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Short communication

Simultaneous determination of five anthraquinones in medicinal plants and pharmaceutical preparations by HPLC with fluorescence detection

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ABSTRACT

A reversed-phase high-performance liquid chromatography (RP-HPLC) method with fluorescence detection for simultaneous determination of five anthraquinones in *Rhubarb* collected from nine different locations in China, *Polygonum cuspidatum*, *Polygoni multiflori* and three pharmaceutical preparations is proposed and validated. Chromatography was carried out at 25 °C on a Hypersil C18 column with the isocratic mobile phase of methanol–0.1% aqueous formic acid (85:15, v/v) at a flow rate of 1.0 ml/min. The excitation and emission wavelengths were set at 440 and 540 nm, respectively. A comprehensive validation of the method included tests of sensitivity, linearity, precision and accuracy. The linear regressions were acquired with $r > 0.999$. Satisfactory intra- and inter-day precisions were achieved with R.S.D.s less than 3.95% and the average recovery factors obtained were in the range of 93.2–103.8%.

1. Introduction

Anthraquinones are known to be present in many different families such as Polygonaceae, Leguminosae, Rubiaceae, Liliaceae and Rhamnaceae [1]. The anthraquinones are considered the active constituents of the *Rhubarb*, *Polygonum cuspidatum sieb.et zucc* and Radix *Polygoni multiflori* belonging to the family Polygonaceae [2,3]. They are officially listed in Chinese Pharmacopoeia [4]. These traditional Chinese medicines have been used to treat various diseases. *Rhubarb*, *P. cuspidatum sieb.et zucc* and their preparations have been used as laxative, antiphlogistic and hemostatic drugs in the treatment of obstipation, gastrointestinal indigestion, diarrhea and jaundices in China and other Asian countries for thousands of years [5,6]. Radix *P. multiflori* possesses many pharmacological functions including tonifying the blood, liver and kidney, strengthening the bones and muscles, preventing premature graying of the hair, and treatment of seminal emission and menstrual and menopausal complaints [7]. The anthraquinone derivatives including emodin, physcion, aloe-emodin, rhein and chrysophanol were accepted as important active components in these medicinal plants, which are often used as criteria in the quality control of these medicinal plants. Recently, a number of pharmacological tests revealed that the anthraquinone derivatives present various biological activities including antifungal [8], antimicrobial [9], anticancer [10–12], antioxidant [13,14], and antihuman cytomegalovirus activity [15].

A literature survey reveals that there have been a number of various analytical methods including HPLC-UV [16–19], UPLC [20], capillary zone electrophoresis (CZE) [21–24], micellar electrokinetic chromatography (MEC) [25–28], capillary electrochromatography (CEC) [29], thin-layer chromatography (TLC) [30] and high-speed counter current chromatography (HSCCC) [31–33] published on the separation and determination of all or some of the five anthraquinones. Although these methods have been routinely used for the analysis of these anthraquinones, they are relatively insensitive. In these medicinal plants, the anthraquinones have been analysed mostly by reversed-phase HPLC-UV detection. However, it is usually difficult to achieve a high sensitivity and baseline separation of analytes from sample matrix because of the great variety of species present and the wide variations in their levels in herbal samples. On the other hand, HPLC–MS [34] and GC–MS [35,36] methods are highly sensitive and reliable. However, these instruments are expensive, which limits availability. Therefore, there is a need for the development of other simpler and reliable methods for the analysis of anthraquinones in medicinal plants and their pharmaceutical preparations.

In general, fluorescence detection is sensitive and selective. The five anthraquinones are known to possess natural fluorescence, but it is difficult to analyse and determine their contents by conventional fluorimetry due to their similar molecule structures. Although HPLC-fluorescence detection has been used to measure other compounds [37], very few applications have been reported

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for the analysis of anthraquinones. Recently, HPLC-fluorescence detection has been used to measure single aloe-emodin in plasma within 12 min [38]. However, this method was not suitable for the simultaneous analysis of five anthraquinones in herbal extracts and related preparations because of its long runtime. Hitherto, no HPLC-fluorescence detection method has been developed for the simultaneous determination of the five anthraquinones.

