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HPTLC Method for Shanzhiside Esters: Simultaneous Quantitative Analysis of Barlerin, Acetylbarlerin and Shanzhiside Methyl Ester in *Barleria* Species

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Abstract

A simple, selective, precise, robust, rapid and reliable high-performance thin layer chromatographic method of analysis for simultaneous determination of acetylbarlerin, barlerin and shanzhiside methyl ester (the major iridoids of *Barleria*) was developed and validated. The three iridoid markers were chromatographed on aluminium base TLC plates precoated with silica gel $60F_{254}$ using chloroform-ethylacetate- methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v) as mobile phase having pH of 5.01. The compounds were quantified by quantitative analysis in absorbance mode at 233 nm. The system gave compact spots for acetylbarlerin, barlerin and shanzhiside methyl ester with optimum resolution (R_f 0.71, 0.61 and 0.50 respectively) in single development. The linear regression analysis data with 95 % confidence limits by the Software package for Statistical Analysis (SPSS software, version 16) for the calibration plots for acetylbarlerin, barlerin and shanzhiside methyl ester showed good linear relationship with r^2=0.997, 0.995 and 0.992 in the concentration range of 1.42-4.95, 0.28-1.67 and 0.60-3.60 µg/spot respectively for the three markers. The mean value of slope and intercept were 0.98 ± 0.013 and -203.14 ± 42.7 for acetylbarlerin, 2.29 ± 0.04 and 249.52 ± 40.5 for barlerin and 0.96 ± 0.021 and 53.82 ± 49.89 for shanzhiside methyl ester respectively. The method was validated for precision, recovery, repeatability and robustness as per the International Conference on Harmonisation (ICH) guidelines. The limit of detection and limit of quantification were 0.07, 0.21; 0.05, 0.15 and 0.05, 0.15 µg/spot respectively for acetylbarlerin, barlerin and shanzhiside methyl ester respectively. Statistical analysis showed the method to be repeatable and selective for the estimation of the three iridoid markers. Since the proposed mobile phase effectively resolves acetylbarlerin, barlerin and shanzhiside methyl ester, the developed method can be successfully applied for the identification and simultaneous quantification of these markers. The method will be of particular use for crude drug/herbal extracts quality testing etc in especially resource constrained countries and laboratories of Asia and Africa where this plant is known to grow in abundance.

Keywords: Iridoids; Shanzhiside esters; Barleria; HPTLC

Introduction

Barleria is a reputed plant of Ayurveda and enjoys high status for its versatile use in several ailments including inflammation [1,2]. The genus *Barleria* of family Acanthaceae comprises of small shrubs or under shrubs, which are distributed in warmer parts of the world. It has about 300 species, of which nearly 32 are reported to occur in India [3] and many are grown as low hedge plants in gardens [4,5]. The important medicinal species are *B. buxifolia* Linn., *B. courtallica* Nees., *B. cristata* Linn., *B. longifolia* Linn., *B. prionitis* Linn., *B. lupulina* Lindl. and *B. strigosa* Willd [4,6].

The well documented traditional uses of *Barleria* employs whole plant for various ailments like catarrhal affections of children which are accompanied by fever and much phlegm, leaves to relieve toothache, roots for boils and glandular swellings, bark for cough and anascara and decoction of the plant in dropsy and as an anti-inflammatory [1,6-14]. Some of the important Ayurvedic formulations of *Barleria* are Sahacaradi taila, Nilikadya taila, Astavarga kvatha curna and Rasnarandadi kvatha curna [14]. The genus *Barleria* has gained importance in recent years for the treatment of various diseases such as liver disorders, diabetes, neurological disorders, immunodeficiency, inflammation, ulcers, HSV-2 viral diseases, etc. [6,7,11].

The genus is reported to contain iridoids, anthraquinones, flavonoids, sterols, and fatty acids [14]. Iridoids constitute the major class of compounds isolated from *Barleria* and important bioactive iridoids are acetylbarlerin, barlerin and shanzhiside methyl ester [14]. Besides *Barleria*, these iridoids are also reported to be present in *Gentian* [15], *Melampyrum* [15,16], *Morinda* [17], *Phlomis* [15,18], *Stilbe* [15] and *Valerian* [15,19] species etc. Principally, iridoids are cyclopentan-[c]-pyran monoterpenoids [15]. There is a continuing interest in iridoids as many of them have shown a host of biological and pharmacological activities like cardiovascular, antihepatotoxic, choleretic, hypoglycemic, analgesic, anti-inflammatory, antimutagenic, antispasmodic, antitumor, antiviral, immunomodulator and purgative properties [17,19,20]. The broad diversity of biological activities exhibited by the iridoids has always interested the chemists to explore new methods of isolation and determination. Various techniques for e.g. HPLC [21,22], capillary HPLC [23]/HPLC [24,25] coupled with Photodiode Array Detector and Electron Spray Ionisation Mass Spectrometric (DAD-ESI-MS), densitometric determination [26] and micellar electrokinetic capillary chromatography [27] are employed to analyse various groups of iridoids. After a bibliographic search, it was found that HPLC is the most common reported method for the

