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Research Article
Development and Validation of an HPTLC Method for Estimation of Berberine in *Berberis aristata*

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Abstract

Background and Objective: High Performance Thin Layer Chromatographic (HPTLC) is a planner chromatographic technique used for the detection and quantification of natural products. An HPTLC densitometric method was developed and validated for the estimation of berberine in *Berberis aristata* stem bark accessions from the different sub-Himalayan region in India and Nepal is described. **Materials and Methods:** Sample preparation involved extraction of the powdered sample with the water-ethanol mixture (30:70 v/v) containing HCl (5%) at 50°C for 6 hrs, followed by filtration and partitioning with equal volumes of chloroform thrice. The organic layer was pooled, dried under reduced pressure and redissolved in methanol for analysis by HPTLC. Ascending development on precoated silica gel G F_{254} plates was carried using toluene-ethyl acetate-formic acid-methanol (9:9:2:3 v/v) as mobile phase. Detection and quantitation of berberine were performed by densitometry at 350 nm. **Results:** The HPTLC densitometric method was developed and validated as per ICH guidelines for the estimation of berberine in different accessions. The developed method gave well resolved, compact bands of berberine at R_F 0.52±0.11. The berberine content among the accessions from India was highest for Tamil Nadu (12.08±1.16%) followed by Jammu and Kashmir (7.28±0.44%), Uttarakhand (6.98±0.31%) and Himachal Pradesh (5.34±0.23%). The specimens from Nepal contained 8.44±0.73% of berberine. **Conclusion:** The developed method was found precise, robust and accurate and was successfully used for the detection and quantification of berberine in different accessions.

Key words: *Berberis aristata*, Berberidaceae, berberine, HPTLC, ICH guidelines

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INTRODUCTION

Berberis aristata DC. (Berberidaceae), commonly-known as Indian Barberry, is a widely distributed in sub-Himalayan regions at an altitude of 1500-3000 m. The plant is used in traditional medicines as a mild laxative, bitter, antipyretic, stomachic and liver tonic[1,2]. It is reported to have hepato-protective[3], anti-osteoporotic[4], anti-hyperglycaemic and antioxidant[5] activities. The chief constituent of the roots and stem of *B. aristata* are berberine, berbamine, aromoline, palmatine, oxyacanthine and oxyberberine[6].

Berberine is an isoquinoline alkaloid found in more than nine plant families but occurs most frequently in Berberidaceae[7]. It is found in *Berberis aquifolium* (Oregon grape), *Berberis vulgaris* (Barberry), *Berberis aristata* (Indian barberry), *Hydrastis canadensis* (Goldenseal), *Phellodendron amurense* (Amur cork tree), *Coptic chinensis* (Chinese goldthread) and *Tinospora cordifolia* and to a smaller extent in *Argemone mexicana* (Prickly poppy) and *Eschscholzia californica* (Californian poppy). Pharmacological effects reported for berberine have been compiled earlier[8]. These include hepatoprotective[9,10], anti-proliferative[11] and anti-diabetic activities[12,13].

HPTLC is a planner chromatographic technique used for the detection and quantification of analytes. The advantages of HPTLC over other analytical methods are accurate sample application and *in-situ* scanning that facilitates reliability, rapidity and accuracy of the analysis. It also allows simultaneous estimation of several samples utilising only a small quantity of a mobile phase, hence minimising the analysis time and cost. A thorough review of the literature revealed that quite a few analytical techniques have been reported for the determination of berberine in plant extracts and their formulations[14-16]. These reports are greatly varied concerning the berberine content reported for the samples. These discrepancies can be mainly attributed to improper sample preparation and the low specificity of the methods employed. To the best of our knowledge, this is also the first study related to the determination of berberine in *B. aristata* accessions from different geographical locations. This research aimed to provide an optimized sample preparation method and a validated HPTLC method for the estimation of berberine in crude drug samples and finished formulations.

MATERIALS AND METHODS

Study area: *Berberis aristata* stem bark from five sub-Himalayan regions were studied and they are Palampur, Himachal Pradesh, India (HP), Ganderbal, Jammu and Kashmir, India (J and K): Tehri Garhwal, Uttarakhand, India (UK), Ooty Nilgiris, Tamil Nadu, India (TN), and Dang, Nepal (NP). The research project was conducted from May, 2017 to June, 2018 in the Department of Pharmacognosy, School of Pharmaceutical Education and Research, Jamia Hamdard, New Delhi, India.

