL) direction and the basolateral-to-apical (BL-to-AP) direction were calculated and shown in Figure 19 in Appendix. In the AP-to-BL direction, verapamil had no significant effect on the

transport of rhodamine 123. Papp $_{AP-BL}$ values of rhodamine 123 in the absence and presence of verapamil were 2.03 ± 0.42 × 10^{-6} cms^{-1} and 2.33 ± 0.38 × 10^{-6} cms^{-1}, respectively (Figure 15). On the contrary, the Papp $_{BL-AP}$ values of rhodamine 123 was significantly reduced from 13.03 ± 1.53 × 10^{-6} cms^{-1} to 4.86 ± 0.41 × 10^{-6} cms^{-1} in the presence of verapamil (Figure 15). In this study, the efflux ratio of rhodamine 123, a known P-gp substrate, was 6.73 ± 0.78. This ratio was in agreement with the USFDA recommendation that compounds being P-gp substrate should have the efflux ratio ≥ 2 (USFDA, 2012). In addition, the efflux can be inhibited by one or more P-gp inhibitor (USFDA, 2012). In this study, the presence of verapamil, a known P-gp inhibitor, was able to inhibit the transport of rhodamine 123 from the basolateral side to the apical side. Hence, the results suggested that the Caco-2 monolayers expressed functional P-gp at an appreciable level.

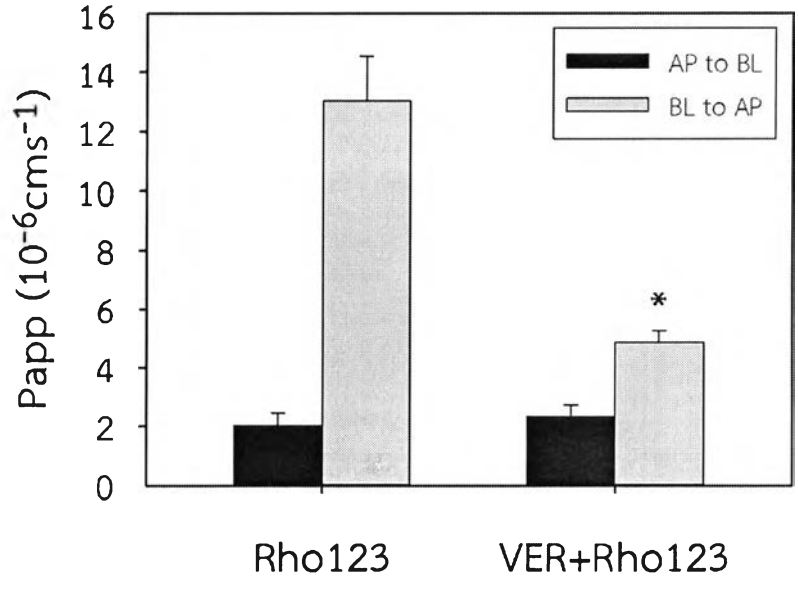

Figure 15. Apparent permeability coefficients (Papp) of rhodamine 123 (Rho123) across the Caco-2 monolayers in the absence and presence of verapamil (VER). Papp values were calculated in both the apical-to-basolateral (AP-to-BL) direction and the basolateral-to-apical (BL-to-AP) direction. Data represent mean ± S.E. of three independent experiments.

*$p < 0.05$, significant difference comparing between the presence and absence of verapamil.

5. Transport studies of phyllanthin

5.1 Unidirectional transport

Theophylline and antipyrine (high permeable compounds; BCS class I) and furosemide (low permeable compound; BCS class IV) were used as permeability markers in this study (Wu and Benet, 2005). The Papp values across the monolayer in the AP to BL direction of phyllanthin and three permeability markers including theophylline, antipyrine, and furosemide were listed in Table 6. The Papp (AP-to-BL)

values of the highly permeable theophylline and antipyrine were markedly higher than that of a poorly permeable furosemide by at least 21 fold. The presence of phyllanthin in the cocktail of permeability markers had no effect on their transport rates. Hence, phyllanthin had no interference on the permeability of the internal markers. The Papp of phyllanthin in the absorptive direction was $34.90 \pm 1.18 \times 10^{-6}$ cms^{-1} which was in the rank order of highly permeable theophylline and antipyrine.