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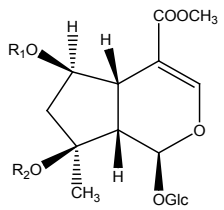
analysis of shanzhiside esters [21,22,24]. To our knowledge, three reports are known where analysis of *B. prionitis* has been done by TLC densitometery using the solvent system chloroform-methanol (8:2, v/v) [28-30]. The first report was from our laboratory and this solvent system was developed for the estimation of single iridoid marker barlerin and the method was not validated. Subsequently in another publication 10-50 times high per cent content of barlerin (10.03 ± 1.69) along with exceptionally high content of shanzhiside methyl ester (21.55 ± 2.40 per cent) has been reported employing this solvent system. This indicated that the earlier reported method is not specific when more than one marker is desired to be quantified simultaneously. Therefore, in the present study, a battery of solvent systems were tested with an aim (i) to resolve the underlying spots interfering with the markers, (ii) to validate the developed method as per ICH guidelines for the drug quality assurance and (iii) to obtain the most accurate values of per cent content of the three iridoid markers. Hence in this study, a validated HPTLC method was developed employing a unique combination of solvents and was used as a rapid method of analysis for the simultaneous determination of shanzhiside iridoids in *Barleria* species. The proposed method was successfully used for the simultaneous estimation of acetylbarlerin, barlerin and shanzhiside methyl ester in at least 5 different *Barleria* species and varieties *viz.*, *B. prionitis* Linn., *B. lupulina* Lindl, *B. cristata* var. *dichotoma*, *B. cristata* (pink flower variety) and *B. cristata* (blue flower variety) without any other component interfering in the analysis. It is expected that the developed method could easily and successfully be applied with precise results for the estimation of shanzhiside esters in other *Barleria* species/related plant species, herbal extracts, formulations etc.

Experimental

Materials

Standard acetylbarlerin (purity 99.04%), barlerin (purity 99.31%) and shanzhiside methyl ester (purity 99.83%) were isolated in the laboratory from *B. cristata* Linn. whole plant, procured during February to March 2009 from the Medicinal Plants Garden of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, using column chromatography, PTLC and flash chromatography as the preferred isolation techniques [31]. The authenticity of the samples was duly confirmed by National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (Ref. NISCAIR/RHMD/Consult/-2008-09/1127/158) and voucher specimens of the same have been deposited in the Museum-cum-Herbarium of University Institute of Pharmaceutical Sciences-Centre of Advanced Study, Panjab University, Chandigarh, India, under the voucher numbers 1460, 1461,1462, 1463 and 1464 for *B. prionitis, B. cristata* var. *dichotoma* (white flower variety), *B. cristata* (pink flower variety), *B. cristata* (blue flower variety) and *B. lupulina* respectively. *B. prionitis* was collected from August to September 2008 from the Medicinal Plants Garden of University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh. All the chemicals and reagents used were of analytical grade procured from E. Merck chemicals, India.



	R_1	R_2
Acetylbarlerin	Ac	Ac
Barlerin	H	Ac
Shanzhiside methyl ester	H	H

Figure 1: Structures of shanzhiside esters.

Isolation and characterization of shanzhiside esters

The three iridoids (Figure 1) were isolated from the whole plant of *B. cristata* using various chromatographic methods and were duly characterized with the help of different spectroscopic techniques like UV, IR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS. These were identified as acetylbarlerin, barlerin and shanzhiside methyl ester.

Characterization of acetylbarlerin: $UV_{\lambda max}$ = 233 nm; ^{1}H NMR (400 MHz, CD_3OD) (δ) : 1.45 (H-10) [*s*], 1.92 ($OCOOCH_3$) [*s*], 1.95 ($OCOOCH_3$) [*s*], 1.99 (H-7) [*d, J* = 5.52 Hz], 2.03 (H-7) [*d, J* = 5.56 Hz], 2.84 (H-9) [*dd*, J=8.56 and 3.44 Hz], 3.09 (H-5) [*dd, J* = 7.98 and 1.12 Hz], 3.30 - 3.60 (H-2' - H-6') [*m*], 3.58 ($COOCH_3$) [*s*], 4.77 (H-I ') [*s*], 5.16 (H-6) [*d, J*= 5.56 Hz], 5.74 (H-I) [*d, J* = 3.48 Hz], 7.41 (H-3) [*d, J* = 1.48 Hz]; ^{13}C NMR (75 MHz, CD_3OD) (δ) : 93.96 (C_1), 153.1 1 (C_2), 107.10 (C_4), 38.52 (C_5), 77.55 (C_6) , 43.5.9 (C_7), 88.27 (C_8), 48.87 (C_9), 29.39 (C_{10}), 20.88 (CH_3), 20.43 (CH_3), 50.58 (CH_3O), 167.17(CO), 171.62 (CO), 171.00 (CO), 98.84 (C_1'), 73.27 (C_2'), 76.52 (C_3'), 76.96 (C_5'), 70.23 (C_1') and 61.55 (C_6').

