

Serum Concentrations of Anthraquinones after Intake of Folium Sennae and Potential Modulation on P-glycoprotein

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Key words

- *Cassia angustifolia*
- Fabaceae
- aloe-emodin
- rhein
- glucuronides
- P-glycoprotein

Abstract

Folium Sennae (leaves of *Cassia angustifolia* or senna) is a laxative and a component in diets for weight control. It contains a variety of anthranoids such as sennosides, aloe-emodin, and rhein. In order to measure the serum concentrations of senna anthranoids, Sprague-Dawley rats were orally administered with single dose and multiple doses of Folium Sennae. The concentrations of anthranoids in serum were determined by HPLC method before and after hydrolysis with sulfatase and β -glucuronidase. The results showed that in the serum, aloe-emodin glucuronides and rhein glucuronides were the major metabolites. Traces of rhein free form were present transiently during the early phase, whereas the free form of aloe-emodin was not detected. We also evaluated the modulation effect of Folium Sennae on P-glycoprotein by using the LS 180 cell model which showed that it significantly inhibited P-glycoprotein by 16–46%. In conclusion, senna anthranoids were rapidly and extensively metabolized to rhein glucuronides and aloe-emodin glucuronides in rats. Folium Sennae ingestion inhibited the efflux function of P-glycoprotein in the intestine.

Abbreviations

AUC _{0-t} :	area under concentration-time curve to the last time
C _{max} :	maximum serum concentration
CV:	coefficient of variation
DMEM:	Dulbecco's Modified Eagle Medium
F:	free form
FS:	Folium Sennae
G:	glucuronides
HBSS:	Hank's buffered salt solution
HEPES:	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
IS:	internal standard
LOD:	limit of detection
LLOQ:	lower limit of quantitation
MRT:	mean residence time
P _{app} :	permeability coefficient
PSG:	penicillin-streptomycin-glutamine
P-gp:	P-glycoprotein
rho 123:	rhodamin 123
S+G:	sulfates plus glucuronides
SDS:	sodium dodecyl sulfate

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Introduction

FS, the leaves of *Cassia angustifolia* Vahl (Fabaceae), synonymous with *Senna alexandrina* Mill., has been commonly used as a laxative worldwide for the treatment of diet-, stress-, or medication-related constipation [1,2]. Nowadays, it is a component in diets for weight control [3]. FS contains polyphenolic anthranoids including sennosides and a variety of anthraquinones, such as aloe-emodin, rhein, and chrysophanol, which exist in

both glycoside and aglycone forms [4,5] (● Fig. 1). Sennosides have been found to be hydrolyzed by intestinal enzymes first to sennidin monoglucosides and sennidins, which are further transformed to rhein, rhein anthrone, and aloe-emodin [6–8]. Till now, although the information regarding the pharmacokinetics of senna anthranoids in FS has been reported, only plasma levels of the total amount of S+G from rhein or aloe-emodin were determined [9], while detailed investigation on the serum concentrations of anthraquinone sulfates or glucuronides has yet to be conducted.

* These authors contributed equally to the study.

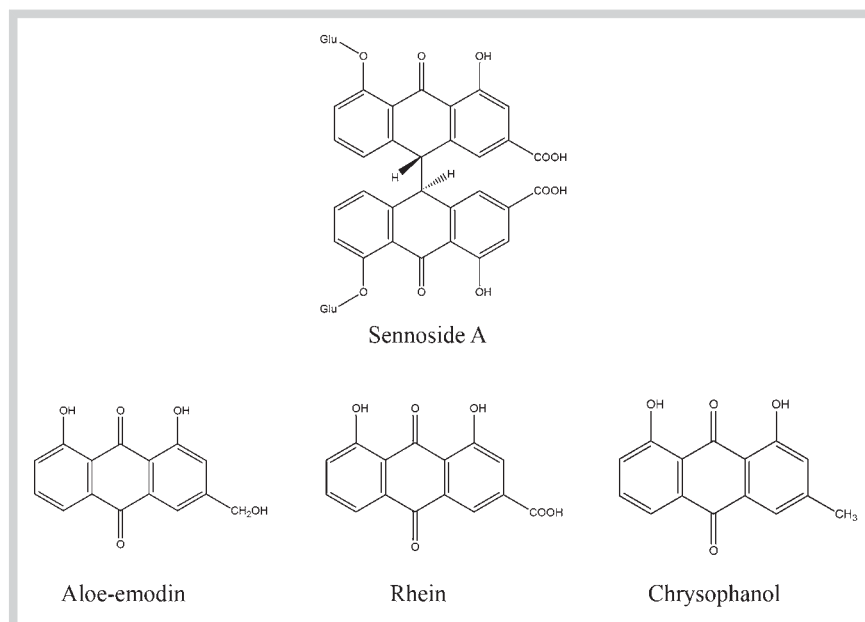


Fig. 1 Chemical structures of anthraquinones in *Folium Sennae*.