The aim of the present study was to develop and validate a simple, rapid and sensitive reversed-phase high-performance liquid chromatography (RP-HPLC) method, using fluorescence detection, to simultaneously quantify aloe-emodin, emodin, rhein, chrysophanol and physcion (Fig. 1) in medicinal plants and their pharmaceutical preparations.

2. Experimental

2.1. Chemicals and solvents

Aloe-emodin (>99%), rhein (>98%), emodin (>99%), chrysophanol (>99%) and physcion (>99%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The chemical structures of the hydroxyanthraquinones are shown in Fig. 1. The stock standard solutions of aloe-emodin, rhein, emodin, chrysophanol and physcion were separately prepared in methanol using reference standards to obtain concentrations of 150, 70, 250, 225 and 44 μg/ml, respectively. A series of working standard solutions with gradient concentration were obtained by diluting the stock solution with methanol. All the solutions were stored at −40 °C.

HPLC grade methanol, acetic acid glacial and formic acid were purchased from Tedia (Fairfield, Ohio, USA). Analytical grade phosphoric acid was obtained from Kelong (Chengdu, China). Water was purified using a Milli-Q purification system (Millipore Bedford Corp., Bedford, MA, USA) and used to prepare all buffer and sample solutions.

2.2. Instruments

Chromatography was carried out using a Shimadzu system (Tokyo, Japan) composed of two LC-10ADvp pumps, SIL 10ADvp autosampler, CTO-10ASvp column oven, RF-10Axl fluorescence detector, LC-10PDA UV detector and SCL-10ADvp controller. A Hypersil C18 column (200 mm × 4.6 mm, 5 μm, Dalian, P.R. China) was used. The data was acquired by CLASS-VP software, v.5.03.

A sonication bath (KQ3200B, Shanghai Branson Ultrasound Co. Ltd., China) working at 40 kHz frequency and 150 W input power was employed as extraction and degassing device.

2.3. Sample collection and preparation

P. cuspidatum sieb.et zucc, Radix *P. multiflori* and rhubarbs collected from nine different geographical locations in China were identified by Professor Ke-Ming Liu, Department of Botany, Hunan Normal University. Three herbal preparations, namely Sanhuangpian, Huanglianshangqingwan and Marenwan were purchased from drug stores in Changsha.

The medicinal plant materials were dried at room temperature and then pulverized in an electric grinder. The sugar coating of the pharmaceutical preparations was removed and the residue was ground in a mortar. The powder was screened through 250 μm sieves. The fine powdered herbal sample or herbal preparation (100 mg) was treated by ultrasonic extraction using methanol/water (25.00 ml, 90:10, v/v) for 30 min at room temperature, then centrifuged for 5 min at 2000 × *g*. The supernatant was filtered through a 0.45 μm nylon membrane before use. Chromatographic peaks of the samples were identified by comparing the retention time with those of the standard compounds and were subsequently quantified using the external standard method.

2.4. HPLC analysis

The HPLC isocratic mobile phase was methanol–0.1% formic acid (85:15, v/v) at a flow rate of 1.0 ml/min at room temperature (25 °C). The sample injection volume was 10 μl. For the HPLC-UV detection method, the system was operated at 254 nm. For the HPLC-fluorescence detection method, the system was operated at an excitation wavelength of 440 nm and an emission wavelength of 540 nm. The total analysis time was 15 min. Three injections were performed for each sample. Some sample solutions were diluted with the mobile phase in order to obtain concentrations within the range of the calibration curve.

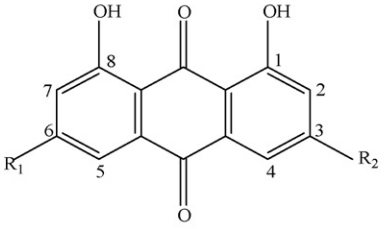
2.5. Method validation

The method validation was performed to ensure its compliance with FDA guideline, such as selectivity, sensitivity (limits of detection and quantification), linearity and linear range, accuracy, precision and stability.