Characterization of barlerin: $UV_{\lambda max}$ = 233 nm; ^{1}H NMR (400 MHz, CD_3OD) (δ) : 1 .41 (H-10) [*s*], 1.90 (H-7) [*d, J* = 5.36 Hz], 1.93 (H-7) [*d, J* = 5.4 Hz], 2.08 ($OCOOCH_3$) [*s*], 2.09 (H-9) [*dd, J* = 15.92 and 2.9 Hz], 3.08 (H-5) [*dd, J* = 8.5 and 0.92 Hz], 3.20 - 4.42 (H-2' - H-6') [*m*], 3.62 ($COOCH_3$) [*s*], 3.80(H-6) [*dd, J* = 12.08 and 6.04 Hz], 4.8 (H-1') [s], 5.82 (H-I) [*d, J* = 2.32 Hz], 7.35 (H-3) [*d, J* = 1.36 Hz]; ^{13}C - NMR (75 MHz, CD_3OD) (δ) : 94.29 (C_1), 152.40 (C_2), 108.37 (C_4), 40.85 (C_5), 74.53 (C_6), 46.29 (C_7), 88.42 (C_8), 48.49 (C_9), 29.41 (C_{10}), 20.8 (CH_3), 50.57 (CH_3O), 167.75 (CO), 172.02 (CO), 98.88 (C_1'), 73.24 (C_2'), 76.54 (C_3'), 76.88 (C_5'), 70.18 (C_4') and 61.47 (C_6').

Characterization of shanzhiside methyl ester: $UV_{\lambda max}$ = 233 nm; m.p. = 98-100°C; ^{1}H NMR (400 MHz, CD_3OD) (δ) : 1.27 (H-10) [s], 1.84 (H-7) [*dd, J* = 7.24 and 6 Hz], 2.12 (11-7) [*dd, J* = 6.84 and 6.4 Hz], 2.63 (H-9) [*dd, J* = 7.64 and 2.54 Hz], 3.01 (H-5) [*dd, J* = 7.04 and 3.06 Hz], 3.20 - 3.66 (H-2' - H-6') [m], 3.75 ($COOCH_3$) [*s*], 4.05 (H-6) [m], 4.97 (H-I') [*s*], 5.59 (H-I) [*d, J* = 2.68 Hz], 7.42 (H-3) [*s*]; ^{13}C - NMR (75 MHz, CD_3OD) (δ) : 93.44 (C_1), 151 .44 (C_2), 110.04 (C_4), 39.97 (C_5), 76.06 (C_6), 47.83 (C_7), δ 76.95 (C_8), 50.34 (C_9), 23.31 (C_{10}), 50.55 (CH_3O), 168.42 (CO), 98.41 (C_1'), δ 73.23 (C_2'), 76.56 (C_3'), 76.95 (C_5'), 70.22 (C_4') and 61.43 (C_6').

HPTLC instrumentation

The samples were applied as spots keeping a distance of 10 mm between the spots, on precoated silica gel G aluminium plate $60F_{254}$ (20 cm × 10 cm, 0.2 mm thickness; Cat. no. 1.05554.0007, E. Merck, Darmstadt Germany, Ltd.) using Linomat 5 (Camag, Switzerland). The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The mobile phase consisted of chloroform-ethyl acetate- methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v) and 20 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm × 20 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with mobile phase. The optimum chamber saturation time for mobile phase was 10 min at room temperature (25°C ± 2) and a relative humidity of 60% ± 5. The development distance

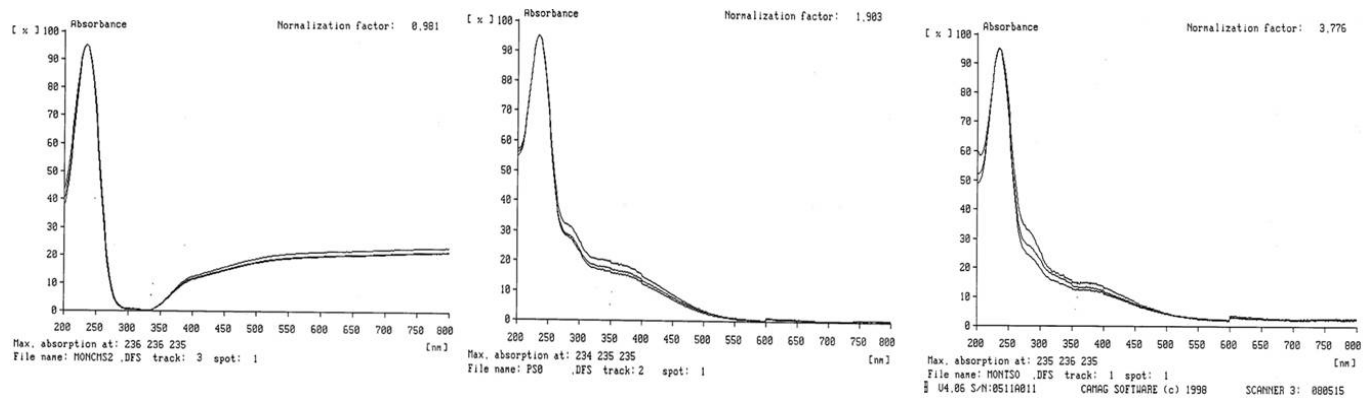


Figure 2: UV spectrum of: A. acetylbarlerin; B. barlerin; C. shanzhiside methyl ester.

was kept at 80 mm. Subsequent to the development, TLC plates were dried in a current of air using an air dryer. Densitometry scanning was performed on Camag TLC scanner 3 in the reflectance absorbance mode at 233 nm with slit dimension of 6 × 0.45 mm and operated by WINCATS software (Camag, version 4.06). Concentrations of the compounds chromatographed were determined from the intensity of diffusely reflected light and evaluation was done *via* peak areas with linear regression. The spectra of three iridoid markers developed in chloroform-ethyl acetate-methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v, pH - 5.01) was taken at peak start, peak apex and peak end of respective spot as a reference for comparison in the test samples and is shown in Figure 2.