A previous study reported that FS infusion decreased the apparent P_{app} of propranolol, verapamil, and rho 123 in Caco-2 cells, which are all substrates of P-gp (ABCB1) [10], a drug efflux pump. P-gp expels a variety of hydrophobic and cationic drugs, including critical medicines such as digoxin, cyclosporine, tacrolimus, everolimus, etc [11]. In this study, we measured the serum concentrations of anthranoids and relevant metabolites after administration of single dose and multiple doses of FS decoction to rats. In addition, the modulation effect of FS on P-gp was further investigated by using a newly developed model of LS 180 cells, a human colon adenocarcinoma cell line.

Results

FS decoction and its acid hydrolysate were analyzed by HPLC method with a gradient elution, and the chromatogram is shown in **Fig. 2**. Before hydrolysis, the concentrations of sennoside A, aloe-emodin, and rhein were 295.8, 242.8, and 1622.1 μM , respectively, whereas chrysophanol, a relatively nonpolar anthraquinone, was not detected in FS decoction. In order to estimate the contents of anthraquinone glycosides, FS decoction was hydrolyzed with hydrochloric acid. After hydrolysis, the concentrations of aloe-emodin and rhein were increased by 62% and 28%, respectively, but chrysophanol was still not detected, indicating that the glycosides of aloe-emodin and rhein were present, but in lower concentrations than their aglycone form, whereas chrysophanol glycosides were not present in the FS decoction.

Fig. 3 shows the HPLC chromatograms of a serum specimen before and after hydrolysis with sulfatase after intake of FS. The analysis of a serum sample took 20 min. Good linear relationships were obtained in the concentration ranges of 0.03–1.0 $\mu\text{g/mL}$ ($Y = 4.231 X - 0.030$, $r = 0.9993$) for aloe-emodin, and 0.6–20.0 $\mu\text{g/mL}$ ($Y = 1.648 X - 0.176$, $r = 0.9995$) for rhein in serum. Validation of the analytical method confirmed that all CVs and the relative errors of intra-day and inter-day analysis were below 14.7% and 11.6%, respectively. The recoveries were 80.3–104.6% and 82.5–85.9% for aloe-emodin and rhein, respectively. The

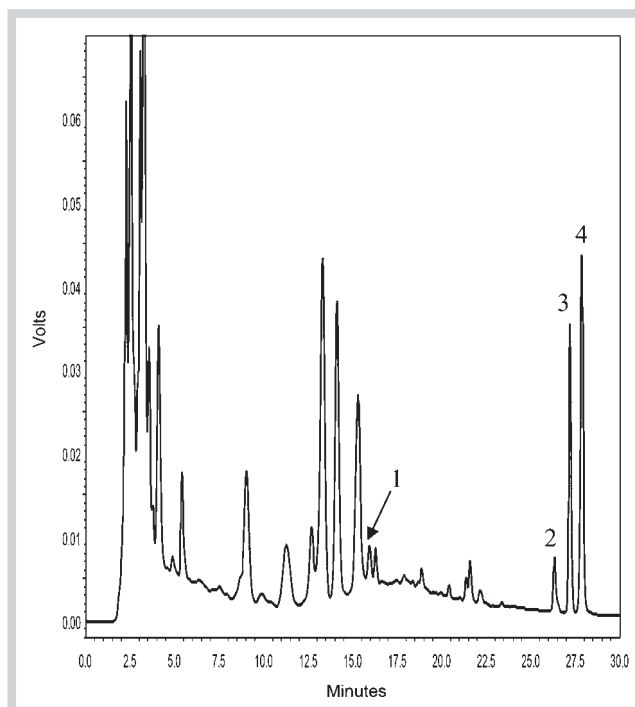


Fig. 2 HPLC chromatogram of *Folium Sennae* decoction. 1: sennoside A, 2: aloe-emodin, 3: amylparaben (IS), 4: rhein.

