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Development and Validation of a Densitometric HPTLC Method for Simultaneous Analysis of Wedelolactone and Asiaticoside in a Polyherbal Formulation

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Summary. A sensitive, accurate, and robust high-performance thin-layer chromatographic (HPTLC) method has been established for simultaneous analysis of wedelolactone (WED) and asiaticoside (ASI) in *Eclipta alba* and *Centella asiatica* Linn., respectively. Chromatography was performed on silica gel with toluene–acetone–methanol–formic acid 3.0:2.0:2.0:0.05 (*v*/*v*) as mobile phase. Densitometric scanning at 317 nm for WED and at 530 nm, after derivatisation with 10% methanolic sulphuric acid, for ASI was used. The method was validated in accordance with the guidelines of the International Conference on Harmonization (ICH). R_F values of 0.26 and 0.75 were obtained for ASI and WED, respectively. The linear ranges were 50–250 and 150–550 ng per band for WED and ASI, respectively, with good correlation coefficients (r^2 = 0.999 and 0.9989, respectively). Accuracy was 99.29% and 99.45% for WED and ASI, respectively. The method was found to be precise, robust, and suitable for routine quality-control analysis of plant extracts and polyherbal formulations.

Key Words: wedelolactone, asiaticoside, HPTLC, *Centella asiatica* Linn., *Eclipta alba*

Introduction

Eclipta alba Hassk. (Family: Asteraceae) is a well known herb with marked pharmacological activity, for example anti-inflammatory, anti-hepatotoxic, and anti-mycotoxic effects, which is used in a variety of disease conditions, for example hepatic and splenic disorders and skin diseases [1, 2]. The important constituents of the plants are reported to be the coumestan derivatives wedelolactone (WED) and demethylwedelolactone. Thiophene derivatives, flavonoids, alkaloids, polypeptides, and triterpenoids have also been reported [2].

Analytical methods available for the quantitative estimation of wedelolactone include UV spectrometry [3], spectrofluorimetry [4], and high-performance liquid chromatography (HPLC) [2].

Centella asiatica (Linn) Urb. (Apiaceae), commonly called 'mandukaparani', is an important medicinal plant which finds its use in the Indian traditional system of medicine as a diuretic and anti-pyretic and is used in the treatment of skin inflammations [5]. It is a mild adaptogen, anti-inflammatory, antiulcerogenic, and anxiolytic [6, 7]. The plant contains a variety of constituents including saponins, phytosterols, essential oils, and fatty acids. The active constituent of the plant was reported to be the triterpene asiaticoside (ASI) [8].

Several methods have been reported for identification ASI, for example TLC coupled with high-speed countercurrent chromatography [9, 10], TLC with mass spectroscopy [11], and high performance thin layer chromatography (HPTLC) [12].

These two plants are of major important as key ingredients in many herbal formulations for treatment of a variety of skin disorders. Commercially available formulations are not standardized products, because of a lack of suitable analytical methods. It is essential to standardize herbal formulations with regard to active constituents, in accordance with ICH requirements for safe use. Literature search revealed the only method reported for simultaneous analysis of WED *(Fig. 1)* and ASI *(Fig. 2)* was spectrophotometric analysis of a polyherbal formulation [13]. Spectrophotometric methods are not always best for analysis of herbal markers, because of a lack of accuracy and reproducibility; the need for a large amount of sample also make them inappropriate for herbal standardization. There is, hence, a need for suitable accurate, sensitive, and robust analytical method for simultaneous analysis of WED and ASI as markers in polyherbal formulations containing *Centella asiatica* and *Eclipta alba*.

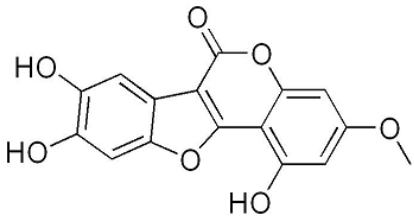

Fig. 1. The structure of wedelolactone

Standardisation of herbal products is difficult, because of the complex nature of the constituents, including many with structural similarity, although substantial developments in analytical techniques, especially chro-