Plant materials and chemicals: The accessions of *Berberis aristata* stem bark from five sub-Himalayan regions were procured through Universal Biotic, Farash Khana, Delhi, India. The samples were identified by Taxonomist, Department of Botany, Faculty of Science, Jamia Hamdard and the voucher specimen [PRL/JH/2015/21(1-5)] were deposited in the Phytochemistry Research Laboratory, Faculty of Pharmacy, Jamia Hamdard, New Delhi, India. Berberine was purchased from Sigma (St. Louis, Mo., USA). Toluene, ethyl acetate and formic acid were of analytical grade obtained from Merck (Mumbai, India). Ethanol was obtained from Fisher Scientific (Leicestershire, UK). All other chemicals were of analytical grade and procured from S.D. Fine Chemicals Ltd. (Mumbai, India).

The extraction procedure: The sample preparation involved the extraction of powdered plant matrix (*B. aristata* stem) and formulations with hydro-alcoholic solvent by maceration. The material (10 g) was macerated with200 mL solvent mixture thrice each of two hours duration. The optimum extraction conditions were selected by a one-variable test, where the effect of one factor on extraction efficiency was determined by keeping the other factors constant. When ethanol content in the extraction solvent was changed (50-90% v/v), the other extraction conditions such as acid content (HCl 5%), temperature (60°C) and extraction time (6 hrs i.e., three cycles) were kept constant. Similarly, the effects of acid content (Hcl 3-7%), temperature (40-80°C) and extraction time (2-10 hrs) on extraction efficiency were also assessed. The menstruum from each extraction was filtered and partitioned separately with di-iso-propyl ether (1:1) thrice. The organic layer was pooled, evaporated to dryness *in vacuo* and weighed for determination of extractive value (% w/w on a dry weight basis). The dried residue of each extract was redissolved in methanol, filtered and applied on pre-coated silica gel plates for analysis by HPTLC.

HPTLC instrumentation and chromatographic conditions: HPTLC was performed on 20×10 cm aluminium backed plates coated with a 0.2 mm layer of silica gel 60 F_{254}

(Merck, Mumbai, India). Dilutions of standard and test solutions were applied to the plates as bands 5.0 mm wide, 10.0 mm apart and 10.0 mm from the bottom edge on the same chromatographic plate by using a CAMAG (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100 μL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28±2°C), with toluene-ethyl acetate-formic acid-methanol (9:9:2:3 %v/v) as mobile phase, in a CAMAG glass twin-trough chamber previously saturated with mobile phase for 20 min. After development, the plates were dried in air and scanned at 350 nm with a CAMAG TLC scanner with Win cat software and using a deuterium lamp. The slit dimensions were 4×0.2 mm and the scanning speed was 20 mm s^{-1}.

Method development: Various solvent systems were tried for the development of a suitable TLC densitometric method for the estimation of berberine in hydro-ethanolic extracts of *B. aristata*. The mobile phases tried for these purposes were: toluene-ethyl acetate-formic acid-methanol (10:8:2:3% v/v), toluene-ethyl acetate-formic acid-methanol (12:7:2:4%v/v), toluene-ethyl acetate-formic acid-methanol (9:8:2:4%v/v), toluene-ethyl acetate-formic acid-methanol (9:9:2:3%v/v), ethyl acetate-methanol-water (6:3:1 % v/v), ethyl acetate-methanol-water (7:2:1 %v/v) and ethyl acetate-methanol-water (8:1.5:0.5 %v/v).

Calibration curve: A stock solution of standard berberine was prepared in methanol at a concentration of 0.1 mg mL^{-1}. The standard dilutions containing 5, 10, 20, 30, 40 and 50 ng mL^{-1} of berberine were prepared from the stock solution that was applied on a plate in 2 μL aliquots to provide 10, 20, 40, 60, 80 and 100 ng of berberine per band, respectively. After the development of the plates, peak height, peak area and concentration data were treated by linear regression analysis. All the experiments were carried out in triplicate. Calibration curves were obtained as plots of peak areas of standard versus the amount spotted.