LLOQs were 0.03 and 0.60 $\mu\text{g/mL}$, and LODs were 0.02 and 0.08 $\mu\text{g/mL}$ for aloe-emodin and rhein, respectively.

Following oral administrations of single dose and the 7th dose of FS, the serum profiles of S+G and glucuronides of aloe-emodin and rhein as well as rhein free form are shown in **Fig. 4**. It revealed that the conjugated metabolites of aloe-emodin and rhein emerged rapidly after FS dosing. The pharmacokinetic parameters of aloe-emodin and rhein and their metabolites are listed in **Table 1**, from which it can be seen that AUC_{0-t} of S+G and G of either aloe-emodin or rhein were comparable. Moreover, the pa-

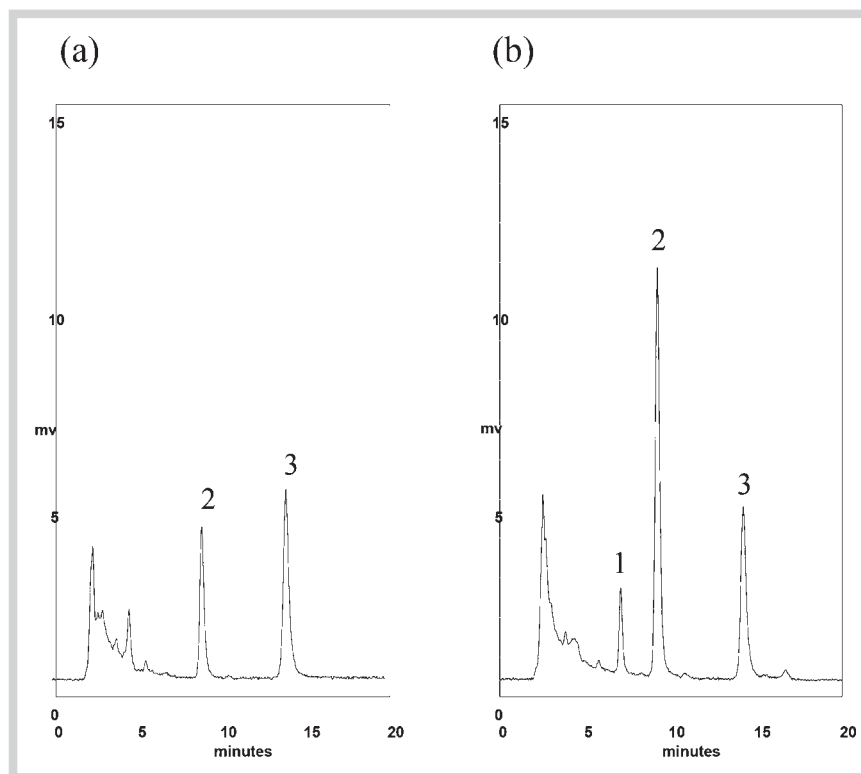


Fig. 3 HPLC chromatograms of a serum sample before (a) and after hydrolysis with sulfatase (b). 1: aloe-emodin, 2: rhein, 3: 1,8-dihydroanthraquinone (IS).

Table 1 Pharmacokinetic parameters of aloe-emodin sulfates plus glucuronides (S+G), aloe-emodin glucuronides (G), rhein free form (F), rhein sulfates plus glucuronides (S+G), and rhein glucuronides (G) in six rats after oral administration of single dose and multiple doses of *Folium Sennae* (10 g/kg).

Parameters	C _{max}	AUC ₀₋₇₂₀	MRT ₀₋₇₂₀
Single dose			
Aloe-emodin S+G	1.2 ± 0.1	252.9 ± 7.4	262.5 ± 22.4
Aloe-emodin G	1.2 ± 0.1	242.0 ± 7.2	265.4 ± 22.8
Rhein F	8.4 ± 1.3	–	–
Rhein S+G	15.9 ± 1.0	3056.7 ± 217.1	205.2 ± 36.6
Rhein G	13.3 ± 1.4	2844.2 ± 290.0	207.7 ± 38.1
7th dose			
Aloe-emodin S+G	1.1 ± 0.2	264.2 ± 57.3	192.6 ± 36.9
Aloe-emodin G	1.0 ± 0.2	209.9 ± 42.6	179.8 ± 41.8
Rhein F	10.2 ± 3.5	–	–
Rhein S+G	13.4 ± 2.6	3047.2 ± 806.0	157.6 ± 39.6
Rhein G	11.0 ± 2.5	2541.3 ± 626.7	163.1 ± 40.9