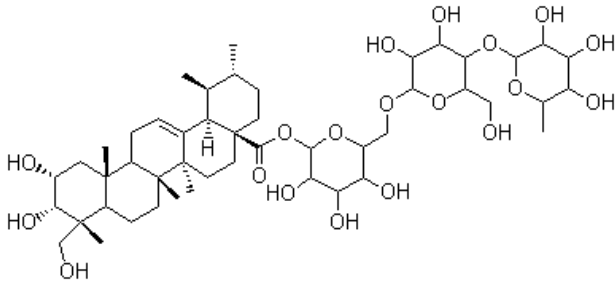

Fig. 2. The structure of asiaticoside

matography, have made this task easier. One chromatographic technique, high-performance thin-layer chromatography (HPTLC), has several advantages in herbal analysis because of its tolerance of crude extracts and the possibility of separating samples in parallel [14]. It is also cost-effective, and enables high sample throughput and in-situ recording of spectra, thus enabling more simple and reliable identification and quantification of components. HPTLC fingerprinting of herbal products furnishes a comprehensive range of data including results from UV and fluorescence scanning, spectra for identity and purity determination, and photo documentation at 254 and 366 nm and in visible light [15]. This technique is approved by the WHO for standardization and quality evaluation of herbal medicine [16].

For these reasons, an attempt was made to establish and validate a sensitive, precise, accurate, and robust densitometric TLC method for simultaneous analysis of WED and ASI and to test its applicability to a commercial formulation.

Experimental

Materials, Reagents, and Solutions

Crude plant material containing ASI and WED was obtained commercially, locally from the Green Pharmacy (Pune, India), and authenticated by the Botanical Survey of India (BSI; Pune, India). Voucher specimens (SLAS-1 and SLWE-1) were deposited in the BSI herbarium. A formulation containing *Centella asiatica* and *Eclipta alba* was procured commercially, locally, and was designated MF. Plant material was washed, dried in the shade, ground in a grinding machine to a coarse powder, and stored at 25°C in an air-tight container.

ASI reference standard (98% (*w*/*w*) purity, by HPLC) was obtained from Indus Biotech (Pune, India) and WED reference standard (98% (*w*/*w*)

purity) was obtained from Sigma–Aldrich (Mumbai, India). Other chemicals and reagents were of analytical grade.

Separate stock solutions (500 μg mL^{-1}) of WED and ASI were prepared by dissolving 5 mg of each, accurately weighed, in methanol and diluting to 10 mL with methanol. Standard solutions were prepared by dilution of the stock solutions with methanol to achieve the concentration ranges 50–250 μg mL^{-1} for WED and 150–550 μg mL^{-1} for ASI. Complete dissolution of the markers was ensured by ultrasonication for 15 min.

HPTLC Analysis

HPTLC was performed on 20 cm × 10 cm plates coated with 0.2 mm layers of with silica gel 60 F_{254} (E. Merck, Darmstadt, Germany; #1.05548). Before use, the plates were prewashed by development with methanol and dried in fume hood. Standard and sample solutions were applied to the plates by means of a Camag (Muttenz, Switzerland) Linomat IV automated spray-on band applicator equipped with a 100-μL syringe (Hamilton, Bonaduz, Switzerland) and operated with the settings: band length 6 mm, application rate 10 s μL^{-1}, distance between bands 6 mm, distance from the plate edge 10 mm, and distance from the bottom of the plate 10 mm.

Plates were developed to 8 cm beyond the origin with toluene–acetone–methanol–formic acid 3.0:2.0:2.0:0.05 (v/v) as mobile phase in a 20 cm × 10 cm × 4 cm twin-trough glass chamber after saturation of the chamber with mobile phase vapour for 20 min (the optimum chamber-saturation time) at room temperature (30.5°C). After development, mobile phase was evaporated from the plate by use of an air-dryer for 10 min.

Densitometric scanning for WED was then performed in absorbance mode at 317 nm using the deuterium lamp as the source of radiation. After scanning for WED the plate was sprayed evenly with 10% methanolic sulphuric acid, incubated at 110°C for 10 min, then scanned for ASI, in reflectance–absorbance mode at 530 nm, using the tungsten lamp as the source of radiation. A Camag model III TLC scanner linked with CATS (V 3.5, Camag) integration software was used. The slit dimensions were 5 mm × 0.45 mm and the scanning speed 10 mm s^{-1}.

For calibration, appropriate volume of each standard solution was applied to a plate to furnish 50–250 ng $band^{-1}$ for WED and 150–550 ng $band^{-1}$ for ASI. Peak area and concentration data were treated by linear least-squares regression analysis.

