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Quality assessment and evaluation of *Oroxylum indicum* through HPLC fingerprint and QAMS for important flavonoid components

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Oroxylum indicum is an important Ayurvedic medicinal plant used in medicinal formulations to treat many diseases. In this research, an effective, sensitive, reliable, cost effective and comprehensive assessment of quality of *Oroxylum indicum* has been developed based on high performance liquid chromatography (HPLC) fingerprint analysis combined with the quantitative analysis of multi-components by a single marker (QAMS) method. The contents of four components i.e., Scutellarin, Hispidulin, Baicalein and Biochanina-A, has been determined, simultaneously, and Baicalein is used as internal reference standard. It was established that there is no major difference between the QAMS method and the traditional external standard method (ESM) (RSD<2.00%). This signifies that QAMS is a consistent and expedient method for the content determination of multiple components, particularly when there is non-availability of multiple reference standards. This method was also validated in terms of linearity, precision, stability, recovery and reproducibility. Hence it can be effectively applied for quality assessment of *Oroxylum indicum* in various Ayurvedic formulations.

KEYWORDS

Oroxylum indicum; ayurveda; QAMS; quality evaluation; HPLC fingerprint.

Introduction

Ayurveda is a holistic native medical system which has flourished widely in India for more than 5000 years. Nowadays, Ayurvedic medicine attracts attention ever more owing to its extensive clinical application and consistent therapeutic efficiency [1]. Regardless of advances in modern medical science, Ayurveda continues to play a pivotal role in prevention and healing of various diseases [2]. Hence, its quality control is of prime importance, since it directly affects the therapeutic potential of Ayurvedic formulations.

Oroxylum indicum Vent., also known as *Shyonaka* in Sanskrit [3] is among the ten plants whose roots are used for preparation of Ayurvedic formulation, *Dashamoola* (means 'ten roots'). Dashmoola is regularly prescribed as *Dashamoolarishta*, *Chyawanprash*, *Dashamoola Kalpa, Dashamoola Churna*, *Dashamoola Ghrita*, and *Dashamoola* Oil [4]. Almost all plant parts like seed, ripened fruit, stem bark, root bark and leaves are used for the preparation of these formulations [5,6].

O. indicum belongs to Bignoniaceae family, widely found in Tropical Asia. The chemical composition includes baicalein, chrysin, hispidulin, scutellarin, biochannin-A, oroxylin A, oroxylin B etc. [7].

Preparations of *O. indicum* have been reported to treat nerve, muscle, bone and joint-related problems due strong anti-inflammatory and analgesic properties [8]. Previous studies have also reported, anti-diabetic [9], hepatoprotective [10], anti-adipogenesis [11], anti-cancerous [12], properties for *O. indicum* and its isolated compounds. *Shyonaka* is used as drug for treating rheumatoid arthritis, inflammation and various other disorders as internal administration or external application [13].

O. indicum is used in ayurveda and folk medicine for treating cancer, diarrhea, diabetes, fever, bone pain, ulcer, and jaundice [14]. Fruit pods have been extensively reported for inhibition of adipogenesis and lipase activity [15]. The seed contains many flavonoids, including Chrysin, scutellarin, baicalein-7-*O*-gentiobioside, Baicalein [16]. Leaves are affirmed for their antioxidant and antiviral activities, particularly for treating chikungunya and reducing oxidative stress. Leaves contain important flavonoids, namely, Chrysin, baicalein, baicalein-7-O-glucoside, scutellarin and Chrysin-7-O-glucuronide [17]. Root and root bark are reported to contain important flavonoids like baicalein, chrysin, oroxylin A, biochanin and ellagic acid [18].

As for bark or stem bark, many biochemical activities have been assessed like antimicrobial, antidiarrheal, analgesic, cytotoxic, hepatoprotective, gastroprotective, antiproliferative, antimetastatic, antiobesity potential, and antioxidant activities [5]. Various flavonoids, namely scutellarin, baiclalein, hispidulin and 5,7,4-trihydroxyflavone, have been identified and separated from the stem bark of *O. indicum* [19]. A number of prominent flavonoids compounds like baicalein, chrysin, ellagic acid, oroxylin A, chrysin, biochanin-A had been separated and identified especially from bark (both stem and root), leaves and seeds [20].

