Quantitative HPLC Determination and Extraction of Anthraquinones in *Senna alata* Leaves

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Abstract

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of four anthraquinones: rhein, aloe-emodin, emodin, and chrysophanol in *Senna alata* leaves. The method involves the use of a TSK-gel ODS-80Tm column (5 µm, 4.6 × 150 mm) at 25°C with the mixture of methanol and 2% aqueous acetic acid (70:30, v/v) as the mobile phase and detection at 254 nm. The parameters of linearity, precision, accuracy, and specificity of the method were evaluated. The recovery of the method is 100.3–100.5%, and linearity (r^2 > 0.9998) was obtained for all anthraquinones. A high degree of specificity as well as repeatability and reproducibility (relative standard deviation values less than 5%) were also achieved. The solvent for extraction of anthraquinones from *S. alata* leaves was examined in order to increase the anthraquinone content of the leaf extract. It was found that a solution of 5% hydrochloric acid (v/v), 5% ferric chloride (w/v), and 15% water in methanol (v/v) was capable of increasing the anthraquinone content in the leaf extract up to 1.67% (w/w).

Introduction

Senna alata (L.) Roxb. (*Cassia alata* L.) is a plant belonging to the family Leguminosae. In Thailand, *S. alata* leaves have traditionally been used for the treatment of constipation and dermatophyte infections (1). Anthraquinone glycosides were demonstrated as the active constituents for the laxative properties (2), and the aglycones, including aloe-emodin, rhein, emodin, and chrysophanol, exhibited antifungal activity (3,4). Its leaf extract possessed antifungal activity against *Trichophyton mentagrophytes*, *T. rubrum,* and *Microsporum gypseum* with minimum inhibitory concentration (MIC) values of 125 mg/mL and against *Microsporum canis* with a MIC value of 62.5 mg/mL (5). Recently, the poor quality of *S. alata* leaves due to the content of anthraquinones being lower than the standard value in the monograph (6) has been a major problem in the production of the herbal medicines from *S. alata*. Analytical methods for the quantitative determination of anthraquinones in *S. alata,* including spectrophotometry (6) and high-performance liquid chromatography (HPLC) (7), have been reported. However, the spectrophotometric method is time consuming because it requires several sample preparation steps. Although an HPLC method has been developed, validation of the analytical procedure has not yet been established. In an effort to develop an improved method for the determination of anthraquinone content in *S. alata* leaf extract and its quality control, an extraction method with simultaneous quantification of the anthraquinones in *S. alata* leaves through HPLC was developed. The HPLC method was also validated.

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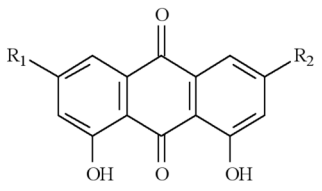
Experimental

Plant material

Senna alata leaves were collected from Songkhla province, Thailand. The voucher specimen (specimen no. SKP 097.1 03 01 01) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Chemicals and reagents

Rhein, aloe-emodin, emodin, chrysophanol, and anhydrous ferric chloride were purchased from Sigma-Aldrich (Germany). Methanol (HPLC grade and analytical grade) was purchased from Labscan Asia (Thailand). Acetic acid was purchased from J.T. Baker (Phillipsburg, NJ). Water was purified in a Milli-Q system (Millipore, Bedford, MA).



Aloe-emodin : $R_1 = CH_2OH$; $R_2 = H$

Chrysophanol : $R_1 = CH_3$; $R_2 = H$

Emodin : $R_1 = CH_3$; $R_2 = OH$

Rhein : $R_1 = COOH$; $R_2 = H$

Figure 1. Chemical structures of anthraquinones.

Standard solution

Separate stock solutions of the reference standards, rhein, aloe-emodin, emodin, and chrysophanol were made in methanol. A working solution of the combined standards were subsequently prepared in methanol and diluted to provide a series of the standards ranging from 12.5–200 µg/mL for use in constructing calibration curves for each of the target analytes.