Table 6. Permeability of phyllanthin, theophylline, antipyrine, and furosemide across the Caco-2 cell monolayers.

Compound	Conc. (μM)	$P_{app\ (AP \rightarrow BL)}$ (10^{-6} cms^{-1})		Percentage absorption (in human)
		with phyllanthin	without phyllanthin	
Phyllanthin	75	34.90 ± 1.18	-	-
Theophylline	100	24.46 ± 0.47	24.50 ± 0.26	100[a]
Antipyrine	100	31.31 ± 0.62	30.56 ± 0.33	100[b]
Furosemide	100	1.24 ± 0.41	1.12 ± 0.42	61[c]

P_{app} values show the apparent permeability of compounds across the Caco-2 monolayer from the apical-to-basal direction. Each value represents the mean ± S.E. of three independent experiments.

[a] Percentage human absorption reported in Yamashita et al., 2000; Zhu et al., 2002.

[b] Percentage human absorption reported in Lennernäs, 1998; Yamashita et al., 2000; Zhu et al., 2002; Ungell and Karlsson, 2003.

[c] Percentage human absorption reported in Benet, 1979; Yamashita et al., 2000; Zhao et al., 2001; Zhu et al., 2002.

5.2 Bidirectional transport

The transport rate of rhodamine 123 in the BL-to-AP direction was significant higher than that in the AP-to-BL direction. The Papp values of rhodamine 123 in the AP-to-BL direction and the BL-to-AP direction were 2.03 ± 0.42 × 10^{-6} cms^{-1} and 13.03 ± 1.53 × 10^{-6} cms^{-1}, respectively (Figure 16). The mean efflux ratio of rhodamine 123 was 6.73 ± 0.78 (n = 3). On the contrary, the transport rates of phyllanthin in both directions were comparable. The Papp values of phyllanthin in the AP-to-BL direction and the BL-to-AP direction were 40.09 ± 1.04 × 10^{-6} cms^{-1} and 47.88 ± 1.40 × 10^{-6} cms^{-1}, respectively (Figure 16). The efflux ratio of phyllanthin was 1.19 ± 0.01 (n = 3). These results suggested that P-gp had no influence on the transport of phyllanthin.

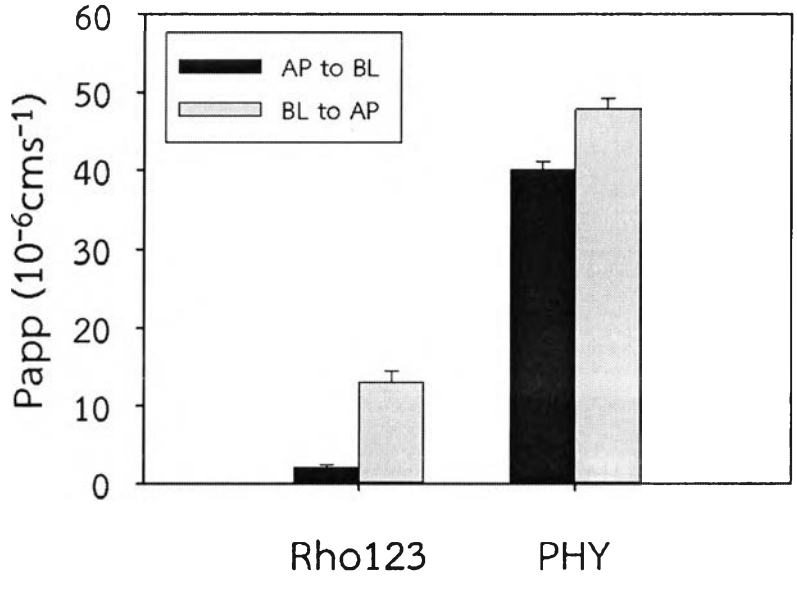

Figure 16. Permeability of rhodamine 123 and phyllanthin across the Caco-2 cell monolayers from the apical-to-basolateral (AP-to-BL) direction and the basolateral-to-apical (BL-to-AP) direction. Data represent mean ± S.E. of three independent experiments.