3. Results and discussion

3.1. Optimization of the HPLC conditions

In general, the fluorescence intensity of substance was inclined to be affected by the polarity and pH of solution. The chromatographic behaviour of analytes was investigated with the following isocratic mobile phases: (a) methanol–water (75:25, v/v), (b) methanol–water (85:15, v/v), (c) methanol–water (90:10, v/v), (d) methanol–0.1% phosphoric acid (85:15, v/v), (e) methanol–0.1% acetic acid (85:15, v/v), (f) methanol–0.1% formic acid (85:15, v/v) and (g) methanol–0.05% formic acid (85:15, v/v), respectively. The total runtime was shortened with the increase of the concentration of methanol in mobile phase. The runtime is the shortest using mobile phases (c), but the peaks of aloe-emodin and rhein were



	R_1	R_2
Aloe-emodin	H	CH_2OH
Rhein	H	COOH
Emodin	OH	CH_3
Chrysophanol	H	CH_3
Physcion	OCH_3	CH_3

Fig. 1. Chemical structures of aloe-emodin, rhein, emodin, chrysophanol and physcion.

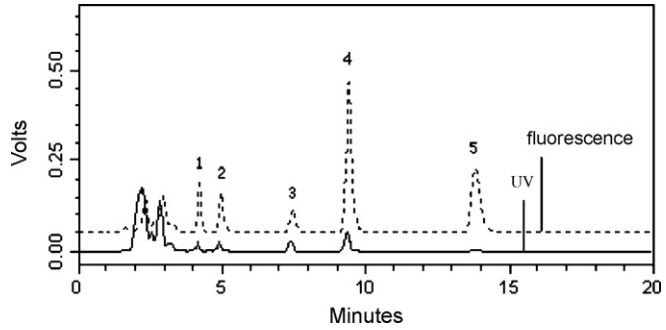


Fig. 2. The chromatograms of the five anthraquinones (1, aloe-emodin; 2, rhein; 3, emodin; 4, chrysophanol; 5, physcion) with HPLC-fluorescence detection and HPLC-UV method from rhubarb in, Datong, Qinghai.

overlapped. Although the fluorescence intensity of the analytes is stronger in mobile phases (a), (b), (c), the peaks of rhein and aloe-emodin showed significant tailing. Mobile phases (d), (e), (f) and (g) gave adequate resolutions, and mobile phase (f) gave a good resolution for the target compound, satisfactory analysis time and fluorescence intensity, so mobile phase (f) was selected for the further experiments.

The five anthraquinones showed maximum UV absorption at 254 nm which was selected for the HPLC-UV analysis. For the HPLC-fluorescence detection method, optimal excitation and emission wavelengths for the five anthraquinones were at 440 and 540 nm, respectively. Fig. 2 shows the typical HPLC profiles of the five anthraquinones with HPLC-fluorescence detection and HPLC-UV method in one run. The peaks of the analytes are completely separated and the shapes are symmetrical.

3.2. Optimization of ultrasound-assisted extraction

Extraction by sonication is a powerful technique in phytochemical research. Refluxing was not applied because it required a long period of time (at least 10 h), as reported by Koyama et al. [24]. Furthermore, Soxhlet extraction often causes thermal degradation of the analytes. So ultrasound-assisted extraction has been used for the extraction of anthraquinones from plant material and preparations. The extraction conditions were optimized on *Rheum palmatum* L. in Shaanxi. and the peak areas of the analytes were used to evaluate extraction efficiency.

The extraction solvent is a critical factor affecting extraction efficiency. Based on literature reports [20,24], aqueous methanol was selected as the extraction solvent and the concentration of methanol was optimized for 100 mg sample powder sonication with 25 ml methanol–water in different ratios (10:0, 9:1, 8:2, 7:3, 5:5, v/v) for 30 min. The results showed that methanol–water (9:1, v/v) provided the best yield. Therefore, methanol–water (9:1, v/v) was used as extraction solvent.

The optimum solvent/sample ratio was obtained by extracting 100 mg sample powder with different volumes (5, 15, 25, 30, 40 ml) of 90% methanol for 30 min. The results showed that an increase of extraction efficiency of analytes could be observed with increasing solvent volume from 5 to 25 ml. Further increase in solvent volume did not result in significant enhancement of anthraquinone yield. Hence, 250/1 (ml/g) was selected as optimum solvent/sample ratio.