Data expressed as mean ± SE; C_{max} (nmol/mL): maximum serum concentration; AUC₀₋₇₂₀ (nmol · min/mL): area under concentration-time curve to the last time; MRT₀₋₇₂₀ (min): mean residence time

rameters including C_{max}, AUC_{0-t}, and MRT were not significantly different between single-dose and multiple-dose treatments. Given that the highest serum level of rhein free form was detected at the first sampling time (10 min) in all rats, and the later serum levels of rhein in most rats were below LLOQ, the AUC_{0-t} and MRT of rhein F could not thus be calculated either after single-dose or multiple-dose treatments.

The modulation of FS on P-gp was investigated by using LS 180 cell model. MTT assay indicated that FS and verapamil (a positive control) exerted no significant influence on cell viability at the tested concentrations. In transport study, the intracellular accumulations of rho 123, a typical P-gp substrate, measured after

4-h incubation with various tested agents are shown in **Fig. 5**. FS at 0.125–5.0 mg/mL significantly enhanced the intracellular accumulation of rho 123 by 16–46% while verapamil at 100 μM, as a positive control, significantly enhanced the intracellular accumulation of rho 123 by 49%.

Discussion

Quantitation of the FS decoction prepared by using boiling water as extraction solvent indicated that rhein was the major anthranoid (corresponding to 0.046% of the dried FS), whereas sennoside A and aloe-emodin were comparably minor. In contrast to previous studies reporting that sennosides A and B were the major anthranoids (corresponding to 1–3% of the dried FS) in the methanol-water extract of FS [4,12,13], this discrepancy could be accounted for by the use of different extraction solvents employed in the studies. Based on the solubility character of sennosides, using hot water to prepare FS decoction should solubilize a lower amount of sennoside A from FS.

For the quantitation of anthranoids in serum specimens, an HPLC system with fluorescence detection was developed and optimized in this study. Validation of the analytical method of rhein and aloe-emodin in serum confirmed that the precision, accuracy, and recovery were satisfactory.

In order to quantify both the free form and conjugated metabolites of anthraquinones, serum samples were assayed before and after hydrolysis with β-glucuronidase and sulfatase, individually. Prior to hydrolysis, the free form of rhein was detected in a few specimens. After hydrolysis with sulfatase or glucuronidase, the peaks of aloe-emodin and rhein emerged or enhanced in the chromatogram, suggesting the presence of aloe-emodin S+G and rhein S+G in the bloodstream. In turn, through hydrolysis with β-glucuronidase or sulfatase, the concentration of G or