Calibration curves of acetylbarlerin, barlerin and shanzhiside methyl ester

The standard solutions of acetylbarlerin (14.15 mg/10 ml), barlerin (5.55 mg/10 ml) and shanzhiside methyl ester (12 mg/10 ml) were prepared in methanol in a 10 ml volumetric flask to give concentration of acetylbarlerin (1.42 µg/µl), barlerin (0.56 µg/µl) and shanzhiside methyl ester (1.20 µg/µl). The standard solutions were spotted on the TLC plate in triplicate in increasing volumes of 1.0 to 3.5 µl (acetylbarlerin) and 0.5 to 3.0 µl (barlerin and shanzhiside methyl ester), in increments of 0.5 µl, to obtain final concentration range of 1.42-4.95 µg/spot for acetylbarlerin, 0.28-1.67 µg/spot for barlerin and 0.60-3.60 µg/spot for shanzhiside methyl ester. Each concentration was spotted three times on the TLC plate.

Method validation

Validation of the developed HPTLC method was carried out as per the International Conference on Harmonization (ICH, 1996) guidelines for specificity, sensitivity, accuracy, precision, repeatability and robustness.

Specificity: The specificity of the developed method was established by analysing various extracts and partitioned fractions of mother extract of different *Barleria* species *viz.*, *B. cristata*, *B. prionitis*, and *B. lupulina* containing three bioactive iridoid markers namely acetylbarlerin, barlerin and shanzhiside methyl ester. The spots of the three iridoids in extracts and fractions were confirmed by comparing the relative front (R_f) values and UV spectrum in test samples with spots of the standard markers at three different levels of peak start, peak apex and peak end position.

Sensitivity: The values of limit of detection (LOD) and limit of quantification (LOQ) were ascertained using diluted and known concentrations of all the three markers in replicate and comparing the results with a blank sample of methanol spotted six times following same method as mentioned in HPTLC instrumentation. Sensitivity of the method was determined with respect to limit of detection (LOD) and limit of quantification (LOQ). Series of concentration of acetylbarlerin (0.02-2.83 µg/spot), barlerin (0.03-1.11 µg/spot) and shanzhiside methyl ester (0.03-1.20 µg/spot) were applied on plate and analysed to determine LOD and LOQ.

Linearity: Linearity was determined using six concentration levels with calibration curves plotted over a wide concentration range of 1.42-4.95 µg/spot for acetylbarlerin, 0.28-1.67 µg/spot for barlerin and 0.60-3.60 µg/spot for shanzhiside methyl ester, respectively. The calibration curves were developed by plotting peak area versus concentration (n=6) with the help of WINCATS software.

Accuracy: Accuracy of the method was evaluated by carrying the recovery study at three levels. The recovery experiments were performed by spiking the sample of whole plant of *B. prionitis* with three different amounts of each marker at 50%, 100% and 150% of their pre-analysed content. The experiment was conducted in triplicate.

Precision: Precision of the developed method was evaluated in terms of intra-day and inter-day precision. Intra-day precision was established by analysing analytical concentrations of acetylbarlerin (2.12 µg/spot), barlerin (0.56 µg/spot) and shanzhiside methyl ester (1.20 µg/spot) in triplicate, for three times on the same day (Instrumental and manual precision). Inter-day precision was determined by analyzing three times measurement of same concentrations of acetylbarlerin, barlerin and shanzhiside methyl ester over a period of three days (n=3) for both system and manual precision. The intra-day and inter-day variation for the three iridoids were expressed in terms of the mean and % RSD (Relative Standard Deviation) values of the peak areas.

Repeatability: Repeatability of measurement of peak area was determined by analysing the spot of acetylbarlerin (2.12 µg/spot), barlerin (0.56 µg/spot) and shanzhiside methyl ester (1.20 µg/spot) of the calibration curve, nine times without changing the position of the plate.

Robustness: Small changes in mobile phase composition, its volume, chamber saturation time and slight change in the solvent migration distance were introduced to study their effect on the results. Robustness of the method was determined from triplicate measurement at an analytical concentration of 2.12 µg/spot of acetylbarlerin, 0.56 µg/

