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Original Article

Rapid validated high performance thin layer chromatography method for simultaneous estimation of mangiferin and scopoletin in *Canscora decussata* (South Indian *Shankhpushpi*) extract

Neeraj K. Sethiya*, Ashish Trivedi, Shri Hari Mishra

Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M. S. University of Baroda, Vadodara, India

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ABSTRACT

Mangiferin (polyphenolic xanthone) and scopoletin (phenolic coumarin) are well-studied biological markers present in *Canscora decussata* (Roxb.) Roem. & Schult., Gentianaceae. The objective set for the present studies is to establish and develop a new, simple, selective, sensitive, and precise high performance thin layer chromatography method for the simultaneous estimation of mangiferin and scopoletin in hydroalcoholic extract of *C. decussata*. The thin layer chromatographic separation of these biomarkers was carried out on aluminum plate pre-coated with silica gel $60F_{254}$, eluted with ethyl acetate:acetic acid:formic acid:water (10:0.5:0.5:1.5). The plate was then dried and densitometric scanning was performed at 254 nm using a Camag TLC scanner III. The system was found to give compact spots for mangiferin (R_F 0.22) and scopoletin (R_F 0.78). A good relationship of linear precision between the concentrations (100–600 ng/spot) and peak areas was obtained with correlation coefficient (r) of 0.9979 (mangiferin) and 0.9962 (scopoletin), respectively. The limits of detection and limit of quantification were determined to be 46 and 94 ng/spot for mangiferin and 31 and 78 ng/spot for scopoletin respectively. The percentage of recovery was found from 99.91 to 99.94% for mangiferin and 99.75 to 99.86% for scopoletin. Results obtained from recovery studies showed excellent reliability and reproducibility of the method. Present communication on validated high performance thin layer chromatography method may provide a new, selective, sensitive, and precise method to estimate mangiferin and scopoletin as phytomarkers in the hydroalcoholic extract of *C. decussata* used in Ayurvedic formulations.

Introduction

Canscora decussata (Roxb.) Roem. & Schult., Gentianaceae, is popularly known as "*Shankhpushpi*" in southern India and is well known for its medicinal values. The fresh juice of the whole plant is used traditionally for the treatment of insanity, epilepsy and cognition debility (Sethiya et al., 2012). This plant is reported to contain several types of phenolics, xanthones, bitter substances, oleoresin, triterpenoids, loliolide, sterols and flavanoids. *C. decussata* has proven its therapeutic potential in acetylcholinesterase inhibition, CNS stimulation, memory enhancement, antioxidant, hypertension, convulsions, tuberculosis, immunomodulation, inflammation, hepatoprotection, spermatogenesis and post-menopausal osteoporosis (Sethiya et al., 2010a). Scopoletin (**1**) and mangiferin (**2**) are recognized as the major active principles largely responsible for the bio-potency of *C. decussata* (Sethiya et al., 2013; Sethiya and Mishra, 2014). Mangiferin is a polyphenolic xanthone, whereas scopoletin is a coumarin, and they are responsible for the antioxidant and memory enhancing activities (Sethiya et al., 2009a). There are some reports on the application of colorimetry (Jubert et al., 2012), spectrophotometry (Krivut et al., 1976), spectrofluorimetry (Nahata and Dixit, 2008; Sethiya et al., 2008), thin layer chromatography (TLC) fingerprinting (Sethiya et al., 2009b), high performance thin layer chromatography (HPTLC), liquid chromatography–mass spectrometry (LC–MS) and high performance liquid chromatography (HPLC) methods (Risner, 1994; Kapadia et al., 2006; Rastogi et al., 2007; Suryawanshi et al., 2007; Xia et al., 2007; Shastry et al., 2009; Upadhyay et al., 2013) for analysis of mangiferin and scopoletin. Among these analytical methods, mangiferin and scopoletin were analyzed either alone or in combination with one or two other secondary plant metabolites, but attempts for simultaneous quantitative analysis of both by HPTLC in a herbal drug and formulation to establish distinct chemical profiling of *C. decussata* from other related herbs are

* Corresponding author.
E-mail: drsethiya@regenbiocorps.com (N.K. Sethiya).