Currently, the most common method for quality control of Ayurvedic medicines is External Standard Method (ESM). In an ESM, a known data from calibration standard and an unknown data from a random sample are pooled to calculate quantitative data. This method involves simple evaluation of instrument response for target compounds in a sample to the calibration curve [21]. Yet, this assessment is far from effective for assessing other components in Ayurvedic formulations, since high-purity reference standards are expensive and insufficient. Considering these two bottlenecks for multi-component analysis in routine quality control, a rational method known as Quantitative Analysis of Multi-components by a Single marker (QAMS) is adopted [22].

In ESM, the contents of all reference standards corresponding to components in the sample should be determined. Whereas in QAMS method, only the internal standard needs to be determined, thereby reducing the time and cost of detection [23]. This study aimed to establish a reliable chromatography fingerprint method with HPLC analysis and for quality authentication and assessment of components of *Oroxylum indicum*, a single marker (QAMS) method was used to guarantee its clinical safety and effectiveness. Also, comparing content of targeted compounds in different parts of *O. indicum* through QAMS will aid quality control of Ayurvedic formulations, since varied plant parts are taken for wide-ranging preparation.

Experimental

Plant material and chemicals

Twenty one different samples of *Oroxylum indicum* were collected from varied locations of India from October to November, 2019 as shown in Table 1. All the standards for internal reference, viz., Scutellarin (S_1), Hispidulin (S_2), Baicalein (S_3) and Biochanin-A (S_4) (Figure 1) were purchased from Sigma Aldrich. Purity of all these standards were >98%. Water, Methanol and Formic acid for HPLC analysis were purchased from Merk.

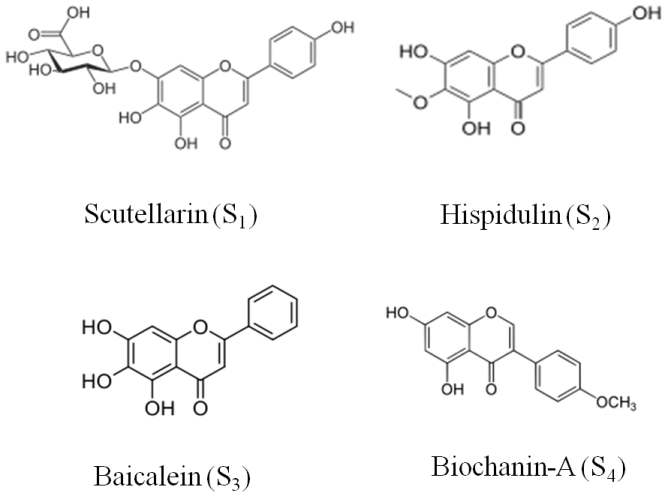

FIGURE 1 Structure of examined Compounds.

TABLE 1 Different locations and plant parts of *Oroxylum indicum* sample in INDIA

Origin	Part of *O. indicum*	Number	Date of Collection
Haridwar	Leaf	Hl	September, 2019
	Flower	Hf	September, 2019
	Bark	Hb	September, 2019
	Seed Pod	Hs	September, 2019
Saharanpur	Leaf	Sl	September, 2019
	Flower	Sf	September, 2019
	Bark	Sb	September, 2019
	Seed Pod	Ss	September, 2019
Rajaji	Leaf	Rl	September, 2019
	Flower	Rf	September, 2019
	Bark	Rb	September, 2019
	Seed Pod	Rs	September, 2019
Nazibabad	Leaf	Nl	October, 2019
	Flower	Nf	October, 2019
	Bark	Nb	October, 2019
	Seed Pod	Ns	October, 2019
Bhimtal	Leaf	Bl	October, 2019
	Bark	Bb	October, 2019
Kiorali	Leaf	Kl	November, 2019
	Bark	Kb	November, 2019
	Seed Pod	Ks	November, 2019

Preparation of sample solution

Various plant parts were dried to constant weight in a hot air oven at 50 ^{0}C and then ground to a fine powder. 2.0 g of sample powder was accurately weighed and extracted with 80% (*v/v*) methanol, in a rotary shaker at 120 rpm. The extracted solution was filtered using a Whatman no. 1 filter paper. The filtrate was dried to constant weight. The dried extract was dissolved in HPLC grade methanol, at a concentration of 10 mg/mL. The solution was filtered through 0.22 µm membrane and 10 µLwas injected for analysis.