Temperature and time were main parameters that affect the extraction efficiency. High temperature and prolonged time should be avoided because of evaporation of solvent. The effect of time was investigated by analysing the amount of anthraquinone extracted with 90% methanol at room temperature (25 °C) for 15, 30, 45, and 60 min. The results showed that the extraction yield became nearly constant from 30 to 60 min (data not shown); so 30 min was chosen as the optimal extraction time.

3.3. Parameters for method validation

The linearity, LOD and LOQ for anthraquinones of standard solution by HPLC-UV and HPLC-fluorescence detection methods were investigated under the above optimum analysis conditions. A good linear relationship between the corresponding peak areas and the concentrations of the analytes was achieved. The linear ranges, slopes (mean value ± S.D.), intercepts, correlation coefficients (r), LOD and LOQ by HPLC-UV and HPLC-fluorescence detection methods are summarized in Table 1. These results indicate that the LOD of HPLC-fluorescence detection method was 100 (aloe-emodin), 12 (emodin), 100 (rhein), 12 (chrysophanol) and 64-fold (physcion) lower, when compared with those derived using HPLC-UV detection, respectively. HPLC method with fluorescence detection should be more suitable for the analysis of the anthraquinones than HPLC-UV methods because of broad linear range and high sensitivity.

The precision of the chromatographic system was evaluated with a standard mixture solution of the anthraquinones under the optimal conditions five times in 1 day for intra-day variation and five injections each day on three consecutive days for inter-day variation. The results are described in Table 2 that shows the intra- and inter-day %R.S.D. values of retention times and peak areas.

The precision of the extraction procedure was tested by repeating the extraction procedure of the same sample of *R. palmatum* L. in Shaanxi. An aliquot of each extract was then injected and quantified. The intra- and inter-day %R.S.D. data are shown in Table 3.

The accuracy of the analytical method was evaluated using the recovery test. This involved the addition of known quantities of reference standard compounds to the appropriate sample weight of rhubarb plant material. The reference standards were added at three different concentration levels (approximately equivalent to 0.5, 1.0 and 1.5 times of the concentration of the rhubarb collected from Datong, Qinghai) with five parallels at each level. The spiked samples were then extracted and analysed by the proposed HPLC method. The obtained mean recoveries are shown in Table 4.

Stability was evaluated with stock solutions of reference standard and extract of *R. palmatum* L. in Shaanxi that were stored at 4 °C and at room temperature (about 25 °C) and analysed every 8 h. The analytes in stock solutions and extract did not show any appreciable change in chromatographic profile over 72 h at 4 °C and within 72 h at room temperature. Other constituents of the extract did not interfere in the determination of the analytes and no degradation products of preservatives were found in Fig. 2.

The validation data highlighted the suitability of the proposed HPLC method for the analysis of anthraquinones.

3.4. Sample analysis

The method developed as described above was tested by application in the analysis of the contents of five anthraquinones in 11 medicinal plants and 3 preparations. The samples were extracted under the optimum extraction conditions and the extracts were analysed by the proposed HPLC-fluorescence detection method. The mean contents of aloe-emodin, rhein, emodin, chrysophanol and physcion in 11 medicinal plant samples and 3 preparations from 3 parallel determinations are summarized in Table 5.

As shown in Table 5, there is high degree of variability in the individual anthraquinone content in rhubarb from the different geographical locations, which could be due to environmental factors such as altitude, growing conditions or the age of the plants during sample collection. Quantitatively, chrysophanol and physcion were the most abundant anthraquinones in rhubarb and Zhangye rhubarb collected from Shaanxi displayed a higher concen-

Table 1
Calibration results, LOD and LOQ values of the five anthraquinones analysed by HPLC-UV and HPLC-fluorescence detection.