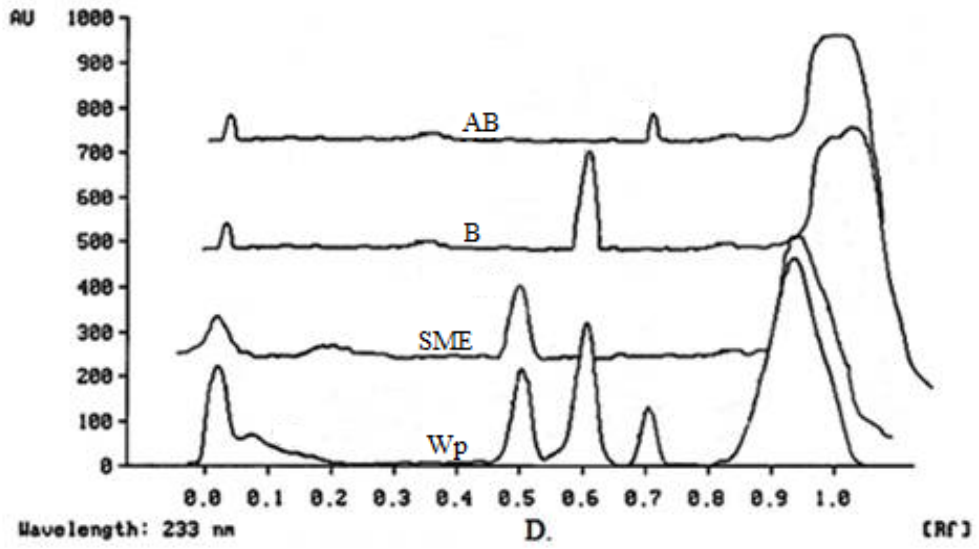


Figure 3: TLC densitometric scan of methanolic extract of *B. prionitis* using chloroform-ethyl acetate-methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v); Wp = whole plant; AB = acetylbarlerin; B = barlerin; SME = shanzhiside methyl ester.

spot of barlerin and 1.20 µg/spot of shanzhiside methyl ester and was represented as mean and % RSD of peak area.

Statistical analysis: The linear regression analysis data was statistically analysed using Software package for Statistical Analysis (SPSS software, version 16) and the mean and % RSD using Microsoft Excel 2010.

Analysis of shanzhiside esters in *Barleria* species/varieties

The test solutions were prepared by extracting accurately weighed 5 g of coarsely powdered material of whole plant, leaf, stem and root of *B. prionitis*, *B. cristata* var. *dichotoma*, *B. cristata* (blue flower variety), *B. cristata* (pink flower variety) and *B. lupulina* with methanol for 4 h using Soxhlet apparatus. The extracts were filtered and concentrated under reduced pressure. The final volume was adjusted to 50 ml with methanol. However, in species with too low content of iridoids, the quantities were further concentrated accordingly.

Another 5 g of coarsely powdered material of whole plant of *B. prionitis* and *B. cristata* var. *dichotoma* was similarly extracted with 50 ml methanol for 4 h in Soxhlet, filtered and the solvent was removed under reduced pressure. The residue was suspended in 15 ml of distilled water and successively partitioned with hexane, chloroform, ethyl acetate and saturated butanol (each 7 ml × 3). The three washings of each solvent were combined, filtered and concentrated under reduced pressure, and the final volume of residue was adjusted to 5 ml with the respective solvent. The solutions were either diluted or concentrated further and an appropriate quantity of each test solution was applied in triplicate on a precoated TLC plate and the plate was developed in solvent system chloroform-ethyl acetate-methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v, pH - 5.01) up to a distance of 80 mm and scanned following the same procedure as used for the preparation of the standard plot. The average AUC of the peak corresponding to acetylbarlerin, barlerin and shanzhiside methyl ester was noted for each test sample and their concentration was calculated from the respective standard plots.

Results and Discussion

High-performance thin layer chromatography (HPTLC) is increasingly gaining popularity as one of the accepted techniques of analysis of pharmaceuticals and herbal drugs. With the advancements in instrumentation of this technique, it is now possible to accomplish more precise and reliable results comparable to HPLC. A validated HPTLC method of analysis was developed for simultaneous estimation of three iridoids, the shanzhiside esters in *Barleria* species. The TLC procedure was first optimized using a range of solvent systems with varying compositions of chloroform-methanol; ethyl acetate-methanol-formic acid; ethyl acetate-methanol-acetic acid; chloroform-ethyl acetate-methanol-acetic acid etc. The reported solvent system of chloroform-methanol (8:2, v/v) gave variable results ranging from a low barlerin content of 0.18% to extremely high content of 10.03%. This clearly indicated the possibility of interference from other components of extract in the analysis as the exceedingly high values could be the result of other underlying spots. Most of the listed solvent systems gave either poor or no resolution of the three markers. The optimum solvent system that gave compact spots with most selective resolution was chloroform-ethyl acetate-methanol-acetic acid (3.0:3.0:3.0:1.0, v/v/v/v, pH - 5.01) with chamber saturation of 10 min at 25°C and solvent migration distance of 80 mm. Further, the desired resolution was obtained with single development having significantly different R_f values of 0.50, 0.61 and 0.71for shanzhiside methyl ester, barlerin and acetylbarlerin and none of the desired components had any underlying or interfering spots (Figure 3). The optimum resolution was obtained using unmodified silica layer as stationary phase on precoated plates and slit-scanning densitometry with UV-Visible light as the detection technique. The iridoid markers namely acetylbarlerin, barlerin and shanzhiside methyl ester were examined directly on the stationary layer. It did not involve any pretreatment or derivatization and optical densitometric scanning was used for the *in situ* measurement of compounds directly on the layer.