Table I. Anthraquinone Content of *S. alata* Leaf Extracts Analyzed by HPLC

Anthraquinone	Anthraquinone content (%w/w) Mean ± S.D.	
	Methanol extract	5% HCl in methanol extract
Rhein	0.02 ± 0.002	0.15 ± 0.009
Aloe-emodin	0.03 ± 0.008	0.04 ± 0.011
Emodin	n.d.*	0.13 ± 0.008
Chrysophanol	n.d.	n.d.

* n.d. = not detected.

Sample Preparation

S. alata leaf powder (100 mg) was extracted with methanol (20 mL) under reflux conditions for 1 h. The extract was then filtered and concentrated under reduced pressure. The sample was reconstituted and adjusted to 5 mL with methanol. Samples were analyzed immediately after extraction in order to avoid possible chemical degradation. The experiments were run in triplicate.

HPLC conditions

HPLC analysis was carried out using an Agilent 1100 series equipped with a Agilent 1100 series photodiode-array detector (PDA) and autosampler. Data analysis was performed using Agilent software [Agilent (Palo Alto, CA)]. Separation was achieved isocratically at 25°C on a 150 mm × 4.6 mm i.d. TSK-gel ODS-80Tm column (Tosoh Bioscience, Tokyo, Japan). The mobile phase consisted of methanol–2% aqueous acetic acid (70:30, v/v) and was pumped at a flow rate of 1 mL/min. The injection volume was 20 µL. The quantitation wavelength was set at 254 nm.

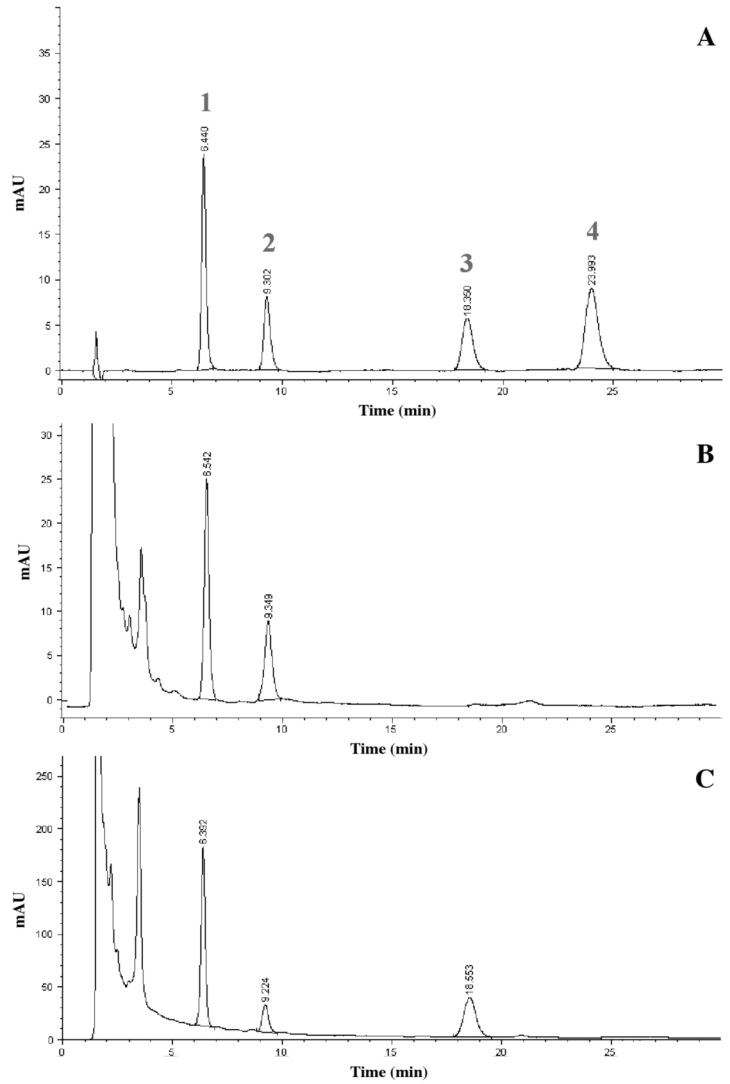


Figure 2. HPLC-chromatograms of authentics (A): aloe-emodin (1), rhein (2), emodin (3), and chrysophanol (4); methanol extract (B), and 5% HCl in methanol extract of *S. alata* leaves (C).