Reference standards	Slope ± S.D.[a]	Intercept ± S.D.	r	Linear range (μg/ml)	LOD (ng/ml)	LOQ (ng/ml)
HPLC with UV detection						
Aloe-emodin	52449 ± 2674	−182.6 ± 17.6	0.9998	0.75–37.50	375	750
Rhein	44455 ± 2044	−36350 ± 1672	0.9995	1.40–28.00	350	1400
Emodin	45523 ± 2458	−12671 ± 671	0.9995	1.25–75.00	156	1250
Chrysophanol	46352 ± 2410	−35477 ± 1703	0.9998	0.28–67.50	141	280
Physcion	2825.1 ± 176	−333 ± 69.71	0.9991	1.40–33.00	140	1400
HPLC with fluorescence detection						
Aloe-emodin	124958 ± 5110	−29198 ± 1394	0.9992	0.09–37.50	3.75	90
Rhein	71635 ± 1741	−17943 ± 822	0.9994	0.07–28.00	3.50	70
Emodin	43669 ± 253	−2243.4 ± 126	0.9998	0.08–75.00	12.50	80
Chrysophanol	125401 ± 157	−63116 ± 1213	0.9994	0.14–56.25	11.20	140
Physcion	36302 ± 773	19973 ± 403	0.9992	0.14–33.00	2.20	140

Construction of calibration curves was performed by least squares linear regression of anthraquinone peak area (y in mVs) on anthraquinone concentration (x in μg/ml).
[a] $n = 3$.

Table 2
Intra- and inter-day precision values for aloe-emodin, rhein, emodin, chrysophanol and physcion.

Compound[a]	Intra-day precision ($n = 5$, mean)						Inter-day precision ($n = 15$, mean)	
	Day 1		Day 2		Day 3		t_R (min)	R.S.D. (%)
	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)		
Aloe-emodin	4.185	0.90	4.125	0	4.120	0.11	4.137	0.60
Rhein	4.996	0.13	4.952	0.26	5.042	0.71	4.997	0.90
Emodin	7.439	0.07	7.479	0.39	7.397	0.45	7.438	0.55
Chrysophanol	9.447	0.60	9.175	1.03	9.716	0.15	9.446	2.86
Physcion	13.905	0.54	13.833	0.12	13.689	0.59	13.809	0.80

Compound	Area	R.S.D. (%)	Area	R.S.D. (%)	Area	R.S.D. (%)	Area	R.S.D. (%)
Aloe-emodin	684,505	1.30	661,716	1.10	661,782	1.28	669,334	2.03
Rhein	403,572	1.29	376,122	1.32	387,229	1.05	388,973	3.55
Emodin	189,998	2.64	175,893	1.59	180,629	2.02	182,173	3.95
Chrysophanol	2,213,283	2.27	2,125,698	2.48	2,146,428	2.00	2,161,803	2.12
Physcion	399,295	1.02	377,581	1.06	382,895	0.96	386,590	2.93

[a] The concentration (μg/ml) of each compound was: aloe-emodin (6.25), rhein (5.84), emodin (10.4), chrysophanol (18.75) and physcion (11.0).

Table 3
Intra- and inter-day precision data for the extraction of anthraquinones from *Rheum palmatum* L. in Shaanxi.

Compound	Intra-day precision ($n = 5$, mean)						Inter-day precision ($n = 15$, mean)	
	Day 1		Day 2		Day 3		Content (mg/g)	R.S.D. (%)
	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)	Content (mg/g)	R.S.D. (%)		
Aloe-emodin	1.01	0.57	1.00	0.68	1.02	0.65	1.01	0.66
Rhein	0.21	1.06	0.21	1.08	0.20	1.04	0.21	1.07
Emodin	1.28	1.48	1.27	1.41	1.30	1.50	1.28	1.29
Chrysophanol	12.12	1.06	12.08	1.08	12.09	1.10	12.10	0.88
Physcion	33.95	0.38	33.97	0.46	33.92	0.50	33.95	0.17

Experimental conditions as in Section 2.4.

Table 4
Recovery data of anthraquinones from the rhubarb collected from Datong, Qinghai.

Compound	Added (mg/g)	Detected (mg/g)[a] ($n = 5$)	Mean recovery (%) ($n = 5$)	R.S.D. (%)
Aloe-emodin	0.13	0.13 ± 0.003	100	2.10
	0.25	0.25 ± 0.002	100	0.8
	0.38	0.36 ± 0.004	94.7	1.2
Rhein	0.44	0.41 ± 0.011	93.2	2.88
	0.88	0.85 ± 0.008	96.6	0.99
	1.32	1.23 ± 0.012	93.2	1.02
Emodin	0.15	0.14 ± 0.002	93.4	1.51
	0.30	0.30 ± 0.003	100	1.01
	0.45	0.45 ± 0.005	100	1.10
Chrysophanol	0.37	0.36 ± 0.007	97.3	1.96
	0.74	0.76 ± 0.007	102.7	1.03
	1.11	1.12 ± 0.012	100.9	1.06
Physcion	1.88	1.93 ± 0.003	102.7	1.49
	2.76	2.78 ± 0.028	100.7	1.04
	4.14	4.30 ± 0.051	103.8	1.21

[a] Calculated by subtracting the total amount after spiking from the amount in the herb before spiking. Data were expressed as means of five experiments.