Under the experimental conditions employed, the minimum amount of markers that could be detected was found to be 0.07 µg for acetylbarlerin and 0.05 µg for both barlerin and shanzhiside methyl ester; the lowest quantifiable amount of markers was 0.21 µg/spot for acetylbarlerin, 0.15 µg/spot for barlerin and shanzhiside methyl ester (Table 1). The method was confirmed to be particularly specific as

Parameter	Acetylbarlerin	Barlerin	Shanzhiside methyl ester
Limit of detection (LOD) (µg ± SD)	0.07 ± 3.10	0.05 ± 2.69	0.05 ± 2.08
Limit of quantification (LOQ) (µg ± SD)	0.21 ± 1.5	0.15 ± 2.8	0.15 ± 3.1
Linearity range (µg/spot)	1.42 – 4.95	0.28 – 1.67	0.60 – 3.60
r^2	0.997	0.995	0.992
Slope ± SD	0.98 ± 0.013	2.29 ± 0.04	0.96 ± 0.021
95 % Confidence interval of slope	0.956 - 1.009	2.209 - 2.368	0.917 - 1.007
Intercept ± SD	-203.14 ± 42.7	249.52 ± 40.5	53.82 ± 49.89
95 % Confidence interval of intercept	-293.60- (-112.68)	163.73 - 335.32	-51.94 - 159.58

Table 1: Limit of detection, quantification and linear regression data for the calibration curves of shanzhiside esters.

Original marker content in the sample (µg)	Level of spiking in %	Theoretical concentration after spiking (µg ± SD)	Total amount of drug analysed in spiked sample (µg ± SD)	% Average recovery ± SD	% RSD
Acetylbarlerin (0.35)	50 100 150	0.53 ± 23.8 0.70 ± 15.1 0.88 ± 4.5	0.50 ± 7.3 0.68 ± 27.0 0.84 ± 39.3	95 ± 3.10 97 ± 4.02 96 ± 4.46	3.25 4.15 4.65
Barlerin (0.81)	50 100 150	1.21 ± 19 1.62 ± 29 2.02 ± 37	1.19 ± 14 1.59 ± 55 1.99 ± 32	99 ± 2.62 98 ± 2.36 99 ± 1.04	2.65 2.41 1.05
Shanzhiside methyl ester (1.36)	50 100 150	2.04 ± 22 2.72 ± 28 3.39 ± 81	2.01 ± 20 2.66 ± 115 3.35 ± 146	99 ± 1.29 98 ± 3.77 99 ± 1.95	1.30 3.86 1.98

Table 2: Recovery studies.

Parameter	Average amount detected (µg ± SD), % RSD		
	Acetylbarlerin	Barlerin	Shanzhiside methyl ester
Reproducibility (*n*=9)	2.12 ± 11, 0.51	0.56 ± 11.0, 1.98	1.20 ± 2, 0.15
Manual precision Intra-day Inter-day	 2.12 ± 33, 1.56 2.13 ± 6, 0.29	 0.55 ± 17.0, 3.03 0.55 ± 9.7, 1.75	 1.20 ± 20, 2.04 1.201 ± 14, 1.15
Instrumental precision Intra-day Inter-day	 2.12 ± 40, 1.89 2.12 ± 31, 1.47	 0.56 ± 13.8, 2.49 0.56 ± 5.91, 1.06	 1.19 ± 28, 2.34 1.20 ± 28, 2.37

Table 3: Reproducibility and precision studies.

no interference of matrix was observed for any of the markers. The method was also linear in a concentration range of 1.42-4.95 µg/spot for acetylbarlerin, 0.28-1.67 µg/spot for barlerin and 0.60-3.60 µg/spot for shanzhiside methyl ester (n=6), with respect to peak area. The linear regression data shown in (Table 1) using SPSS software with 95% confidence limits revealed a good linear relationship over the concentration range studied demonstrating its suitability for analysis. Results of accuracy from recovery studies as shown in (Table 2), showed the method accuracy in the desired range. The results of reproducibilty, intra-day and inter-day precision are shown in (Table 3) and robustness of the method for acetylbarlerin, barlerin and shanzhiside methyl ester is shown in (Table 4) respectively.

Analysis of the selected iridoids in Barleria species/varieties - Barleria should be italicised

The quantitative analysis of the three selected markers, *viz.*, acetylbarlerin, barlerin and shanzhiside methyl ester was done in various *Barleria* species and extracts using the technique of TLC-densitometry. Comparative chromatograms (Figure 4) of various species and varieties of *Barleria* were prepared and analyzed for the per cent content of three selected iridoids. The details are given in (Table 5). The estimation of the three iridoids was accomplished in all the parts and partitioned fractions for two abundantly growing species of *Barleria* i.e. *B. prionitis* and *B. cristata* var. *dichotoma*. In case of *B. lupulina*, the quantitative estimation was restricted only to different parts due to limited quantity of the available material. There was no interference in analysis of the three shanzhiside esters from any other components present in the matrix. The three iridoids resolve in the chromatogram at significantly different R_f values. The highest content of acetylbarlerin was found to be 3.82%, w/w in leaf of *B. lupulina*, that of barlerin was 0.97%, w/w in stem of *B. prionitis* and shanzhiside methyl ester was 2.62% w/w in leaf of *B. prionitis*. The iridoids were found to be absent in two varieties of *B. cristata* (pink and blue flower variety) as indicated by flat chromatogram in (Figure 4). Statistical evaluation of the results was performed with respect to accuracy and precision using SPSS software (version 16) at 95% confidence limit. The low % RSD value indicated the suitability of this method for routine analysis of shanzhiside methyl esters.

The HPTLC method for simultaneous analysis of three shanzhiside esters has been developed and validated. As of now, HPLC is the most common reported method for the analysis of these esters. The two reports on HPTLC technique employ estimation of single or two markers, but the methods are not validated. Moreover the findings of Ghule et al. [29] report very high content of barlerin and shanzhiside methyl ester indicating interference from other components and hence cannot be considered for analysis. The HPTLC method in present study may provide a suitable alternative for the reported HPLC technique by providing fast and reliable analysis. The method will be of special interest in resource constrained countries and laboratories of Asia and Africa, where large number of species and varieties of this plant are known to grow in abundance.