Method validation

Calibration curve

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the standard compounds at five concentrations and plotting peak area against the concentration of each reference standard. The linearity of the detector response for the standards was determined by means of linear regression. The curves showed coefficient of correlation (r^2) ≥ 0.9995.

Accuracy

Sample portions were fortified with known quantities of the standard analytes in order to assay accuracy data. Prior to analyte fortification, the background levels of rhein, aloe-emodin, and emodin in extracts of *S. alata* were determined so as to calculate actual recoveries. The amount of each analyte was determined in triplicate, and percentage recoveries were then calculated.

Precision

Precision experiments were conducted for intra- and inter-day. The solution of one sample was used to achieve repeatability testing. The repeatability data was the content of six injections separately on the same day. The data used to calculate the % RSD of interday precision was the content of three samples analyzed on different three days (three injections on each successive each).

Specificity

Peak identification was carried out using the standards and a diode-array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

Solvent for extraction

Determination of optimum hydrochloric acid concentration

S. alata leaf powder (20 mg) was extracted with methanol (20 mL) consisting of several concentrations of hydrochloric acid (0%, 3%, 5%, and 10% v/v) under reflux conditions for 1 h. The extract was then filtered and concentrated under reduced pressure. The sample was adjusted to 5 mL with methanol and subjected to HPLC analysis. The experiments were done in triplicate.

Determination of optimum ferric chloride concentration

S. alata leaf powder (20 mg) was extracted with methanol (20 mL) consisting of 5% hydrochloric acid and several concentrations of ferric chloride (0%, 2.5%, 5%, and 10% w/v) under reflux conditions for 1 h. The extract was then filtered and concentrated under reduced pressure. The sample was adjusted to 10 mL with methanol and subjected to HPLC analysis. The experiments were done in triplicate.

Determination of optimum water concentration

S. alata leaf powder (20 mg) was extracted with methanol (20 mL) consisting of 5% hydrochloric acid, 5% ferric chloride, and several concentrations of water (0%, 5%, 10%, 15%, and 20% v/v) under reflux conditions for 1 h. The extract was then filtered and concentrated under reduced pressure. The sample was adjusted to 10 mL with methanol and subjected to HPLC analysis. The experiments were run in triplicate.

Table II. Linear Ranges and Correlation Coefficients of Calibration Curves

Anthraquinone	$y = ax + b$ linear model*	r^2	concentration (µg/mL)
Rhein	$y = 6349.8x - 0.52$	1.0000	12.5–200
Aloe-emodin	$y = 8397.7x - 1.34$	0.9999	12.5–200
Emodin	$y = 4800.9x - 7.71$	1.0000	12.5–200
Chrysophanol	$y = 9220.8x - 11.37$	0.9998	12.5–200

* y = peak area and x = concentration.

Table III. Repeatability, Reproducibility, and Recoveries of Rhein, Aloe-Emodin, and Emodin from *S. alata* Leaf Extracts

Anthraquinone	R.S.D. (%) Intra-day ($n = 6$)	R.S.D. (%) Inter-day ($n = 3$)	% recovery (Mean ± S.D.)
Rhein	0.53	0.78	100.3 ± 1.20
Aloe-emodin	0.42	0.62	100.5 ± 1.21
Emodin	0.36	0.57	100.4 ± 0.87

Table IV. Anthraquinone Content in *S. alata* Leaf Extracts Under Reflux with Various HCl Concentrations in Methanol