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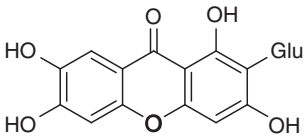
not available. In the last few decades, HPTLC has emerged as an efficient and powerful analytical technique for fingerprinting and quantification of marker compounds in herbal drugs due to its merits of reliability, simplicity, sensitivity, accuracy, suitability for high throughput screening and speed of estimation of the content of phytomarkers in herbs (Mukherjee et al., 2010). HPTLC is currently gaining momentum for marker-based standardization of herbal drugs and is commonly applied for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials and formulated products (Bhatt et al., 2010; Trivedi et al., 2011). Keeping in view the utility of *C. decussata*, and the lack of an appropriate simple TLC method for the simultaneous separation of mangiferin and scopoletin, it is proposed to develop a routine method of analysis for simultaneous qualitative and quantitative estimation of mangiferin and scopoletin in hydroalcoholic extract of *C. decussata* by HPTLC. The proposed method was validated by specificity, range, linearity, accuracy, precision, detection limit, quantitative limit, and robustness according to the ICH guidelines (ICH, 1996, 2005).



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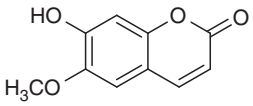

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Material and methods

Plant material

Whole herb of *Canscora decussata* (Roxb.) Roem. & Schult., Gentianaceae, was collected (November, 2011) from the Ninai Ghat (Gujarat, India) and identified by Dr. S.C. Agrawal (Department of Botany, CDRI, Lucknow, India). Voucher specimens of plants (Pharmacy/CD/09-10/13/NS) have been deposited in Herbal Drug Technology Lab, Pharmacy Department, The M. S. University of Baroda, Gujarat (India).

Chemicals and reagents

Mangiferin reference standard (98%) was obtained from Sigma Aldrich (India), and scopoletin reference standard (98%) was obtained as gift sample from Laila Impex Laboratory, Vijayawada (India). Analytical-grade solvents were obtained from E-Merck, Mumbai, India. Pre-coated silica gel $60F_{254}$ TLC plates were purchased from Merck, Darmstadt (Germany).

Isolation and characterization of mangiferin

Extraction and isolation of mangiferin was done according to the method given in our previous studies (Sethiya et al., 2010b). Powdered materials (120 g) were first defatted with petroleum ether (500 ml) and the marc remained after this was dried and extracted with methanol to obtain the methanol extract (yield 4.47%, w/w). The methanol extract of *C. decussata* was subjected to preparative thin layer chromatography using silica gel G TLC plates as adsorbent and n-butanol, acetic acid and water (4:1:2, v/v) as solvent mixture. A yellow amorphous powder was obtained after recrystallization of fraction obtained from preparative chromatography. The isolated compound was analyzed by IR, MS, ^{1}H and ^{13}C NMR and identified by comparison of their spectral data with that in the literature and for reference standard of mangiferin (Chaudhuri and Ghosal, 1971; Kim et al., 2006; Dineshkumar et al., 2010; Luo et al., 2012; Sellamuthu et al., 2012; Bhuvaneswari, 2013).

Isolation and characterization of scopoletin

Isolation of scopoletin was done by reported method of Nahata et al. (2010). The methanol extract of *C. decussata* was subjected to column chromatography using silica gel (80–120 mesh) as adsorbent and chloroform, methanol and toluene (8:1:1) as eluent. The fraction was further purified by preparative chromatography and recrystallized with acetone to get crystalline material. The isolated compound was analyzed by IR, MS, ^{1}H and ^{13}C NMR and identified by comparison of their spectral data with that in the literature and with reference standard of scopoletin (Goodwin and Kavanagh, 1949; Silva et al., 2002; Nahata et al., 2010; Bhatt et al., 2011; Darmawan et al., 2012).