Preparation of standard solution

Standard stock solutions were prepared by dissolving 4 mg Baicalein, 2 mg hispidulin, 4 mg biochanin-A and 1 mg scutellarin in 1 mL of HPLC grade methanol. Working solution of mixtures of all standards was prepared by

diluting stock solutions in methanol right before HPLC analysis.

Instrument and conditions

The analysis was performed by isocratic High Performance Liquid Chromatography (HPLC) using Perkin Elmer Flexar HPLC system consisting of a Flexar UV/Vis LC detector, a Flexar binary pump, and equipped with Brownlee, C18 column (5 mm, 4.615 0 mm x 250 mm). Methanol and water were used as mobile phases and the analysis was performed at 280 nm, with a run time of 20 minutes. Isocratic HPLC was performed employing binary mobile phase consisting of methanol and water (2:1 *v/v*), containing 1.3% formic acid. A flow rate of 1.0 mL min^{-1} with column temperature at 40 ^{o}C was used throughout the analysis. 10µLof sample was injected into the column. An equilibrating time of 2 minutes was set between two runs. The compounds from samples were identified by comparing the retention time of standards. Quantification of compounds was done using standard curves.

Calculation of relative conversion factor (F_x)

Availability, stability and ease of separation are the main characteristics of the single marker to determine multi-components in the sample through chromatography. In this study Baicalein was used as a marker, since it is found in high concentration in *O. indicum,* has high stability, low cost and has important pharmacological activities. With Baicalein as a single marker, the relative conversion factor for the other analyte was calculated as F_x (a). Using F_x the concentration of each analyte (C_x) in the same sample can be calculated according to equation (b) and (c):

Relative Correction factor,

$$F_x = \frac{f_x}{f_i} = \frac{A_x/C_x}{A_i/C_i} \quad \text{(a)} \qquad C_x = \frac{C_i}{F_x} \times \frac{A_x}{A_i} \quad \text{(b)}$$

$$w_x = \frac{C_x \times V}{m} \quad \text{(c)}$$

Where, A_x and A_i: peak area of analyte and reference standard, respectively

C_x and C_i: concentration of analyte and reference standard, respectively

m : mass of *O. indicum* extracts (mg)

w_x : mass concentration of flavonoid component in *O. indicum*

v : volume of sample

Hierarchical cluster analysis (HCA)

The similarity between each sample was visually demonstrated with a dendogram in HCA. In any plant system, mature leaves are the source, i.e., they are capable of producing photosynthate in excess of their own needs. Also, the overall pattern of transport in the phloem can be stated simply as a source-to-sink movement [24], hence we considered leaf samples from each location for construction of dendogram.

Results and discussion

Method validation

Calibration curve, Linearity, Limits of Quantification and Detection

Calibration curves were prepared by serially diluting stock solutions of S_1, S_2, S_3 and S_4 and it was found to be linear in a working range of 62.5-500 µg/mL. The correlation coefficient (R^2) were 0.981, 0.993, 0.997 and 0.999 for S_1, S_2, S_3 and S_4, respectively (Table 2). LOQ and LOQ for the four marker compounds were in the range of 1.954-14.630 µg/mL and 0.618-10.979 µg/mL respectively, hence showing high sensitivity of the established chromatographic conditions

TABLE 2 Calibration and sensitivity data for 4 marker compounds (n = 9)

Analyte	Calibration Curve	R^2	%RSE	Linear range (µg/mL)	LOQ (µg/mL)	LOD (µg/mL)
Scutellarein (S_1)	19.24100E+3 x+-67.68006E+4	0.981	39.8	62.5-500	14.630	10.979
Hispidulin (S_2)	19.16151E+3 x+26.22866E+4	0.993	6.8	62.5-500	6.176	4.632
Baicalein (S_3)	42.27251E+3 x+-29.82130E+4	0.997	14.7	62.5-500	8.234	2.605
Biochanin-A (S_4)	33.18216E+3 x+80.87112E+3	0.999	12.0	62.5-500	1.954	0.618