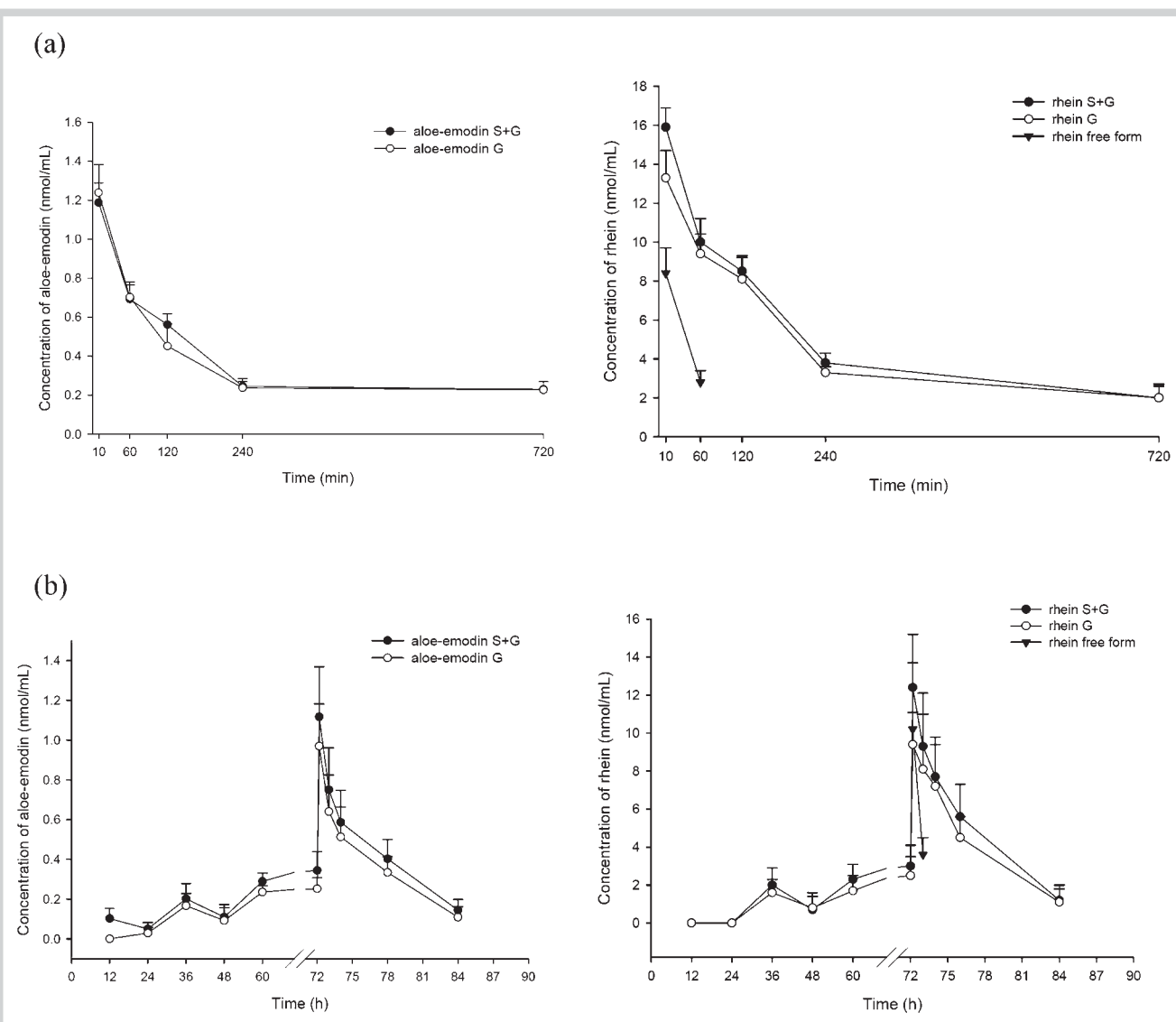


Fig. 4 Mean (\pm SE) serum concentration-time profiles of free form, sulfates and glucuronides (S+G), and glucuronides (G) of aloe-emodin and rhein after

oral administration of (a) single dose and (b) the 7th dose of FS decoction (10 g/kg) to six rats.

S + G was calculated. Owing to considerable amount of glucuronidase present in the sulfatase used in this study (Type H-1), treatment of sulfatase hydrolyzed both S+G. Accordingly, the difference between the concentrations of S+G and G could estimate the concentration of sulfates.

The serum profiles of aloe-emodin S+G and rhein S+G revealed that these conjugated metabolites appeared rapidly in the circulation, suggesting that aloe-emodin and rhein were quickly absorbed and intensively metabolized by conjugation reaction. The profiles of S+G and G of aloe-emodin or rhein were essentially superposable following either single-dose or multiple-dose treatments, implying that the major metabolites of aloe-emodin and rhein were glucuronides, whereas their sulfates were very minor. The total systemic exposure of rhein G was greater than that of aloe-emodin G by 12 folds after either single or repeated dosing of FS, suggesting that rhein glucuronides were the major metabolites. The AUC_{0-t} of S+G and G of aloe-emodin or rhein after single dose and multiple doses were comparable, implying

that these conjugated metabolites were not accumulating in the blood under such dosage regimen.

The free form of rhein was detected at the first sampling time after both single-dose and multiple-dose administrations, indicating that rhein was transiently existing as free form in the circulation during the very early phase and turned over rapidly to its conjugate forms, which was in good agreement with an *in vitro* study reporting relatively low glucuronidation activity of UDP-glucuronyltransferase toward carboxylic acids [14]. Regarding aloe-emodin, the free form was not detected in all serum samples. These results were consistent with a previous study reporting the biological fates of aloe-emodin and rhein after rhubarb administration to rats [15].