For each of the four samples 5.0 μL of the extracts were applied in triplicate so that the sample zone scan areas matched the scan areas of the standards in the middle of the calibration ranges given above. The amounts of

ASI and WED were determined from the respective calibration plots obtained by plotting the concentration of standard against peak area.

Validation of the Method

The method was validated for specificity, linearity, precision, accuracy, and robustness in accordance with ICH guidelines [17–19].

The specificity of the method was ascertained by measurement of peak purity. The identity of the bands of the markers in the sample was confirmed by comparison of the R_F and spectra of the bands with those of the standards. The peak purity of the markers was assessed by comparing spectra acquired at the peak start (S), peak apex (M), and peak end (E) positions of the bands.

Linearity was determined by construction of calibration plots and linear least-squares regression analysis as described above. The limits of detection (LOD) and quantification (LOQ) were determined by diluting standard stock solutions of known concentration until the average responses were approximately three (for LOD) or ten (for LOQ) times the responses of the blank. To determine the blank response, six replicate analyses were performed using methanol alone.

Precision, as RSD (%), was studied by determining the repeatability of sample application by measurement of peak areas. The variability of the method was studied by analysis of standard solutions of WED (50, 150, and 250 ng per band) and ASI (150, 350, and 550 ng per band), on the same day (intra-day precision) and on different days (inter-day precision); again the results were expressed as RSD (%).

The robustness of the method was checked by making intentional changes to the experimental conditions. The composition of the mobile phase was changed slightly (formic acid ±0.01 mL), and the amount of mobile phase and the temperature were varied by ±5%. The plates were prewashed with methanol and activated at 60 ± 5°C for 5, 10, or 15 min before chromatography. Time from band application to chromatography and from chromatography to scanning was also varied (0, 30, 60, or 90 min). Robustness was measured for three different amounts (50, 150, 250 ng band^{-1} for WED and 150, 350, 550 ng band^{-1} for ASI).

The accuracy of the method was tested by performing recovery studies by the method of standard addition. The amounts of the markers present in 250 mg crude extracts was determined from the regression equations, then known amount of the standards were added at three levels (80, 100, and 120% of the amount originally present) and the samples were re-analysed. Percentage recovery and average percentage recovery were calculated.

To determine stability in sample solutions, solutions of two different concentrations, 100 and 200 ng per band for WED and 150 and 300 ng per band for ASI, were prepared from stock solution, stored at room temperature for 0.5, 1.0, 2.0, 4.0 and 24 h, and analysed. To measure band stability, two-dimensional chromatography using the same mobile phase was used to reveal any decomposition occurring during application and development. (If decomposition occurs during development, peak(s) of decomposition product(s) should be obtained in both the first and second directions of development [20].)

Analysis of WED and ASI in the Crude Plant Materials

Plant material powder (500 mg) was extracted separately by ultrasonication with methanol (4 × 25 mL, each time for 45 min). The pooled extracts from each sample were concentrated, individually transferred to 10-mL volumetric flasks, and diluted to volume with methanol. The extracts were centrifuged at 5000 rpm for 10 min and the supernatant was filtered through a 0.45-µm filter membrane before chromatographic analysis for drug content. The analysis was repeated six times.

Analysis of Commercial Formulation

To determine the WED and ASI content of tablets, twenty tablets were weighed, the mean weight was determined, and they were finely powdered. A weight of powder equivalent to one tablet was transferred to a 100-mL volumetric flask containing 50 mL methanol, and the mixture was sonicated for 30 min, then diluted to 100 mL with methanol. The resulting solution was centrifuged at 3000 rpm for 15 min and the supernatant was analysed for the markers by applying 10 µL of the filtered solution (2500 µg mL^{-1}) to an HPTLC plate, followed by development and scanning.

Results and Discussion

Initially, the mobile phase was selected on the basis of previous reports of analysis of ASI and WED [12, 21]. A commonly used mobile phase containing toluene, ethyl acetate, and methanol was tried initially. Several modifications were tried on a trial-and-error basis, because the polarity of the two markers is very different. After several trials, addition of acetone was found to enable movement of ASI, whereas a small amount of formic acid had a large effect on

movement of WED. Hence the final, optimum, mobile phase was toluene–acetone–methanol–formic acid 3.0:2.0:2.0:0.05 (*v*/*v*) which afforded desirable R_F values and symmetrical, well-resolved, reproducible peaks with good shape and baseline separation. The R_F values obtained were 0.26 and 0.75 for ASI and WED, respectively. The identities of the bands from the sample extracts and commercial formulation were confirmed by overlapping the densitograms obtained from standards with those obtained from samples *(Figs 3* and *4)*.