Precision and stability

To assess precision, inter-day and intra-day variations were analyzed. Intra-day precision was determined by analyzing the same sample solution 'Rl', within the same day at 0, 1,3,6,12 and 24 hours. Inter-day precision was validated with the same batch of samples as used above for ten consecutive days. The RSD values for intra-day and inter-day was of the same magnitude and <2% (Table 3), as outlined by Indian Pharmacopoeia. Hence the method can be considered to be precise. To validate repeatability, six independently prepared extracts of 'Rl' were analyzed. The RSD values of the target compounds ranged flanked by 1.3506-2.0509, showing that the chromatogram response did not vary with different attempt of extraction.

TABLE 3 Precision, stability, repeatability of marker compounds (n=9)

Analyte	Intraday RSD (%)	Interday RSD (%)	Repeatability RSD (%)
S_1	1.750380	1.227871	1.350616
S_2	1.393674	1.076377	2.050917
S_3	0.503453	0.728477	1.826953
S_4	1.961999	1.875334	2.115512

Recovery of marker compounds

To perform recovery studies, a known amount of reference compound was spiked in the sample solution. Analysis was performed as mentioned above for nine replicates each. Recovery was calculated using equation (d) and it ranged between 97.81 to 100.807, with RSD <2%, showing that errors had a small effect on the recovery values (Table 4).

$$Recovery\ \% = \frac{Detected\ value}{Calculated\ value} \times 100 \quad \text{(d)}$$

Analysis of multi-component by ESM

The developed HPLC method was then applied to twenty one different samples of *Oroxylum indicum*. The chromatograms for standard solution and *O. indicum* sample are shown in Figure 2 and content of the four components are shown in Table 4.

Table 4 Recovery data for marker compounds (n = 9)

Analyte	Initial amount (µg)	Amount added (µg)	Calculated value (µg)	Detected value (µg)	Recovery (%)	Mean (%)	RSD (%)
		50	3937.75368	3888.13798	98.74		
S_1	3887.753676	100	3987.75368	3957.84552	99.25	99.4133333	0.77266927
		200	4087.75368	4097.97306	100.25		
		50	1406.49229	1401.56956	99.65		
S_2	1356.492288	100	1456.492288	1418.91479	97.42	97.81	1.716915793
		200	1556.492288	1499.83597	96.36		
		50	7380.56738	7393.11434	100.17		
S_3	7330.56738	100	7430.56738	7417.93542	99.83	100.8066667	1.396228946
		200	7530.56738	7712.80711	102.42		
		50	2909.860004	2928.77409	100.65		
S_4	2859.860004	100	2959.860004	2880.53576	97.32	98.95333333	1.683524043
		200	3059.860004	3025.89556	98.89		