P-gp has been thought to play an important role in the barrier function of intestine for drug absorption [16]. In order to evaluate the effect of FS on P-gp activity, transport assay of rho 123, a typical P-gp substrate, was conducted by using LS 180 cells. As shown in **Fig. 5**, similarly to verapamil (a positive control of P-gp inhibitor), FS markedly increased the intracellular accumu-

lation of rho 123, indicating that the efflux function of P-gp was suppressed by FS. This finding of FS as an inhibitor of P-gp was in good agreement with a previous study using Caco-2 cells [10]. This LS 180 model was newly developed and superior to Caco-2 model because of much shorter time needed to reach confluency before a transport study.

It has been reported that senna led to toxicity in digoxin users [17]. Moreover, a recent population-based, nested, case-control study has furnished further evidence that the combined use of digoxin and sennosides for less than 14 days increased the risk of digoxin toxicity in heart failure patients [18]. However, no pharmacokinetic relevant mechanism has been proposed by these investigators. Based on our result showing that the transport of rho 123 was inhibited by FS decoction, the higher toxicity of digoxin in humans can be in part explained by the inhibited efflux of digoxin, a substrate of P-gp, into gut lumen, which then led to enhanced blood level and toxicity. Therefore, the concurrent use of FS with critical medicines which are substrates of P-gp require caution in clinical practice to ensure drug safety. In conclusion, senna anthranoids were rapidly and extensively metabolized to mainly rhein glucuronides and aloe-emodin glucuronides in rats. FS ingestion inhibited the efflux function of P-gp in the intestine.

Materials and Methods

Chemicals and reagents

Sennoside A (purity 96%), aloe-emodin (purity 95%), DMSO, rho 123, SDS, Triton X-100, verapamil (purity 99%), β -glucuronidase (type B-1, from bovine liver), and sulfatase (type H-1 from *Helix pomatia*, containing 14 000 units/g of sulfatase and 498 800 units/g of β -glucuronidase) were purchased from Sigma Chemical Co. Rhein (purity 95%) was purchased from Aldrich Chemical Co. 1,8-Dihydroxyanthraquinone (purity 98%) and amyl paraben (purity 98%) were obtained from Tokyo Chemical Industry Co. Methanol, acetonitrile, acetic acid (glacial, 99%), and ethyl acetate were LC grade and purchased from J.T. Baker, Inc. L(+)-Ascorbic acid (purity 99.7%) was obtained from RdH Laborchemikalien GmbH & Co. KG. Other reagents were HPLC grade or analytical grade. MTT (purity 98%) was obtained from Alfa Aesar. FBS was supplied by Biological Industries Inc. PSG, DMEM, trypsin/EDTA, HBSS, and HEPES were purchased from Invitrogen. Milli-Qplus water (Millipore) was used throughout this study.

Preparation of Folium Sennae decoction

The crude drug of FS was purchased from an herbal drugstore in Taichung, Taiwan. The plant material was identified by Dr. Yu-Chi Hou. A voucher specimen (CMU-P-1905-11) was deposited in the Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University. Water (12 L) was added to 600 g of FS and heated on a gas stove. After boiling, gentle heating was continued until the volume was reduced to less than 600 mL. The mixture was filtered while hot. The filtrate was gently boiled until the volume was reduced to below 600 mL, and sufficient water was added to make 600 mL which was immediately divided into aliquots and frozen at -20°C for later use. Each mL of decoction represents 1.0 g of FS.

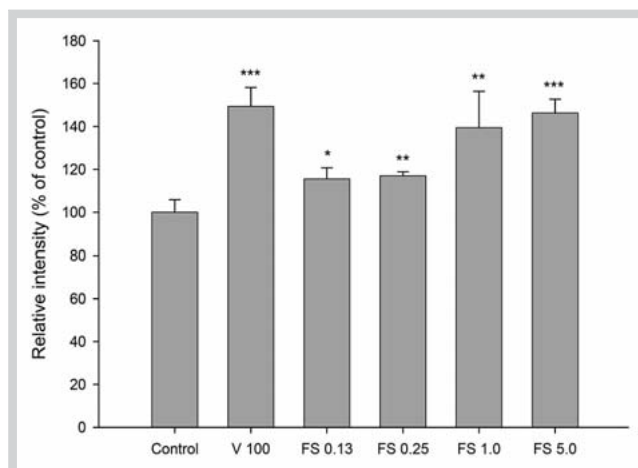


Fig. 5 Effects of Folium Sennae (mg/mL) and verapamil (V, 100 μM) on the accumulation of rho 123 in LS 180 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Quantitation of anthraquinones in Folium Sennae decoction before and after hydrolysis