Parameter	Amount spotted (µg)	Average amount detected (µg ± SD)	% RSD
(A) Acetylbarlerin			
Mobile phase composition 3:3.1:2.9:1	2.12	2.13 ± 51	2.39
Mobile phase composition 3:3:3:1		2.12 ± 43	2.01
Mobile phase composition 3.2:2.8:3:1		2.12 ± 51	2.40
Mobile phase volume 8 ml		2.12 ± 54	2.53
Mobile phase volume 10 ml		2.12 ± 43	2.01
Mobile phase volume 12 ml		2.13 ± 39	1.86
Chamber saturation time: 5 min		2.12 ± 57	2.69
Chamber saturation time: 10 min		2.12 ± 43	2.01
Chamber saturation time: 15 min		2.12 ± 55	2.59
Solvent migration distance: 78 mm		2.12 ± 53	2.51
Solvent migration distance: 80 mm		2.12 ± 43	2.01
Solvent migration distance: 82 mm		2.12 ± 39	1.84
(B) Barlerin			
Mobile phase composition 3:3.1:2.9:1	0.56	0.56 ± 15.1	2.72
Mobile phase composition 3:3:3:1		0.56 ± 9.4	1.69
Mobile phase composition 3.2:2.8:3:1		0.55 ± 9.6	1.74
Mobile phase volume 8 ml		0.56 ± 7.9	1.43
Mobile phase volume 10 ml		0.55 ± 9.4	1.69
Mobile phase volume 12 ml		0.56 ± 15.5	2.80
Chamber saturation time: 5 min		0.55 ± 11.6	2.11
Chamber saturation time: 10 min		0.56 ± 9.4	1.69
Chamber saturation time: 15 min		0.56 ± 14.2	2.56
Solvent migration distance: 78 mm		0.56 ± 11.1	2.00
Solvent migration distance: 80 mm		0.55 ± 9.4	1.69
Solvent migration distance: 82 mm		0.55 ± 10.8	1.95
(C) Shanzhiside methyl ester			
Mobile phase composition 3:3.1:2.9:1	1.20	1.19 ± 32	2.71
Mobile phase composition 3:3:3:1		1.20 ± 18	1.54
Mobile phase composition 3.2:2.8:3:1		1.19 ± 38	3.14
Mobile phase volume 8 ml		1.20 ± 38	3.16
Mobile phase volume 10 ml		1.20 ± 18	1.54
Mobile phase volume 12 ml		1.19 ± 26	2.16
Chamber saturation time: 5 min		1.20 ± 29	2.38
Chamber saturation time: 10 min		1.20 ± 18	1.54
Chamber saturation time: 15 min		1.19 ± 39	3.21
Solvent migration distance: 78 mm		1.20 ± 34	2.82
Solvent migration distance: 80 mm		1.20 ± 18	1.54
Solvent migration distance: 82 mm		1.19 ± 37	3.11

Table 4: Robustness of the method.

Conclusion

The HPTLC method was developed for the simultaneous estimation of three shanzhiside esters (iridoid markers) *viz.*, acetylbarlerin, barlerin and shanzhiside methyl ester. The developed method is simple, sensitive, precise, robust, specific, accurate and reliable for the determination of shanzhiside methyl ester iridoids. Statistical analysis indicates that the method is repeatable and selective for the simultaneous analysis of these three biologically active iridoids in different species/varieties of *Barleria*. The method was validated to ensure that it fits well the intended purpose and meet the strict regulatory requirements for analysis of herbal drugs. It is expected that the proposed method would also be useful for comparing and differentiating more *Barleria* or related plant species especially in drug discovery programmes. Interestingly, the method can detect and quantify very low concentration of iridoids to high per cent content and can be extended to study the degradation of shanzhiside esters under different stress conditions as per recommendation in ICH guidelines. The proposed method will definitely facilitate the drug quality assurance in such laboratories/countries where more sophisticated and costly analytical equipments are insufficiently available especially those of Asia and Africa where this plant is known to grow in abundance.