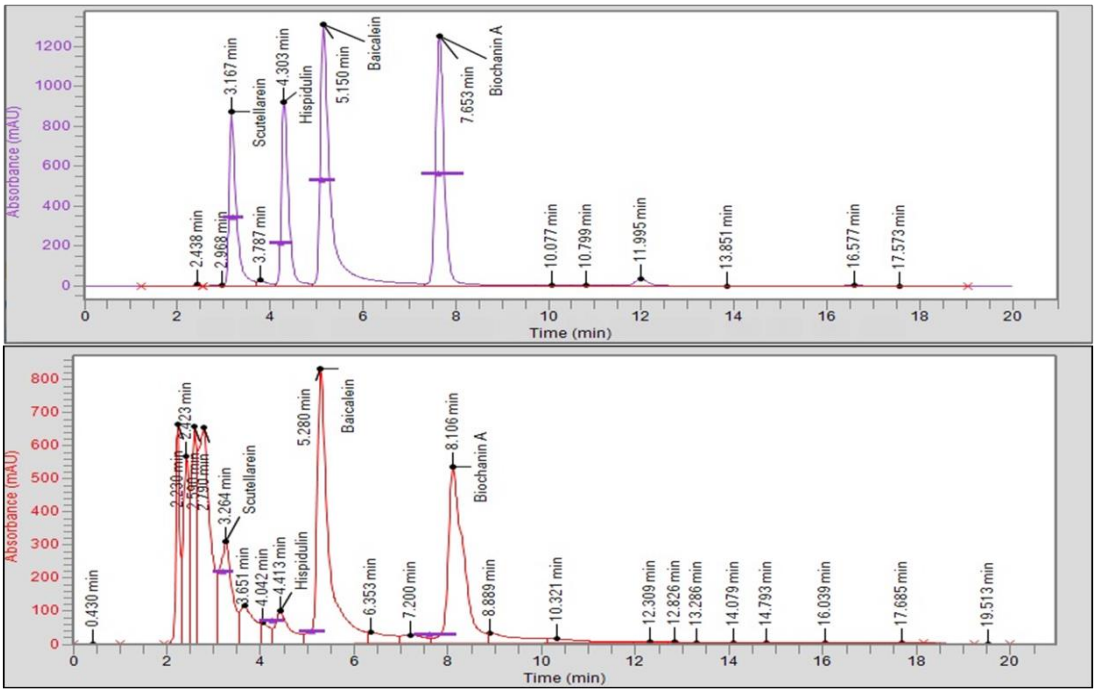

FIGURE 2 HPLC chromatogram of, (A) mixed standard solution containing the four quantitative compounds and (B) a representative sample solution 'Rl' of *Oroxylum indicum*

Quantitative analysis of multi-components by single marker

To determine and authenticate the effectiveness of single marker (Baicalein) for quantitative study of multi components in *O. indicum*, contents of scutellarin, hispidulin and biochanin-A were calculated using ESM and relative correction factors (F_x). The value of F_x was calculated as an average value, under different injection volume (2, 5, 10, 15 and 20 µl) of *O. indicum* sample as shown in Table V.

The values F_x with different column and instrument is shown in Table 5. Relative error was calculated based on equation (e). The relative error signifies that, if variation in different column(s), instruments, laboratories and among analyte are insignificant, the divergence of QAMS from ESM is small too (Table 6). In this manner the effectiveness of both the developed method and QAMS is validated for estimating concentrations of different flavonoids of *O. indicum*.

Relative Error, $RE\% = \frac{QAMS-ESM}{ESM} \times 100$ (e)

TABLE 5 Relative correction factor under different injection volume (n=6)

Injection volume (μL)	Value of RCF		
	F_a	F_b	F_c
2	0.828288	1.049456	1.611816
5	0.827457	1.072765	1.614426
10	0.832952	1.061277	1.664813
15	0.861006	1.071939	1.596424
20	0.820843	1.031432	1.601746
Mean	0.834102	1.05736	1.617845
RSD %	1.875509	1.638436	1.684854

F_a: $f_{scutellarin}/f_{baicalein}$; F_b: $f_{hispidulin}/f_{baicalein}$; F_c: $f_{biochanin\text{-}A}/f_{baicalein}$

TABLE 6 Relative correction factor with different instrument (n=6)

Instrument	Column	Value of RCF		
		F_a	F_b	F_c
Perkin Elmer, Flexar	Brownlee C18	0.828288	1.617198	1.656731
Shimadzu, Nexera	Shim-pack GIST-HP C18	0.746394	1.344291	1.766166

TABLE 7 Assessment of ESM and QAMS (mg/g) in 21 different samples of *Oroxylum indicum*