Method validation: Validation studies ensure the suitability and reproducibility of the method in analysing the desired analyte. The method was validated for linearity, Limit Of Detection (LOD), Limit Of Quantification (LOQ), specificity and precision (repeatability) as per the International Conference on Harmonization guidelines[17,18].

Linearity: Dilutions of standard in the range of 10-100 ng per band were analysed in triplicate to prepare six-point linear calibrations. The plates were developed, scanned and quantitatively evaluated. Calibration curves as plots between peak area and concentration were obtained. Linearity was determined by averaging the values obtained from these plots by regression analysis.

Precision: Precision was determined at two levels according to ICH guidelines (repeatability and intermediate precision). Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of berberine at levels of 40, 60 and 80 ng per band in triplicates.

Robustness: Robustness is a measure of the capability of a method to remain unaffected by little but intentional changes in the method conditions and is an indicator of the stability of the method. The effect of small and deliberate changes in the mobile-phase composition (toluene-ethyl acetate-formic acid-methanol with 9.0:8.5:2.0:3.0,9.0:9.0:2.5:3.0 and 9.5:8.0:2.0:2.5 v/v ratio), volume of mobile phase(18, 20 and 22 mL) and duration of saturation (9, 10 and 11 min) were investigated. The robustness experiments were carried out with 60 ng per band in triplicates.

LOD and LOQ: LOD and LOQ of an analytical procedure represent the lowest amount of analyte that can be detected and quantified with suitable precision and accuracy, respectively. To estimate LOD and LOQ values, a blank solution (methanol) was spotted six times following the same method as explained above. The standard deviation (σ) of the magnitude of analytical response was calculated for six replicates. The LOD was expressed as 3.3 σ/slope of the calibration curve, whereas LOQ was expressed as 10 σ/slope of the calibration curve.

Applications of developed method: The test samples (4 μL) were applied in triplicate and chromatograms were obtained under the same conditions as that of standards. The area under the peak corresponding to that of the standard was recorded and content of the same was calculated from the regression equation obtained from calibration curves.

RESULTS

Method development: The ascending development with toluene-ethyl acetate-formic acid-methanol (9:9:2:3 v/v) to a distance of 8 cm gave a sharp, compact and well-resolved

band at R_F value 0.52±0.11 for berberine. Figure 1 shows bands of berberine in standard solution (S1-S6), different accessions from Himachal Pradesh (HP), Jammu and Kashmir (JK), Uttarakhand (UK), Tamil Nadu (TN) and Nepal (NP) and marketed formulations (F1-F3) at 350 nm. The bands of berberine standard showed maximum absorbance at 350 nm and therefore, it was chosen as the wavelength or densitometry. Figure 1 showed HPTLC chromatograms of *B. Aristata* samples and their formulations together with standard berberine. These results show the quantitative variation of berberine in different geographical accessions and formulations. Figure 2 showed the HPTLC chromatogram of berberine in standard solution at 350 nm at 0.50-0.60 Rf value with 150 AU.