HCl Concentration (% v/v)	Anthraquinone content (% w/w) Mean ± S.D. Rhein	Aloe-emodin	Emodin	Total
0	0.02 ± 0.002	0.03 ± 0.002	n.d.*	0.05 ± 0.004
3	0.05 ± 0.009	0.14 ± 0.008	0.11 ± 0.012	0.30 ± 0.011†
5	0.04 ± 0.011	0.15 ± 0.009	0.13 ± 0.008	0.32 ± 0.007†‡
10	0.03 ± 0.003	0.14 ± 0.010	0.13 ± 0.002	0.30 ± 0.013†

* n.d. = not detected.
† Significant difference ($P < 0.05$) when compared with 0% v/v HCl.
‡ Significant difference ($P < 0.05$) when compared within the same column.

Results and Discussion

We examined the optimal conditions for the simultaneous quantitative determination of rhein, aloe-emodin, emodin, and chrysophanol (Figure 1) in *S. alata* leaf extract using an isocratic reversed-phase HPLC system. As all four compounds have good absorption at 254 nm, this wavelength was used for quantitation. Mixtures of methanol and 2% aqueous acetic acid were examined as the mobile phase and its composition was optimized. The ratio of methanol to 2% aqueous acetic acid was was 70:30. This ratio obtained a good resolution of rhein, aloe-emodin, emodin, and chrysophanol. All four compounds were eluted within 30 min with satisfactory resolution (Figure 2).

The proposed isocratic procedure exhibits simultaneous quantification for the analytes (rhein, aloe-emodin, emodin, and chrysophanol) and is simple, rapid, and selective. The previously reported HPLC method required the use of a gradient elution system and required a longer time; a total run time of approximately 60 min is required, and validation of the analytical procedure is not yet established (7). In addition, the spectrophometric method requires several time-consuming sample preparation steps with anthraquinone content determined as total hydroxyanthraxene derivative (6).

On the basis of HPLC analysis, only rhein and aloe-emodin are the major aglycone of anthraquinone found in the methanol extract of *S. alata* leaves (Table I). However, the leaves extracted with 5% (v/v) HCl in methanol were capable of producing emodin in the extract. This may be due to the occurrence of emodin glycoside hydrolysis. Although chrysophanol has been reported as a constituent in *S. alata* leaves (8,9), it was not detected in both extracts. This may be due to a very small amount of this anthraquinone accumulated in *S. alata* leaves.

Defining the linearity, accuracy, intra- and inter-day precision, and specificity validated the HPLC method. Linearity was evaluated using standard samples over five calibration points (from 12.5–200 µg/mL) with six measurements for each calibration point. Rhein, aloe-emodin, emodin, and chrysophanol exhibited linearity over the evaluated ranges with correlation coefficients 1.0000, 0.9999, 1.0000, and 0.9998, respectively (Table II).

Intra-day precision was estimated by the relative standard deviation of six measurements; for rhein, aloe-emodin, and emodin were each less than 5% (Table III). Analysis of three independently prepared samples of *S. alata* leaf extract determined the interday-precision. The relative standard deviation values for interday analysis of rhein, aloe-emodin, and emodin were also each less than 5% (Table III).

Method accuracy was determined by analyzing *S. alata* leaf extracts fortified with known quantities of each standard analyte. Recoveries in the range of 100 ± 2% were observed for all compounds (Table III).

Table V. Anthraquinone Content in *S. alata* Leaf Extracts Under Reflux with Various Ferric Chloride Concentrations in 5% HCl in Methanol

$FeCl_3$ concentration (% w/v)	Anthraquinone content (% w/w) Mean ± S.D.			
	Rhein	Aloe-emodin	Emodin	Total
0	0.04 ± 0.011	0.15 ± 0.009	0.13 ± 0.008	0.32 ± 0.007*
2.5	n.d.†	0.54 ± 0.044	0.33 ± 0.070	0.87 ± 0.103*‡
5	n.d.	0.99 ± 0.094	0.47 ± 0.011	1.46 ± 0.146
10	n.d.	1.08 ± 0.095	0.51 ± 0.014	1.59 ± 0.093

* Significant difference ($P < 0.05$) when compared with 5% ferric chloride.
† n.d. = not detected.
‡ Significant difference ($P < 0.05$) when compared with 0 % ferric chloride.