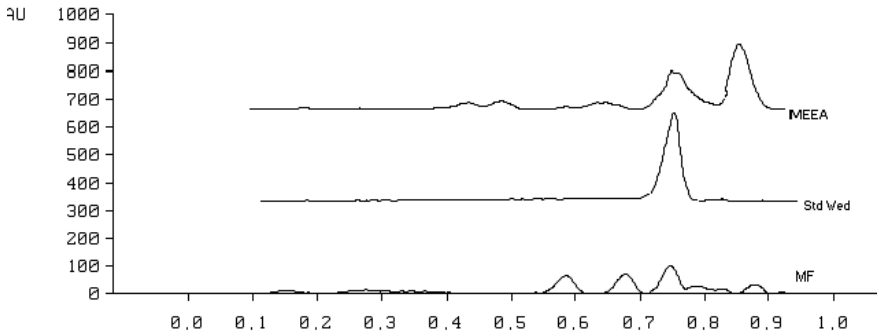

Fig. 3. Overlaid densitograms obtained from analysis of wedelolactone at 317 nm: Std Wed, wedelolactone standard; MF, commercial formulation containing wedelolactone; MEEA, methanolic extract of *Eclipta alba*

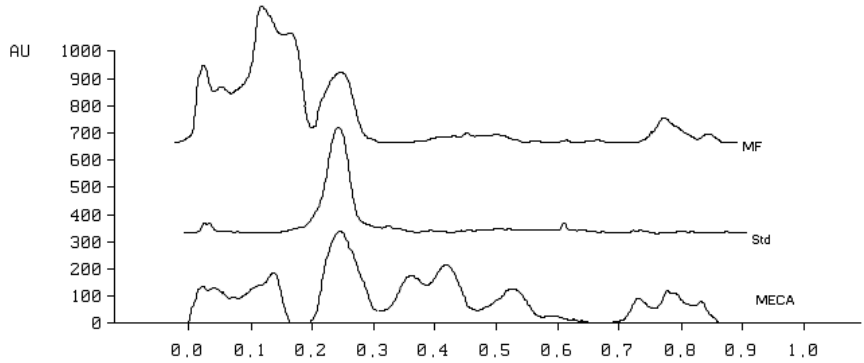

Fig. 4. Overlaid densitograms obtained from analysis of asiaticoside at 530 nm after derivatization with 10% sulphuric acid in methanol: Std: asiaticoside standard; MF, commercial formulation containing asiaticoside; MECA, methanolic extract of C*entella asiatica* Linn.

Validation of the Method

The linear regression data (*Table I*; $n = 6$) revealed good linear relationships between response and amount over the ranges 50–250 ng per band

(r^2 = 0.999) and 150–550 ng per band (r^2 = 0.9989) for WED and ASI, respectively. The limits of detection (LOD) and quantification (LOQ) were 30 and 50 ng band^{-1}, respectively, for WED and 80 and 150 ng band^{-1}, respectively, for ASI. The repeatability of the sample application and measurement of peak area, as RSD, were 1.32 and 1.12%, and 1.62 and 1.87% for WED and ASI, respectively. The results from determination of intermediate precision are listed in *Table II*. The method was found to be precise, because RSD values obtained from repeatability and intermediate precision studies were <2%, as recommended by ICH guidelines.

Table I. Linear regression data for the calibration plots

	WED	ASI
Linear range	50–250 ng per band	150-550 ng per band
r^2 ± SD	0.999	0.9989
Slope ± SD[a]	4.938 ± 0.0922	10.61 ± 0.2017
Intercept ± SD	1229 ± 15.29	137.7 ± 76.15
Confidence limit of slope[b]	4.645–5.231	9.968–11. 25
Confidence limit of intercept[a]	1181–1278	−104.60–380.000
Sy.X[c]	14.58	63.79

[a]p < 0.001 – slope significantly different from zero
[b]95% confidence limit
[c]Standard deviation of residuals from the line