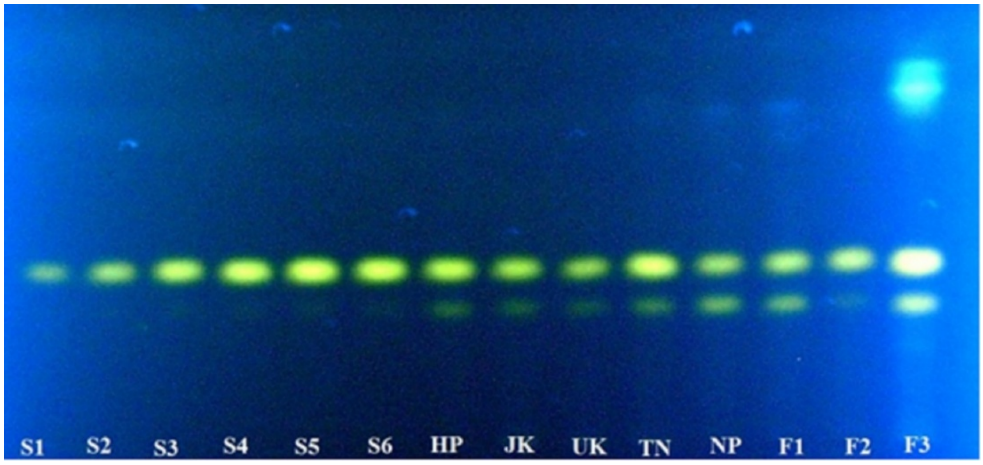

Fig. 1: HPTLC plate showing bands of berberine in standard (S1-S6), accessions from Himachal Pradesh (HP), Jammu and Kashmir (JK), Uttarakhand (UK), Tamil Nadu (TN) and Nepal (NP) and marketed formulations (F1-F3) at 350 nm

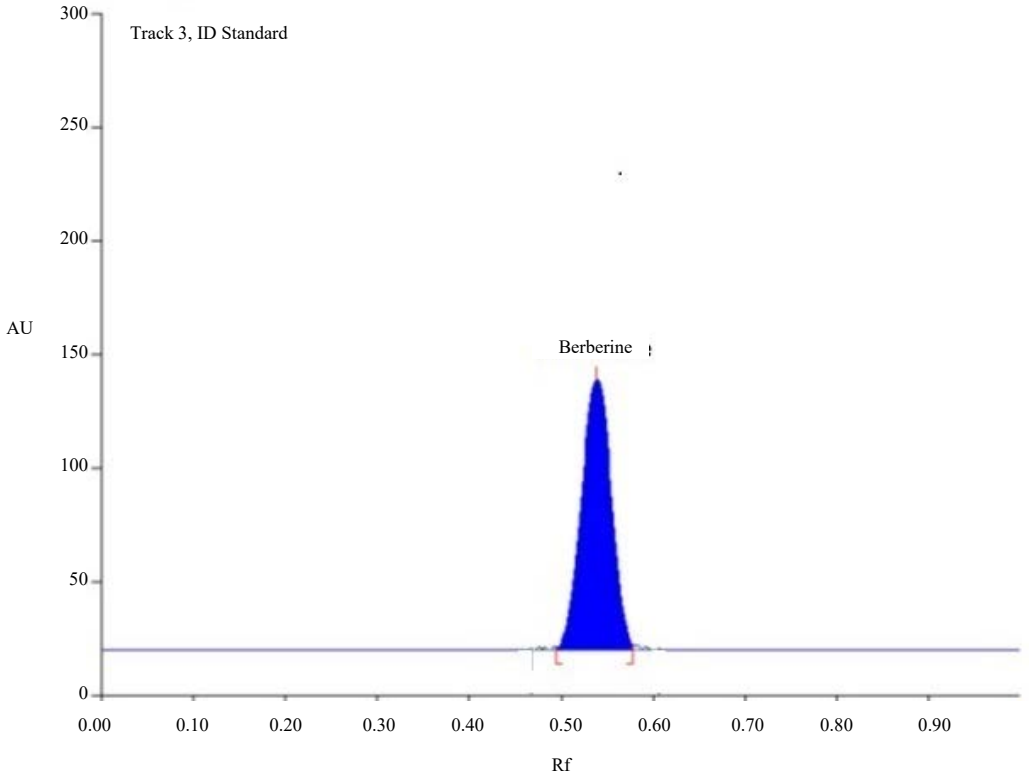

Fig. 2: HPTLC chromatogram of standard berberine

Validation of the developed method

Linearity analysis: The results of regression analysis are shown in Table 1. In this study, linearity was determined by analyzing standard at six levels. A graph was plotted between quantities applied (ng per band) and peak areas. The regression analysis of the calibration plots showed a good linear relationship over the concentration range of 10-100 ng per band of berberine. The linear coefficient (r^2) was found to be 0.9983. The calibration lines were represented by linear equation Y = 28.41+51.53X.

Precision determination: The repeatability of the method was evaluated by analysis of standard levels for berberine at different time intervals within a day and on three different days in a week. Intra-day precision was evaluated by comparing results obtained from analysing three standard levels of berberine at six different times in a day. Results obtained from the intra-day precision analysis are illustrated in Table 2. The data are also presented as relative standard deviation (% RSD) values calculated for berberine. These values were low (≤1.21%), indicating that the analysis performed at different times in a day was precise. Inter-day precision was determined by comparing 18 values obtained from analysis conducted on three days within a week. Results of the inter-day analysis are shown in Table 2. The % RSD was also low(≤0.68%), indicating that the results were still repeatable even when analysed on different days. Data with % RSD≤2.5 are considered repeatable.