Table VI. Anthraquinone Content in *S. alata* Leaf Extracts Under Reflux with Various Water Concentrations in 5% Hydrochloric Acid and 5% Ferric Chloride in Methanol

Water conc. (%v/v)	Anthraquinone content (% w/w) Mean ± S.D.		
	Aloe-emodin	Emodin	Total
0	0.42 ± 0.026	0.71 ± 0.001	1.13 ± 0.025
5	0.64 ± 0.166	0.66 ± 0.014	1.30 ± 0.025
10	0.78 ± 0.056	0.58 ± 0.007	1.36 ± 0.029
15	1.11 ± 0.085	0.56 ± 0.004	1.67 ± 0.016*
20	0.87 ± 0.023	0.34 ± 0.027	1.21 ± 0.005

* Significant difference ($P < 0.05$) when compared within the same column.

Utilizing the PDA makes it possible to obtain the UV spectra. Specificity of the method was evaluated using UV-absorption spectra produced by the diode-array detector. The spectra were taken at three points of the peaks. When they were compared with the standard, homogeneity for spectra of all peaks was observed.

A few different extraction solvents were tried to maximize the anthraquinone content in *S. alata* leaf extract. *S. alata* leaves were extracted under reflux conditions using a mixture of HCl, $FeCl_3$, and water in methanol as the solvent. Water was used for extraction of anthraquinone glycoside, and HCl and $FeCl_3$ were used for hydrolysis and oxidation of anthraquinone glycoside as the aglycone. The concentrations of each component in methanol were varied in order to get a high yield anthraquinone extract. The results showed that emodin was observed when an acidic methanol was used for extraction, and 5% v/v HCl in methanol gave significantly higher total anthraquinone content than those of 0, 3, and 10% v/v HCl in methanol (Table IV). Variations of $FeCl_3$ concentration in methanol with 5% HCl were then examined as the extraction solvents. It was found that only aloe-emodin and emodin were observed in the extract after oxidization with $FeCl_3$. However, the content of total anthraquinones was increased when the concentration of $FeCl_3$ was increase to 5% w/v (Table V). Therefore, 5% w/v $FeCl_3$ in methanol with 5% v/v HCl was appropriately used for the extraction of the anthraquinones from *S. alata* leaves. Natural occurring anthraquinones in *S. alata* leaves are in both glycoside and aglycone forms (10). This suggests that oxidization and hydrolysis of anthraquinone glycoside are required in the extraction process in order to increase anthraquinone content in *S. alata* extract.

Because glycosides are usually dissolved in water, adequate aqueous concentrations may be required to increase the anthraquinone glycoside extraction. An optimization study for water content in the extraction solvent exhibits the greatest extract anthraquinone levels when employing 15% v/v water (Table VI). Thus, the suitable solvent for the anthraquinone extraction of *S. alata* should be comprised of 5% hydrochloric acid, 5% ferric chloride, and 15% water in methanol.

Conclusion

A simple, specific, precise, accurate, rapid, and reproducible HPLC method has been developed to quantify the active principles in *S. alata* leaves. The simultaneous quantitative determination for rhein, aloe-emodin, emodin and chrysophanol yield analyte data that provides useful marker information for the quality control of *S. alata* leaf extract. This study suggests that a mixture of 15% water, 5% hydrochloric acid, and 5% ferric chloride in methanol is a suitable solvent for anthraquinone extraction of *S. alata* leaves.

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Manuscript received June 21, 2007;
revision received October 3, 2007.