Table 5
Concentrations of five anthraquinones in 11 medicinal plant materials and 3 preparations ($n = 3$).

No.	Samples	Aloe-emodin (mg/g)	Rhein (mg/g)	Emodin (mg/g)	Chrysophanol (mg/g)	Physcion (mg/g)
1	Rhubarb in Chengdu, Sichung	0.54 ± 0.001	0.42 ± 0.005	0.46 ± 0.002	2.27 ± 0.010	6.26 ± 0.026
2	Rhubarb in Longnanyuangdong, Gansu	0.85 ± 0.002	0.82 ± 0.018	0.69 ± 0.036	1.70 ± 0.017	4.94 ± 0.006
3	Rhubarb in Xihe, Gansu	1.14 ± 0.01	0.85 ± 0.005	0.74 ± 0.065	10.58 ± 0.097	28.64 ± 0.096
4	Rhubarb in Songxian, Henan	0.07 ± 0.000	0.09 ± 0.000	0.80 ± 0.001	2.51 ± 0.007	5.12 ± 0.071
5	Rhubarb in Datong, Qinghai	0.25 ± 0.006	0.88 ± 0.009	0.30 ± 0.003	0.74 ± 0.011	2.76 ± 0.017
6	Rhubarb in Fenyang, Shaanxi	0.66 ± 0.009	1.25 ± 0.028	0.95 ± 0.008	2.82 ± 0.049	5.29 ± 0.021
7	Rhubarb in Taibai, Shaanxi	0.31 ± 0.006	0.23 ± 0.001	0.18 ± 0.001	0.71 ± 0.018	1.02 ± 0.029
8	Rhubarb in Zhangye, Shaanxi	1.01 ± 0.001	0.21 ± 0.002	1.30 ± 0.007	12.1 ± 0.028	34.09 ± 0.163
9	Rhubarb in Danba, Sichuang	0.20 ± 0.003	0.43 ± 0.021	0.22 ± 0.008	0.65 ± 0.008	1.82 ± 0.010
10	*Polygoni multiflori*	0.028 ± 0.00	0.036 ± 0.00	1.05 ± 0.021	0.024 ± 0.005	0.103 ± 0.002
11	*Polygonum cuspidatum*	0.00 ± 0.000	0.32 ± 0.002	9.45 ± 0.087	0.082 ± 0.000	3.25 ± 0.076
12	Sanhuangpian	0.58 ± 0.04	0.58 ± 0.04	1.49 ± 0.06	11.44 ± 0.03	6.09 ± 0.02
13	Huanglian shangqingwan	0.50 ± 0.003	0.82 ± 0.003	1.47 ± 0.025	13.56 ± 0.255	0.61 ± 0.018
14	Marenwan	0.24 ± 0.002	0.34 ± 0.008	0.52 ± 0.005	5.51 ± 0.163	0.051 ± 0.001

tration of anthraquinones than other rhubarb, which can provide evidence for quality evaluation of different rhubarb. The content of the anthraquinones in the analysed sample obtained in this study is not in good agreement with literature [20,36], which could be due to samples from the different locations.

4. Conclusion

A simple, sensitive and confirmatory HPLC-fluorescence detection method for the simultaneous determination of five anthraquinones in *P. cuspidatum*, *Polygonum multiflori*, *Rhubarb* from the nine different locations and three preparations was developed for the first time. The proposed method was found to be accurate and precise. The highest amount of anthraquinones was found in *R. palmatum* L. collected from Shaanxi whilst *P. multiflori* has the least amount of anthraquinones. This method was suitable for use as a tool for routine quality assurance and standardization of the anthraquinone from the raw material and commercially available pharmaceutical preparations containing rhubarb.

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