Robustness measurement: Table 3 depicts the robustness of the proposed method. The effect of changes in mobile phase composition (toluene-ethyl acetate-formic acid-methanol with following compositions: 9.0:8.5:2.0:3.0; 9.0:9.0:2.5:3.0; and 9.5:8.0:2.0:2.5 v/v); mobile-phase volume (18, 20 and 22 mL) and duration of saturation (9, 10 and 11 min) on peak areas of berberine was studied at 60 ng per band (in triplicate).The relative standard deviation (RSD, %) of the peak areas was calculated. The variation observed in the results was non-significant. The method was found to be robust when mobile phase composition was changed deliberately, the % RSD were0.41,0.45 and 0.52 with toluene-ethyl acetate-formic acid-methanol, 9.0:8.5:2.0:3.0, 9.0:9.0:2.5:3.0, and 9.5:8.0:2.0:2.5 v/v, respectively. The change in mobile-phase volume also did not significantly affect the analysis; the change in mobile phase volume 20±2 mL showed 0.64, 0.61 and 0.53 in % RSD, respectively. The change in saturation time of TLC chamber

Table 1: Linear regression data for the calibration curve

Linearity range (ng per band)	10-100
Regression equation	Y = 28.41+51.53X
Correlation coefficient (R^2)	0.9983
Slope±SD	51.70±0.55
Intercept±SD	29.57±4.43
Slope without intercept±SD	51.71±0.56
Standard error of the slope	0.32
Standard error of intercept	2.56
95% Confidence interval of the slope	50.32-53.09
95% Confidence interval of intercept	18.55-40.58

n = 3, SD: Standard deviation

Table 2: Results of precision analysis

Amount applied	Intra-day precision			Inter-day precision		
(ng/band)	Mean area±SD	Standard error	RSD (%)	Mean area±SD	Standard error	RSD (%)
40	1893.51±23.09	13.33	1.21	1903.86±13.11	7.56	0.68
60	2729.31±20.43	11.79	0.74	2731.64±13.48	7.78	0.49
80	3698.13±19.13	11.04	0.51	3697.80±18.55	10.71	0.50

n = 6, SD: Standard deviation, RSD: Relative standard deviation

Table 3: Results of robustness analysis

Conditions	Mean peak area±SD	RSD (%)	Standard error
Mobile phase composition (v/v)			
9.0:8.5:2.0:3.0	2722.64±11.21	0.41	8.62
9.0:9.0:2.5:3.0	2737.64±10.92	0.45	6.31
9.5:8.0:2.0:2.5	2697.64±12.92	0.52	7.92
Mobile-phase volume (mL)			
18	2713.42±9.14	0.53	14.31
20	2712.33±10.01	0.64	12.98
22	2699.72±13.84	0.61	11.17
Duration of saturation (min)			
9	2766.62±12.91	0.51	8.62
10	2756.87±13.75	0.34	7.94
11	2783.72±13.98	0.42	9.91

Mobile phase: Toluene-ethyl acetate-formic acid-methanol, n = 3, SD: Standard deviation, RSD: Relative standard deviation