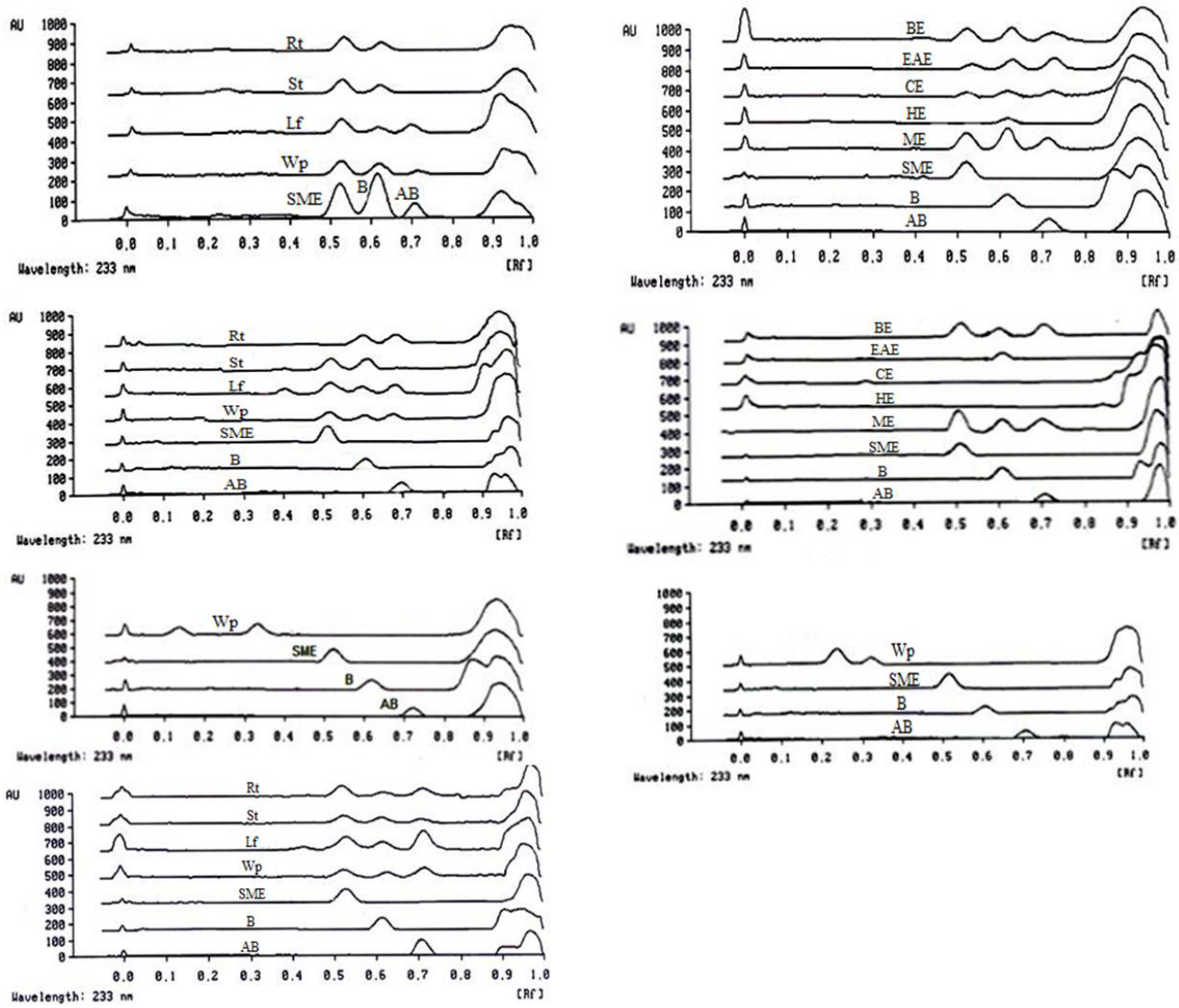


Figure 4: TLC densitometric chromatograms of shanzhiside methyl esters in different *Barleria* species/varieties: **A.** plant parts of *B. prionitis*; **B.** various fractions of methanolic extract of *B. prionitis*; **C.** plant parts of *B. cristata* var. *dichotoma*; **D.** various fractions of methanolic extract of *B. cristata* var. *dichotoma*; **E.** whole plant of *B. cristata* (pink flower variety); **F.** whole plant of *B. cristata* (blue flower variety); **G**. plant parts of *B. lupulina*; **AB:** acetylbarlerin; **B:** barlerin; **SME:** shanzhiside methyl ester; **Wp:** whole plant**; Lf:** leaf; **St:** stem; **Rt:** root; **ME:** mother extract; **HE:** hexane soluble fraction of mother extract; **CE:** chloroform soluble fraction of mother extract; **EAE:** ethyl acetate soluble fraction of mother extract; **BE:** butanol soluble fraction of mother extract.

S. No.	Sample	Acetylbarlerin	Barlerin	Shanzhiside methyl ester
1.	***B. prionitis***			
	Whole plant	0.35	0.81	1.36
	Leaf	0.95	0.25	2.62
	Stem	ND	0.97	1.46
	Root	ND	0.96	1.35
	Hexane soluble fraction	ND	0.02	ND
	Chloroform soluble fraction	0.28	0.08	0.11
	Ethyl acetate soluble fraction	1.28	1.10	1.00
	Butanol soluble fraction	1.68	2.82	3.92
2.	***B. cristata* var. *dichotoma***			
	Whole plant	0.26	0.18	0.52
	Leaf	0.46	0.18	0.70
	Stem	ND	0.23	0.88
	Root	0.16	0.14	ND
	Hexane soluble fraction	ND	ND	ND
	Chloroform soluble fraction	ND	ND	ND
	Ethyl acetate soluble fraction	ND	0.21	ND
	Butanol soluble fraction	0.89	0.59	1.28

3.	***B. cristata* (Pink flower variety)** Whole plant	ND	ND	ND
4.	***B. cristata* (Blue flower variety)** Whole plant	ND	ND	ND
5.	***B. lupulina*** Whole plant Leaf Stem Root	2.20 3.82 0.43 0.72	0.64 0.73 0.59 0.45	0.91 1.30 0.80 1.03

ND: not detected

Table 5: Per cent content of shanzhiside esters in different *Barleria* species/varieties.

Conflict of Interest

There are no conflicts of interest.

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