The HPLC apparatus included a pump (LC-10AT, Shimadzu), an UV spectrophotometric detector (SPD-10AVP, Shimadzu), a Chromatopac (C-R6A, Shimadzu) with an automatic injector (SIL-10A, Shimadzu), and an Alltech Apollo C18 column (5 μm , 4.6 mm \times 150 mm, Alltech).

FS decoction (300 μL) was mixed with 700 μL of methanol and centrifuged to remove the precipitate. The properly diluted decoction (180 μL) was added with 20 μL of amyl paraben solution (50.0 $\mu\text{g/mL}$ in methanol) as IS, and 20 μL was subject to HPLC analysis. The mobile phase consisted of methanol (A) – 0.1% acetic acid (B) and programmed in a gradient manner as follows: A/B: 45/55 (0–5 min), 52/48 (8 min), 65/35 (12 min), 80/20 (22–30 min), and 45/55 (32–35 min). The detection wavelength was set at 270 nm, and the flow rate was 1.0 mL/min.

Acid hydrolysis of 1.0 mL of FS decoction was conducted by adding 25 mg ascorbic acid and 1.0 mL of 1.2 N HCl and incubating in a water bath at 80°C for 30 min. The hydrolysate was then added with sufficient water to make 2.0 mL before HPLC analysis. The glycoside concentration of each anthraquinone was calculated by subtracting the aglycone concentrations in the original decoction from those in the hydrolysate.

Animals

Male Sprague-Dawley rats were purchased from BioLASCO Taiwan Co., Ltd. and housed in conditioned environment with 12-h light/dark cycles. Rats were fasted overnight before drug administration, whereas water was supplied *ad libitum*. Food was offered 3 h after drug administration. All animal experiments adhered to “The Guidebook for the Care and Use of Laboratory Animals” published by the Chinese Society of Animal Science, Taiwan, R.O.C. The experimental protocol had been reviewed and approved on 08/01/2013 (No. 102–144-N) by the Institutional Animal Care and Use Committee of China Medical University, Taiwan.

Drug administration and blood collection

Six rats were orally given a single dose of 10.0 mL/kg of FS decoction (equivalent to 10.0 g/kg of crude drug) via gastric gavage. To another group of six rats, FS decoction was given twice daily for consecutive three days. The 7th dose of FS was given after overnight fasting, and then bioavailability and metabolic study of senna anthraquinones was conducted. The blood samples (0.5 mL each) were withdrawn at 10, 60, 120, 360, 720 min after dosing. The blood was collected in microtubes and centrifuged at 10000 g for 10 min to obtain serum, which was stored at -20°C before analysis.

Quantitation of anthraquinones and their conjugated metabolites in serum

For the assay of the parent forms of anthraquinones, 100 μL of serum was mixed with 50 μL of pH 5 acetate buffer and 50 μL of ascorbic acid (100 mg/mL), then 50 μL of 0.1 N HCl was added and partitioned with 250 μL of ethyl acetate (containing 0.2 $\mu\text{g/mL}$ of 1,8-dihydroxyanthraquinone as IS). The ethyl acetate layer was evaporated under N_2 gas to dryness and reconstituted with 50 μL of the mobile phase, and then 20 μL aliquot was subject to HPLC analysis.

For the quantitation of the glucuronides of rhein and aloe-emodin, 100 μL of serum was mixed with 50 μL of β -glucuronidase (1000 units/mL in pH 5 acetate buffer) and 50 μL of ascorbic acid (100 mg/mL). The mixture was incubated at 37°C for 1 h under anaerobic condition and protected from light. After enzymatic hydrolysis, serum samples were subjected to the same process described above for the assay of parent forms of anthraquinones. For calibrator preparation, 100 μL of serum spiked with various concentrations of rhein and aloe-emodin were added with 50 μL of pH 5 buffer and 50 μL of ascorbic acid. The later procedure followed that described above for serum samples. The calibration curve was drawn by linear regression of the peak area ratios (compounds as IS) against the known concentrations of each anthraquinone.