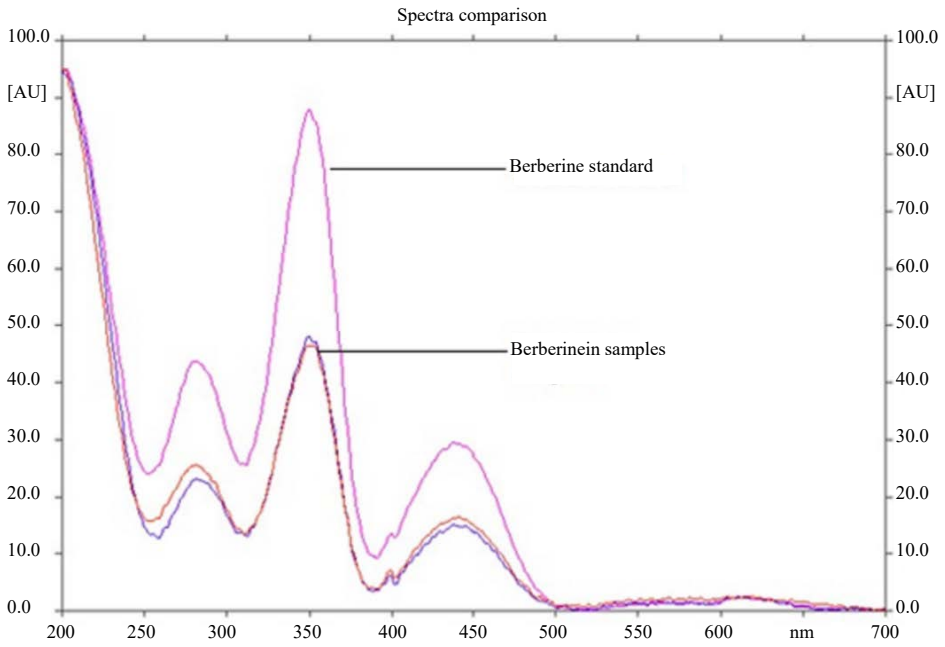

Fig. 3: Overlay of UV spectra of berberine in standard and test samples

Table 4: Extractive values and berberine content in the stem bark of *B. aristata*

Accessions	Code	Extractive value[a]± SD (%)	Berberine content[b]± SD (%)
Himachal Pradesh	HP	9.25±0.71	5.34±0.23
Jammu and Kashmir	JK	6.14±0.41	7.28±0.44
Uttarakhand	UK	8.40±0.52	6.98±0.31
Tamil Nadu	TN	9.44±0.58	12.08±1.16
Nepal	NP	9.22±0.65	8.44±0.73
Formulation 1	F1	4.04±0.23	3.88±0.32
Formulation 2	F2	3.38±0.38	3.32±0.22
Formulation 3	F3	4.17±0.35	4.50±0.35

[a]w/w on a dry weight basis of starting material, [b]w/w of the total extractive

also studied, saturation time 10±1 min shows 0.34, 0.42 and 0.52 in % RSD, respectively. The overall results show that the method was found to be robust with a % RSD less than 2.

Specificity: The specificity of the method was determined by comparing R_F (Fig. 1) and UV spectra in Fig. 3 of peaks of the sample with those of standard. Three-point peak purity, *i.e.,* peak start, apex and end, was compared and was found completely super-imposable. This indicated that the berberine peak was not overlapped by any other component in the samples.

LOD and LOQ determination: LOD and LOQ of the proposed method were determined by the standard deviation method and were found to be 4.0 and 11.2 ng per band, respectively. The calculated LOQ value was lower than the lowest values used for calibration. It indicated that the proposed method has a great potential for the qualitative and quantitative determination of berberine.

Application of the proposed method: The extraction was carried by the maceration method using an ethanol-water mixture containing HCl (5%). The results of extraction studies were summarized in Table 4. The optimal conditions for extraction were: maceration with ethanol-water (70:30 v/v) containing HCl (5%) at a temperature of 50°C for 6 hrs consisting of three successive cycles of 2 hrs duration. The proposed HPTLC method was successfully applied for the detection and quantification of berberine in plant samples and formulation containing *B. aristata* stem bark. The berberine percentage in crude accessions were 5.34±0.23 (HP), 7.28±0.44 (JK), 6.98±0.31 (UK), 12.08±1.16 (TN) and 8.44±0.73 (NP) (Table 4). The berberine content in formulations varied from 3.32-4.5%. Based on these results, it can be concluded that accessions from Tamil Nadu, India were better than the other samples studied here.