6. Effects of phyllanthin on monolayer integrity

The TEER values of Caco-2 monolayers in this study before the transport experiments were in the range of 728.52-1092.78 Ω cm^2 (n = 30). These values were not significantly different between before and after the transport experiments (Table 7). The paracellular flux of Lucifer yellow (LY) at the end of each transport experiment was less than 1% per hour (0.05 ± 0.01% per hour). These results suggested that phyllanthin had no disruptive effect on the Caco-2 permeability model.

Table 7. TEER values at before and after transport experiments.

Compound	TEER values[b] (Ω cm^2)	
	Before	After
Phyllanthin	916.36 ± 46.07	1197.34 ± 27.91
Phyllanthin + Permeability markers[a]	838.53 ± 16.44	973.96 ± 32.23
Permeability markers[a]	871.48 ± 35.19	964.62 ± 51.17

[a] Permeability markers included theophylline, antipyrine, and furosemide.

[b] Transepithelial electrical resistance (TEER) was measured before and after the experiments. Each value represents the mean ± S.E. of three independent experiments.

7. Stability of phyllanthin

The stability of phyllanthin in HBSS was expressed as the percentage remaining with time at 37°C as shown in Figure 17. The amount of phyllanthin did not significantly decrease over 150 min time period. At the incubation period of 180 min, the amount of phyllanthin decreased slightly to 84.06 ± 0.87% in pH 4.5,

88.14 ± 0.41% in pH 6.5, and 89.11 ± 0.28% in pH 7.4. In addition, the percentage phyllanthin remaining in pH 4.5, 6.5, and 7.4 conditions were similar over 180 min time period. The result suggested that phyllanthin was stable in the gradient pH condition used in the transport experiments.

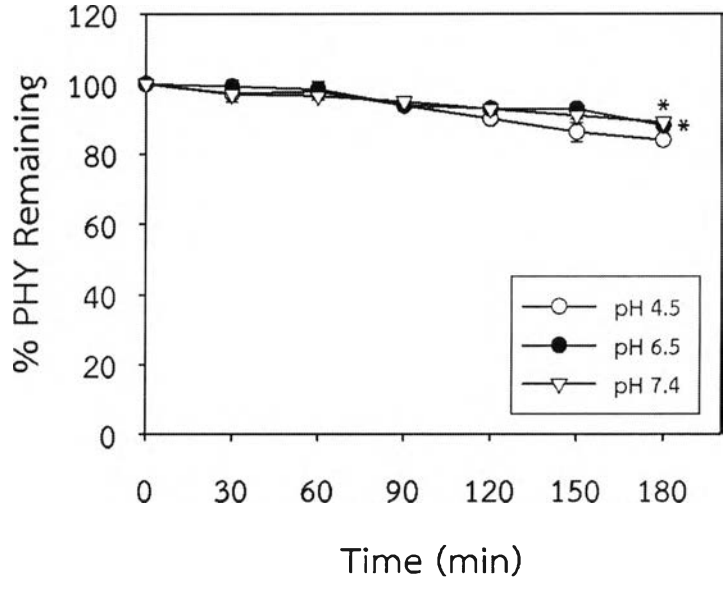

Figure 17. Stability of phyllanthin in HBSS at pH 4.5, 6.5, and 7.4.
Data represent mean ± S.E. of three independent experiments.
*$p<0.05$, significant difference from the value at time 0.

8. Solubility of phyllanthin

The solubility of phyllanthin in aqueous media at various pH conditions (pH 1, 4.5, 6.5, and 7.5) was less than 5 µg/ml at 37 ± 1°C (Table 8). Therefore, phyllanthin could be classified as a practically insoluble or insoluble compound according to Steele and Austin, 2009.

Table 8. The aqueous solubility of phyllanthin in aqueous media at various pH.

pH condition[a]	Phyllanthin concentration (µg/ml)
1	< 5
4.5	< 5
6.5	< 5
7.5	< 5

[a] In aqueous media with a pH range of 1-7.5, at 37 ± 1°C.

Data represent approximate concentration of two independent experiments.