The HPLC apparatus included a pump (LC-10AT, Shimadzu), a fluorescence detector (RF-10AXL, Shimadzu), and an automatic injector (Series 200 Autosampler, Perkin Elmer). The Apollo C18 (4.6×250 mm, 5 μm) column was equipped with a guard column (4.6×50 mm, 5 μm) (GL Science Inc.). The isocratic mobile phase consisted of methanol and 0.1% phosphoric acid at a ratio of 80:20 (v/v). Wavelengths of excitation and emission were set at 440 nm and 520 nm. The flow rate was 1.0 mL/min.

Validation of assay method for serum

The system suitability was evaluated through analysis of precision and accuracy. The precision was analyzed by intra-run and inter-run assays of calibrators three times in one run and over three consecutive runs. The accuracy of the system was expressed by the relative error of the mean calculated concentration to the true concentration of each calibrator. Recoveries were measured by spiking aloe-emodin and rhein into blank serum and water in triplicates to afford 0.5, 0.1 and 0.03 $\mu\text{g/mL}$ of aloe-emodin and 10, 2.5 and 0.6 $\mu\text{g/mL}$ of rhein. The concentrations obtained in blank serum to the corresponding ones in water were compared to obtain recoveries.

LLOQ represents the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, whereas LOD represents the lowest concentration of analysis in a sample that can be detected (with signal/noise > 3).

Cell line and culture conditions

LS-180, human colon adenocarcinoma cell line, was purchased from the Food Industry Research and Development Institute (Taiwan). The cells were cultured in DMEM medium supplemented with 10% FBS (Biological Industries Ltd.), 0.1 mM nonessential amino acid, 100 units/mL of penicillin, 100 $\mu\text{g/mL}$ of streptomycin, and 292 $\mu\text{g/mL}$ of glutamine. Cells were grown at 37°C in a humidified incubator containing 5% CO_2 . The medium was replaced every two days, and cells were subcultured when 80 to 90% confluency was reached.

Cell viability assay

The effect of tested drugs including positive controls (verapamil) on the viability of LS-180 was evaluated by MTT assay [19]. Cells were seeded into a 96-well plate. After overnight incubation, the tested agents were added into the wells and incubated for 24 h, then 15 μL of MTT (5.0 mg/mL) was added into each well and incubated for 4 h. In this period, MTT converted to formazan crystals in the cells. Following removal of the supernatant, SDS solution (20%) was added to dissolve the purple crystal at the end of incubation, and the optical density was detected at 570 nm by a microplate reader (BioTex).

Effect of Folium Sennae on P-glycoprotein activity

Evaluation of the effect of FS on the transport of rho 123 followed a previous method with minor modification [20]. Verapamil was used as a positive control of P-gp inhibitor. LS-180 cells (1×10^5) were cultured using a 96-well plate. After overnight incubation, medium was replaced by rho 123 in HBSS (1 μM) and incubated for 1 h. Following removal of the supernatant, cells were washed twice with ice-cold PBS, then a series of concentrations of FS and verapamil (as a positive control) were added into correspondent wells and incubated for 4 h at 37°C . After the medium was removed, the cells were washed twice with ice-cold PBS. Then 0.1% Triton X-100 (100 μL) was added, and the fluorescence was measured with excitation at 485 nm and emission at 528 nm. To quantify the content of protein in each well, 10 μL of cell lysate was added to 200 μL of diluted protein assay reagent (Bio-Rad), and the optical density was measured at 570 nm. The relative intensity of intracellular accumulation of rho 123 was calculated by comparing with that of control after protein correction.

Data analysis

C_{max} , AUC, and MRT of senna anthraquinones and their metabolites were calculated by noncompartment model of Phoenix WinNonlin® (version 6.3; Pharsight Corporation). C_{max} values were obtained from experimental measurement. AUC_{0-t} was calculated using trapezoidal rule to the last point. The differences among treatments were analyzed by using one-way ANOVA and unpaired Student's t-test for *in vivo* and *in vitro* studies, respectively, taking $p < 0.05$ as significance level.

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Conflict of Interest



The authors report no conflict of interest.

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