HPTLC

Instrumentation conditions

A Camag (Muttenz, Switzerland) HPTLC system including a Linomat V sample applicator, a Camag twin-trough plate development chamber, Camag TLC Scanner 3 and WinCATS integration software was used. Aluminum backed HPTLC plates 10 cm × 10 cm with 0.2 mm layers of silica gel $60F_{254}$ (E. Merck, Darmstadt, Germany), pre-washed with methanol, were used (Agrawal et al., 2013). The length of the chromatogram run was 8 cm. Subsequent to chromatographic development, TLC plates were dried in air with the help of a TLC plate dryer.

Preparation of standard solutions

A common stock solution (1 mg/ml) of mangiferin and scopoletin was prepared in methanol. It was further diluted (10 ml) with methanol to get stock solution of 100 μg/ml. Aliquots (1–6 ml) of this stock solution were transferred to 10 ml volumetric flasks and diluted to volume with methanol to furnish standard solutions containing 10, 20, 30, 40, 50, and 60 μg/ml.

Preparation of sample

Freshly collected whole herb of *C. decussata* was dried under shade and coarsely powdered. The 10 g of powder materials were extracted with methanol (70%, 50 ml) and after standing for 48 h at room temperature, the hydroalcoholic extract was drained off. This process of extraction at ambient temperature was repeated till exhaustive extraction of mangiferin and scopoletin was accomplished, which was thoroughly monitored by TLC analysis. The hydroalcoholic extract obtained was combined, filtered and evaporated to dryness under reduced pressure in a rotary evaporator at 45 °C and finally dried under high vacuum to furnish the final extract. A measured quantity of the dried hydroalcoholic extract of *C. decussata* was dissolved in methanol and filtered through Whatman qualitative filter paper no. 1, pore size 11 μm (Maidstone, UK), and the volume of the solution was adjusted with methanol in a volumetric flask to obtain a final concentration of 1 mg/ml. This solution was used for the HPTLC analysis.

Chromatographic studies and densitometric scanning

Thin layer chromatographic (TLC) studies were performed using different solvent systems, and finally, ethyl acetate, acetic acid, formic acid and water (10:0.5:0.5:1.5, v/v) were found to be the suitable mobile phase for the proper separation of mangiferin and scopoletin in a single track. These markers were further fingerprinted with hydroalcoholic extract of *C. decussata* to ascertain their presence. The plates were densitometrically scanned (254 nm) with slit dimension of 1 mm × 0.1 mm. After the development, bands in the extracts were identified by matching their R_F values with those obtained as standards.

Calibration of mangiferin and scopoletin and their analysis in C. decussata extract

A 10 μl of each concentration (10–60 μg/ml) of 1:1 (w/v) mixture of mangiferin and scopoletin was applied by means of Linomat V sample applicator to get a final concentration of 100–600 ng/spot. This was plotted against peak area to obtain a calibration plot. Further, 10 μl of the extract solution (10 μg/spot) was applied. After applying the chromatography technique, the amounts of mangiferin and scopoletin present in the extract were determined by means of the calibration plot.

Method validation

Accuracy and precision

The repeatability of the sample application and measurement of peak area were carried out using six replicates of the same spot of mangiferin and scopoletin, which were expressed in terms of percentage relative standard deviation (% RSD) and standard error (SE). The intra-day precision was determined at three different concentration levels of different markers, 100, 300 and 500 ng/spot, six times on the same day, and the inter-day precision was determined at three different concentrations of markers, 100, 300 and 500 ng/spot, six times on five different interval days over a period of 1 week (Kumar et al., 2008).

Robustness of the method

By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different compositions such as ethyl acetate, acetic acid, formic acid and water (9:0.5:0.5:2, v/v) and ethyl acetate, acetic acid, formic acid and water (11:0.5:0.5:1, v/v) were tried and the chromatograms were run. The amount of mobile phase, temperature and relative humidity were varied in the range of +5%. The plates were pre-washed by methanol and activated at 60 ± 5 °C for 2, 5 and 7 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20, 40 and 60 min. The robustness of the method was done at three different concentration levels, 100, 300 and 500 ng/spot (Agrawal et al., 2004).

Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of the response and the slope (*S*) of the calibration curve at levels approaching the LOD according to the formulas: LOD = 3.3 (SD/*S*) and LOQ = 10 (SD/*S*). The SD of the response was determined based on the SD of *y*-intercepts of regression lines (Tuzimski and Bartosiewicz, 2003). Three different levels (50, 100 and 200 ng/spot) of the mixed standard stock solution (25 μg/ml) of mangiferin and scopoletin were prepared and used accordingly. Blank methanol was spotted six times following the same method as explained in the instrument and chromatographic conditions and the signal-to-noise ratio was determined. The LOD and LOQ were experimentally verified by diluting the known concentration of mangiferin and scopoletin until the average responses were approximately 3 or 10 times the standard deviation of the response for six replicate determinations.

Specificity

The specificity of the method was ascertained by analyzing the standard drug and sample. The spots for mangiferin and scopoletin in sample were confirmed by comparing the R_F values and spectra of the spot with that of standard. The peak purity of mangiferin and scopoletin were assessed by comparing the spectra at three different levels, *i.e.*, peak start, peak apex and peak end positions of the spot (Abourashed and Mossa, 2004).

Recovery studies

To study the accuracy and precision of the method, recovery studies were performed by the method of standard addition. The recovery of added standard was studied at two different levels, each being analyzed in a manner similar to that described for the assay (Biringanine et al., 2006; Wagner et al., 2008). The hydroalcoholic extract of *C. decussata* used for recovery studies was pre-analyzed by the developed method as mentioned in the experimental section and found to contain 44 mg of mangiferin and 4.5 mg of scopoletin per gram of extract. Thus, 25 μg of the same pre-analyzed extract contained 880 ng/spot of mangiferin and 90 ng/spot of scopoletin, which was used for the recovery studies. The pre-analyzed extract samples were reanalyzed by spiking with an extra 1:2 ratio i.e. 100 and 200 ng/spot of the respective standard of mangiferin and scopoletin. The experiment was conducted six times to check for the recovery of the mangiferin and scopoletin.

Results and discussion

Isolation and characterization of mangiferin

The isolated yellow amorphous compound showed a single spot and exactly matched with R_F of reference standard of mangiferin in TLC plate. It melts between 273 and 276 °C (for reference standard 274 °C). It gave an apricot green yellow color with 1% ferric chloride reagent, a blue quench in UV at 254 nm and a light yellow fluorescence in UV at 366 nm. It was further characterized as mangiferin by UV with absorption maxima of 315 nm, mass spectroscopy [significant peaks at m/e 404 (M-18) (12); 368 (45); 326 (14); 300 (21); 285 (34); 273 (100)], mixed melting point (Superfit Melting Point Apparatus), UV absorption maxima (GBC-Cintra, Australia) and superimposable FTIR (Shimadzu FTIR 8400S) spectral analysis. The structure of mangiferin was confirmed by comparing physicochemical and spectral data with those in the published literature (Chaudhuri and Ghosal, 1971; Kim et al., 2006; Dineshkumar et al., 2010; Luo et al., 2012; Sellamuthu et al., 2012; Bhuvaneswari, 2013).

Isolation and characterization of scopoletin

The isolated crystalline white compound showed a single spot and exactly matched with R_F of reference standard of scopoletin in TLC plate. It gave blue fluorescence in UV (254 and 366 nm) and melted at 204–206 °C (for reference standard 205 °C). It was further characterized as scopoletin by mixed melting point, UV absorption maxima and superimposable FTIR spectral analysis. The FTIR spectrum of the compound is characteristic of 6-methoxy, 7-hydroxy coumarin, i.e. scopoletin. A superimposable FTIR spectrum with the reference standard proved this fact. The structure of scopoletin was confirmed by comparing physicochemical and spectral data with those in the published literature (Goodwin and Kavanagh, 1949; Silva et al., 2002; Nahata et al., 2010; Bhatt et al., 2011; Darmawan et al., 2012).