Table II. Precision of the method

Drug	Conc. (μg mL^{-1})	Intra-day		Inter-day	
		Conc. found ± SD	RSD (%)	Conc. found ± SD	RSD (%)
WED	50	49.6 ± 0.390	0.786	49.4 ± 0.286	0.578
	150	195.2 ± 1.284	0.657	197.8 ± 2.001	1.011
	250	293.4 ± 2.015	0.686	298.2 ± 1.017	0.341
ASI	150	149.5 ± 1.107	0.740	148.95 ± 0.638	0.428
	350	349.6 ± 3.817	1.091	348.6 ± 3.510	1.006
	550	556.0 ± 2.046	0.347	553.85 ± 1.802	0.325

Separation of the drug was similar when analyses were performed using a different chromatographic system on different days. To assess robustness, the standard deviation of peak areas was calculated for each change of conditions, and RSD was <2%. The low values of RSD listed in *Table III* indicate the method was robust. In assessment of specificity, the peak purity,

measured by comparing spectra acquired at the peak start (S), apex (M), and end (E) positions of the bands, were r(S,M) = 0.9975 and r(M,E) = 0.9969 for WED, and r(S,M) = 0.9984 and r(M,E) = 0.9974 for ASI. Good correlations (r^2 = 0.999 and r^2 = 0.997) was also obtained between the standard and sample spectra of WED and ASI (*Figs 5* and *6*). The accuracy of the method was checked by the standard addition method. The results are presented in *Table IV*. Recovery of WED and ASI was 99.29% and 99.45%, respectively.

Table III. Robustness of the method

Condition	SD of peak area		RSD (%)[a]	
	WED	ASI	WED	ASI
Mobile phase composition (formic acid ± 0.01 mL)	1.780	1.125	0.753	0.224
Amount of mobile phase (±5%)	1.541	1.360	0.174	0.317
Time from application to chromatography	1.537	1.611	0.421	0.541
Time from chromatography to scanning	1.749	1.042	0.142	0.328
Temperature (±5%)	1.271	1.141	0.241	0.157
Plate pretreatment	1.012	1.122	0.147	0.258

[a]n = 6; average for three concentrations 50, 150, 250 ng band^{-1} for WED and 150, 350, 550 ng band^{-1} for ASI

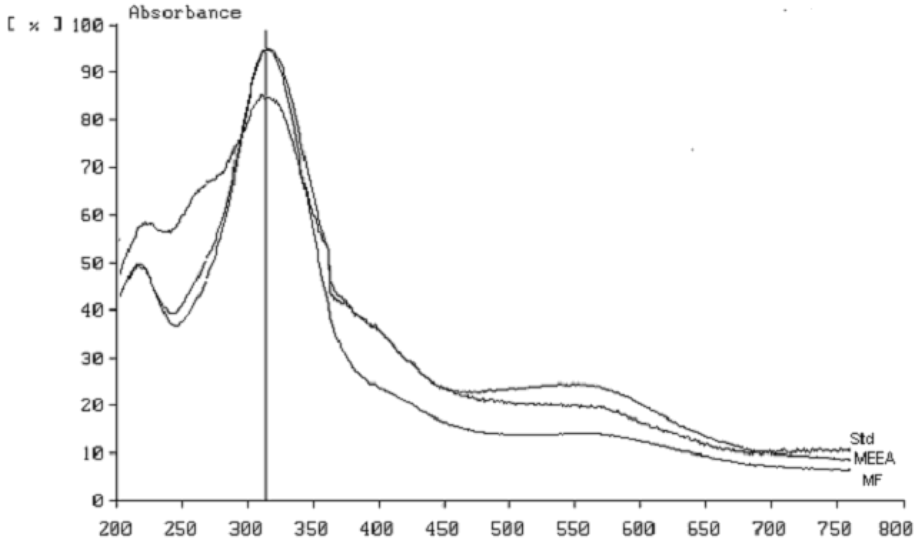

Fig. 5. Overlaid in-situ spectra of wedelolactone obtained from standard and samples: Std, wedelolactone standard; MF, commercial formulation containing wedelolactone; MEEA, methanolic extract of *Eclipta alba*