DISCUSSION

The hydro-alcoholic solvent was used in this work to effect an efficient extraction with a relatively easier workup of extracts for sample preparation for analysis. HCl was added to the solvent mixture that resulted in the formation of salts of berberine (pKa 2.47) while the strongly basic alkaloids such as berbamine (pKa 7.33) remained as alkaloidal base. For selection of optimum extraction parameters like solvent composition, temperature, time and acid content separate experiments were set up in triplicate. The effect of each factor was determined in terms of percent extractives while keeping the other factors constant at their median level. The effect of variation in the composition of the solvent mixture was tested concerning its water content on extraction efficiency. The results showed that the extraction efficiency increased with an increase in water content in extraction solvent until up to 30 % v/v. Further increase in water content did not result in a further increase in extraction yield. As quaternary alkaloidal bases are water-soluble, the water content in the solvent mixture considerably improved the extraction efficiency. The effect of temperature on extraction efficiency was examined over a range of 40-80°C. It was observed that when the temperature was increased to 50°C, the extraction efficiency increased. However, when the temperature increased beyond 50°C, the extraction efficiency did not change significantly. It suggested the optimum temperature for the extraction of berberine is 50°C. The effect of time on extraction efficiency was examined throughout 2-10 hrs. As expected, the yield increased with increasing extraction time and reached maximum at 6 hrs. The effect of acid content was also studied over a range of 3-7%. The maximum extraction was achieved when 5% HCl was added to the menstruum. Based on these results the optimal conditions for extraction were: maceration with ethanol-water (70:30 v/v) containing HCl (5%) at a temperature of 50°C for 6 hrs consisting of three successive cycles of 2 hrs duration.

For the optimization of HPTLC conditions, both low polarity solvent mixtures of toluene-ethyl acetate and high polarity solvent mixtures of methanol-ethyl acetate-formic acid resulted in the poor separation of bands. Moderately polar solvent mixtures of toluene-ethyl acetate-formic acid-methanol (9:9:2:3 v/v)after optimization resulted in well-resolved compact bands for the analytes (Fig. 1).

Linearity ranges for the calibration curves indicated that the method was sensitive for the analytes. The method was found to be robust and able to with stand small deliberate changes[17,18], however, caution should be observed with formic acid in the mobile phase as it is highly volatile and polar. Peak purity of analytes shown by super-imposition of their visible spectra depicted the specificity of the method (Fig. 3). The developed HPTLC method was found sensitive, linear, accurate and robust for the estimation of berberine in different accessions of *B. aristata* bark[19].

Ghosh *et al.*[15], developed and validated an HPTLC method for estimation of berberine in the different formulation and reported 3.96±1.21% w/w berberine content in the formulation. In another study, Kamal *et al.*[16], developed and validated an HPTLC method for the determination of berberine in Berberis extracts and formulation. The berberine content was found to be 0.033 and 0.0089% (w/w), respectively. A simultaneous HPTLC and HPLC method was developed and validated for the estimation of quercetin and berberine in Pushyanuga churna prepared in-house and in various marketed formulations. The group reported 0.138±0.014 and 0.60±0.042% berberine content in in-house and marketed formulations[20]. The method being reported here was successfully applied for the detection and quantification of berberine in plant samples and formulation containing *B. aristata* stem bark. The maximum berberine content was found in Tamil Nadu (12.08±1.16%) and then in Nepal (8.44±0.73%) (Table 4). The berberine content in formulations varied from 3.32-4.5%. Based on these results, it can be concluded that accessions from Tamil Nadu, India were better than the other samples studied here. The berberine content in the current study was found to be relatively higher because of standardized extraction conditions. The present study reports the berberine content in *B. aristata* stem bark accessions from different Himalayan regions. The method can be used in future for the analysis of marketed formulations containing berberine.

CONCLUSION

A validated HPTLC densitometric method with a simple sample preparation was developed and validated and applied for the quantification of berberine in hydro-ethanolic extracts of *B. aristata* from different geo-climatic regions. Berberine content among the accessions from India was highest for a specimen from Tamil Nadu followed by Jammu and Kashmir, Uttarakhand and Himachal Pradesh. The specimen from Nepal was found to contain 8.44±0.73% of berberine. The berberine content in formulations studied here varied from 3.32-4.5%. Based on these results, it can be concluded that accessions from Nilgiris, Tamil Nadu were better than the other samples. The method proved useful for routine analysis of berberine in crude drug extracts and finished formulations without any interference.

SIGNIFICANCE STATEMENT

This research study leads to the development and validation of an HPTLC densitometric method for the estimation of berberine in different accessions of *B. aristata* collected from Himalayan regions. The study results show that the developed HPTLC method was accurate, sensitive and robust.

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