TLC fingerprinting of mangiferin and scopoletin in C. decussata extract

The selected mobile phase resolved mangiferin and scopoletin efficiently from other components of *C. decussata extract*. The R_F of mangiferin and scopoletin was found to be 0.22 and 0.78, respectively. The results obtained by fingerprinting of mangiferin and

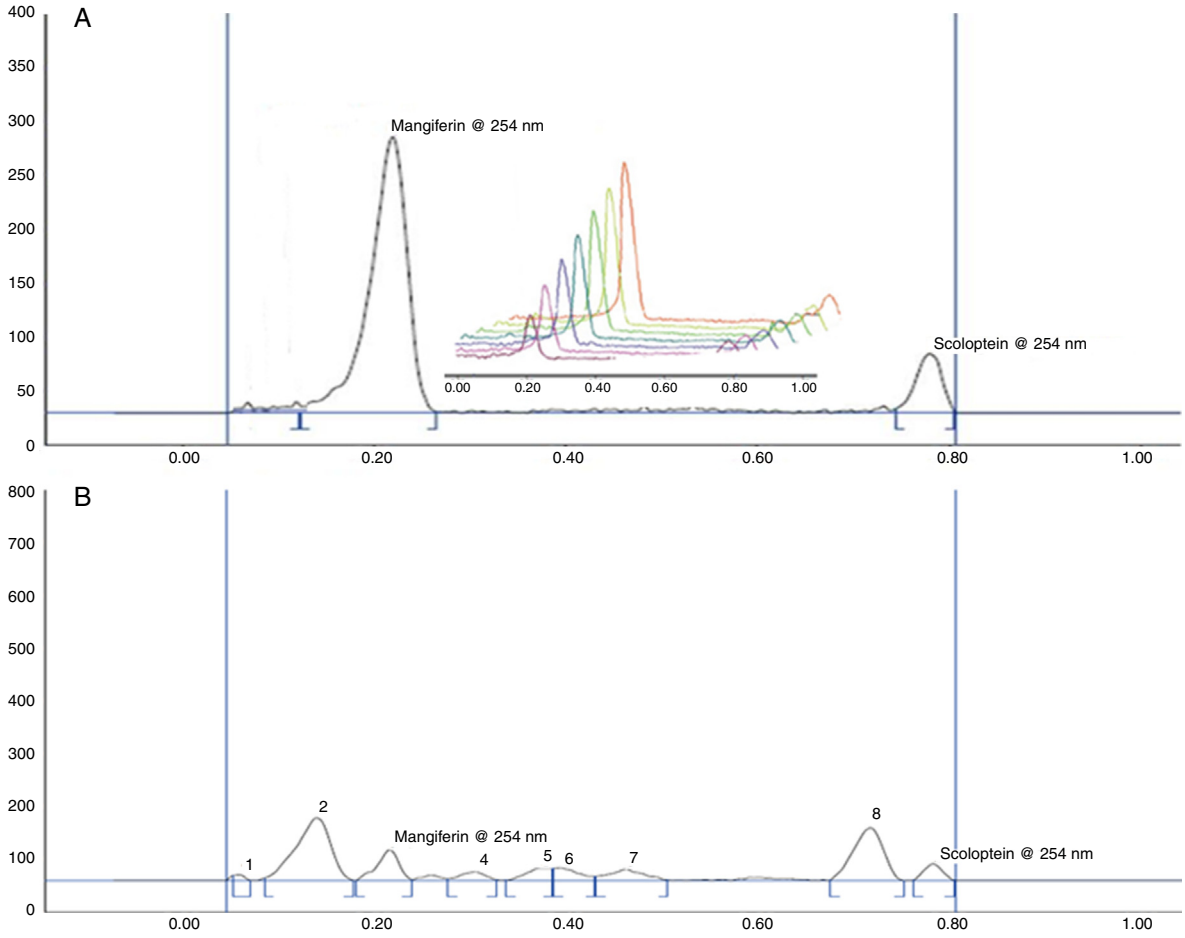

Fig. 1. (A) Chromatogram of combined standards mangiferin and scopoletin (100–600 ng/spot): peak 1 (R_F = 0.22): peak 2 (R_F = 0.78); mobile phase is ethyl acetate:acetic acid:formic acid:water (10:0.5:0.5:1.5, v/v). (B) Chromatogram of *Canscora decussata* extract (10 μg/spot); peaks 1, 2, 4, 6, 7, and 8 belong to other components present in the extract. Peak 3 is mangiferin (R_F = 0.22) and peak 9 is scopoletin (R_F = 0.78); mobile phase is ethyl acetate:acetic acid:formic acid:water (10:0.5:0.5:1.5, v/v).

scopoletin in hydroalcoholic extract of *C. decussata* are shown in Fig. 1.