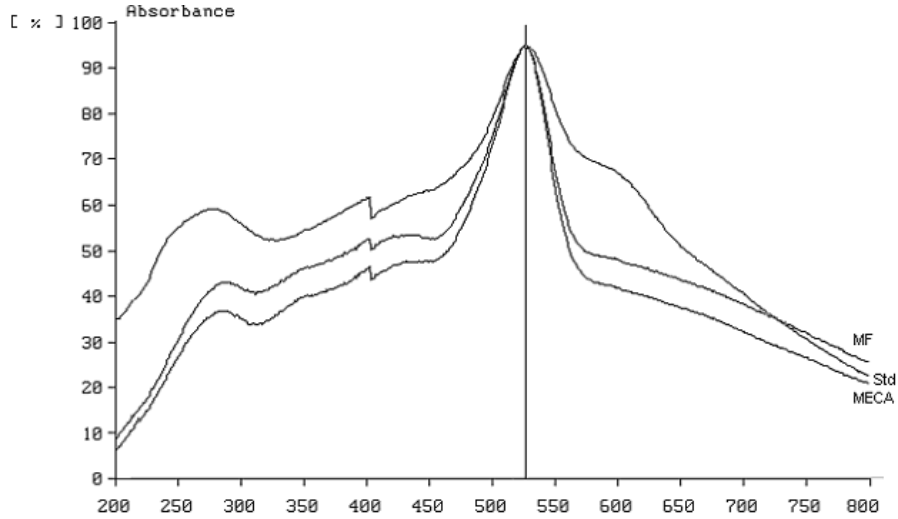

Fig. 6. Overlaid in-situ spectra of asiaticoside obtained from standard and samples: Std, asiaticoside standard; MF, commercial formulation containing asiaticoside; MECA, methanolic extract of *Centella asiatica* Linn.

Table IV. Results from recovery studies

Compound	Amount in sample (μg)	Amount added (μg)	Amount found (μg)[a]	Recovery (%)	Average (%)
WED	4400	3520	7885.33 ± 4.50	99.56 ± 0.05	99.29
	4400	4400	8758.66 ± 9.01	99.53 ± 0.10	
	4400	5280	9580 ± 7.21	98.78 ± 0.28	
ASI	18200	14560	32565.33 ± 5.50	99.34 ± 0.03	99.45
	18200	18200	36250.66 ± 3.05	99.55 ± 0.04	
	18200	21840	39836.66 ± 6.11	99.47 ± 0.02	

[a]Mean ± standard deviation (n = 3)

The average values and the low RSD are indicative of the reliability and reproducibility of the method for tablets.

The absence of additional peaks from chromatograms showed the compounds were stable in sample solutions after storage for 24 hour. Analysis

by two-dimensional chromatography revealed no evidence of decomposition, indicative of band stability.

The results obtained from validation are summarised in *Table V*.

Table V. Summary of validation data

	WED	ASI
Linear range (ng per band)	50–250	150–550
Correlation coefficient	0.999	0.9989
LOD (ng per band)	30	80
LOQ (ng per band)	50	150
Recovery	99.29	99.45
Precision (RSD, %)		
Repeatability of sample application	1.32	1.62
Repeatability of measurement	1.12	1.87
Specificity	0.999	0.997
Robustness	Robust	Robust

Analysis of the Commercial Formulation and Crude Extract

The amounts of WED and ASI in crude extracts and commercial tablets were determined by use of this HPTLC method and the regression equations mentioned above. The WED and ASI content of the crude extract (±RSD) was 1.76 ± 1.005% and 7.28 ± 0.332%, respectively. The amounts in the formulation were 0.662 ± 0.921% and 1.81 ± 0.022%, respectively (*Table VI)*. The smaller amounts in the commercial tablets were possibly indicative of substantial losses during processing. Collection time, origin, and soil conditions may also affect the concentrations of the active constituents, which are, therefore, difficult to predict. Loss of these two ingredients may reduce the therapeutic efficacy of the formulations.

Table VI. Estimation of the drug content of samples

Sample	Drug content (% *w*/*w*)[a]	
	WED	ASI
Crude extract	1.76± 1.005	7.28 ± 0.332
Marketed Formulation	0.662 ± 0.921	1.81 ± 0.022

[a]Mean ± standard deviation (n = 3)

Conclusions

We have established an HPTLC method for simultaneous analysis of wedelolactone and asiaticoside. The method is precise, reproducible, reliable, accurate, and robust, and is therefore suitable for analysis of these markers in polyherbal formulations. The method can be used as a rapid analytical tool for routine analysis to monitor loss or variation of the amounts of these markers in herbal formulations.

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