Calibration of mangiferin and scopoletin and their analysis in C. decussata extract

The calibration plots (Fig. 2) were linear in the range 100–600 ng/spot and the correlation coefficient (r) of 0.9979 (mangiferin) and 0.9962 (scopoletin) were indicative of good linear dependence of peak area on concentration. The calibration curve was represented by the linear equation $y = 486.2 + 0.9846x$ and $y = 522.2 + 4.652x$ for mangiferin and scopoletin, respectively (where y is the response as peak area and x is the concentration). The estimated content of mangiferin was found to be 44 mg/g, whereas the content of scopoletin was found to be 4.5 mg/g of hydroalcoholic extract of *C. decussata*, respectively.

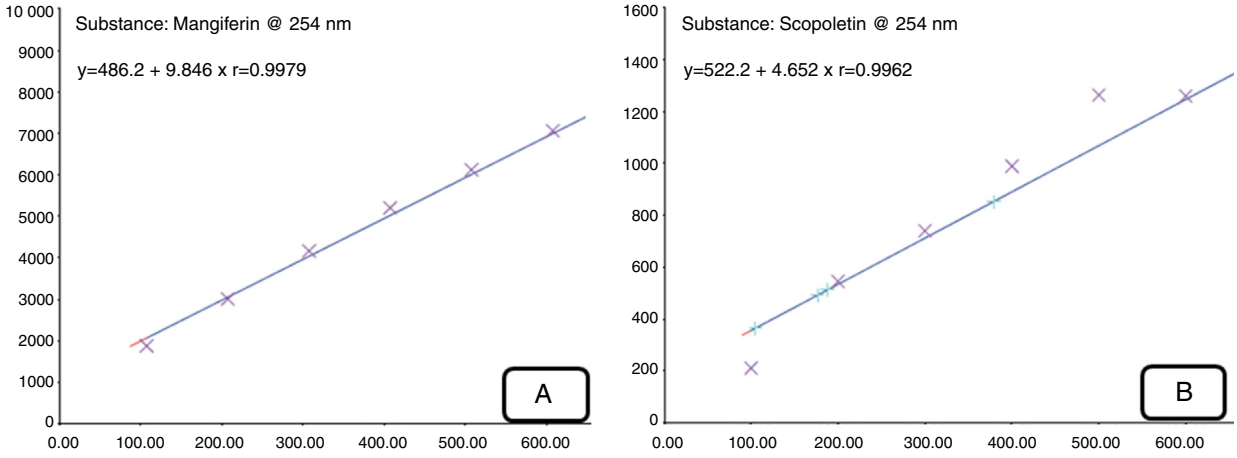

Fig. 2. Calibration plot (A) mangiferin and (B) scopoletin.

Table 1
Intra- and inter-day precision of HPTLC ($n = 6$).

Amount (ng/spot)	Intra-day precision				Inter-day precision			
	Mean area	SD	% RSD	SE	Mean area	SD	% RSD	SE
Mangiferin								
100	4550.6	1.05	0.023	0.61	4467.6	1.05	0.023	0.60
300	5588.4	0.91	0.016	0.52	5483.5	2.47	0.045	1.43
500	6505.3	1.00	0.015	0.57	6423.7	0.60	0.009	0.34
Scopoletin								
100	738.3	0.68	0.092	0.39	688.0	0.50	0.072	0.28
300	988.2	0.78	0.078	0.45	862.0	0.73	0.084	0.42
500	1245.6	1.88	0.150	1.08	1213.9	0.49	0.040	0.28

Table 2
Recovery study of mangiferin and scopoletin ($n = 3$).

Markers	Amount of compound present in plant extract (ng)	Amount of standard added (ng)	Theoretical Amount of standard found in mixture (ng)	Amount of standard found in mixture (ng)[a]	Recovery (%)[a]
Mangiferin	880	100	980	979.20 ± 0.058	99.91 ± 0.006
	880	200	1080	1079.40 ± 0.058	99.94 ± 0.006
Scopoletin	90	100	190	189.54 ± 0.391	99.75 ± 0.206
	90	200	290	289.60 ± 0.289	99.86 ± 0.101

[a] All values are mean ± SEM.

Method validation

Accuracy and precision

The reproducibility of the method was determined by different analysis using the sample from the same homogeneous batch and repeatability was determined by intra-day and inter-day precision. To ascertain the effectiveness of the method, suitability tests were performed on a freshly prepared mixture of standard stock solutions of mangiferin and scopoletin spiked with pre-analyzed hydroalcoholic extract of *C. decussata*. The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and the % RSDs for intra- and inter-day analysis are depicted in Table 1. Intra-day precision (% RSD) on the basis of content of mangiferin and scopoletin was found to be 0.015–0.023 and 0.078–0.15, whereas inter-day precision (% RSD) on the basis of the content was found to be 0.009–0.045 and 0.040–0.084, respectively. So, the TLC densitometric method was found to be precise with RSD for intra-day and for inter-day precision.

Robustness of the method

The SD of peak areas was calculated for each parameter and % RSD was found to be <2%, which shows robustness of the method. This indicates that the proposed method was precise and reproducible.

Limit of detection and quantification

The LOD and LOQ were determined based on the standard deviation of the response of blank and slope estimated from the calibration curve of standard solution of mangiferin and scopoletin. The LOD and LOQ were calculated from the equations LOD = 3.3 (SD/S) and LOQ = 10 (SD/S). The LOD for mangiferin and scopoletin found were 46 and 31 ng/spot, respectively, whereas LOQ were found to be 94 and 78 ng/spot, respectively.

Specificity

The peak purity of mangiferin and scopoletin was assessed by comparing the spectra of standard at peak start, peak apex and peak end positions of the spots, *i.e.*, r (start, middle) = 0.9973 and r (middle, end) = 0.9979. Good correlation ($r = 0.9994$) was also obtained between the standard and sample. It was also observed that the peak of standards of mangiferin and scopoletin did not interfere with the peak of mangiferin and scopoletin in the hydroalcoholic extract of *C. decussata*, therefore the method can be considered specific. The chromatogram of standards mangiferin and scopoletin from the extract was matched in a similar fashion.

Recovery studies

The recovery study was performed by the method of standard addition. The recoveries of added standards (mangiferin and scopoletin) were studied at two different levels. The results of content estimation and recovery studies of mangiferin and scopoletin from hydroalcoholic extract of *C. decussata*, after spiking it with 100 and 200 ng/spot of additional standards, are listed in Table 2. The average percent recoveries at two different levels were found in the range of 99.91–99.94% (mangiferin) and 99.75–99.86% (scopoletin) showing the reliability and reproducibility of the method respectively.

Estimation of the content of marker in Ayurvedic botanicals is of the utmost importance in evaluating the phytochemical entity of the herb. The current study was performed to develop a HPTLC method for the simultaneous determination of mangiferin and scopoletin content in *C. decussata*; one of the botanicals claims used as a source of *Shankhpushpi*. The developed HPTLC method is precise, specific, and accurate for the determination of mangiferin and scopoletin. Statistical analysis proved that the method is inevitable for the analysis of mangiferin and scopoletin. Since the proposed mobile phase effectively resolves mangiferin and scopoletin, the method can be used for routine qualitative, as well as quantitative analysis, of these markers in *C. decussata* extracts used for Ayurvedic formulation. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities which allows the determination of variations in the botanicals of *Shankhpushpi*. It may be extended to study the degradation of mangiferin and scopoletin under different stress conditions as per the recommendations of ICH guidelines.

Authors' contributions

NKS and AT carried out the laboratory work and the data analyses. NKS and SHM wrote the manuscript. SHM designed the study and supervised the laboratory work. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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