Sample	External Standard method (mg/g)				QAMS method (mg/g)					
	S_1	S_2	S_3	S_4	S_1	RE %	S_2	RE%	S_4	RE %
Rf	1953.419	609.4838	4299.154	2258.679	1958.8962	0.2804092	628.107456	3.0556374	2349.237014	4.0093242
Rs	1109.331	241.0999	6828.925	937.0506	1118.863583	0.859268	239.5066241	0.6608397	937.5125985	0.0493031 5
Rb	1272.269	128.3527	322.383	485.0985	1224.728417	3.736649	123.9436565	3.4350924	471.3811576	2.8277361 8
Rl	3887.852	1376.186	7306.757	2864.372	3887.808407	0.001127	1394.717668	1.3465899	2868.751117	0.1528952 4
Sf	1890.597	930.0051	5727.217	2814.646	1795.083839	5.052001 7	980.7735707	5.4589413	2774.045163	1.4424888 2
Ss	1002.184	205.8383	3435.148	426.1418	1020.106426	1.788290 3	221.6927524	7.7023894	418.0855389	1.8905113 6
Sb	447.1428	208.7733	380.1893	749.0912	464.9787965	3.988874 5	204.1651036	2.2072897	776.9268068	3.7159238 6
Sl	7947.431	5674.488	9902	6955.746	7866.035052	1.024183 4	5583.504096	1.6033886	6954.674767	0.0154060 6
Hb	381.6029	65.24421	396.8943	495.9325	340.0346196	6.126668 4	62.89065897	3.6072949	489.3331253	1.3306964
Hs	1669.717	358.3812	9491.606	1258.725	1628.298742	2.480547 1	353.7429572	1.2942081	1222.702133	2.8618179 9
Hf	936.5308	354.8554	2239.498	608.9443	959.0472377	2.404242 7	363.1315975	2.3322651	619.0902153	1.6661464
Hl	2077.837	871.3589	4674.627	2690.264	2104.184937	1.268065 7	890.2218455	2.1647778	2733.298104	1.5996245 9
Nf	2191.854	1102.09	1903.416	937.7425	2249.845212	2.645756 5	1132.985712	2.8033763	972.6086169	3.7180893 4
Ns	1527.16	676.7896	2191.543	20894.32	1756.848683	15.04026 5	715.2173437	5.6779526	21150.6537	1.2267988 7
Nb	1923.354	153.2516	345.9553	1880.691	1792.233123	6.817298 1	151.0315758	1.4486009	1812.166064	3.6435994 1
Nl	1594.521	699.4413	1862.197	1995.723	1646.483491	3.258834 3	737.3564927	5.4207786	2009.838309	0.7072660 1
Bl	7466.277	5625.715	2800.671	42828.01	7423.544788	0.572342 7	5794.69798	3.0037668	42641.69816	0.4350249 6
Bb	2247.281	114.1899	1056.889	7867.808	2764.354427	23.00883 4	121.1602329	6.1042033	8145.066064	3.5239594 3
Kb	1746.96	333.6802	966.3676	641.5023	1342.31578	23.16275 6	271.8542402	18.528504	531.8338147	17.095565 5
Kl	4498.513	3132.334	810.8322	11583.32	4323.639122	3.887374 8	3054.569329	2.4826339	10874.3783	6.1204048 3
Ks	6217.297	1104.261	2803.111	27509.26	6562.876326	5.558347 5	1191.212959	7.8741847	26894.60556	2.2343577 2

HCA and fingerprint analysis of samples

Table 8 shows the proximities amongst the samples. Stacked plot (Figure 5) was used as characteristic chromatogram for evaluating similarity of different samples through HPLC fingerprint. The HCA results showed relationship and distribution pattern among *O. indicum* samples from different locations, which is clearly displayed in the dendogram (Figure 3) and icicles (Figure 4). The dendogram evidently classified the samples into two major clusters. The cluster results were consistent with the icicles. There are many methods which have been developed for flavonoid quantification in *Oroxylum indicum* through High Pressure Liquid Chromatography [25-27]. In another work, chromatographic fingerprint method combined with QAMS was developed by Peng *et al.,* (2019) [28]. But the current proposed method is a swift, cost effective, accurate and cost effective RP-HPLC method using basic UV-Visible detector, developed for co-quantification of important flavonoids. Our method ensures satisfactory resolution and use of QAMS guarantees precise quantification of all targeted phytoconstituents using internal reference only. The calculations for F_x of different plant parts of *Oroxylum indicum* further provide data for precise estimation of phytoconstituents, since different parts are used for preparation of different Ayurvedic formulations. The fingerprint technique focuses on identifying and assessing the stability of samples and is accepted by WHO, 1991 [29], as a policy for quality assessment of herbal medicines. HPLC combined with QAMS plays the most important role among all fingerprint methods.

TABLE 8 Euclidean distance between samples of *Oroxylum indicum* from different locations

	1:RL	2:SL	3:HL	1:RL	5:BL	6:KL
1:RL	.000	.034	.055	.116	.203	1.217
2:SL		.000	.044	.094	.181	1.195
3:HL			.000	.071	.190	1.200
4:NL				.000	.142	1.137
5:BL					.000	1.015
6:KL						.000

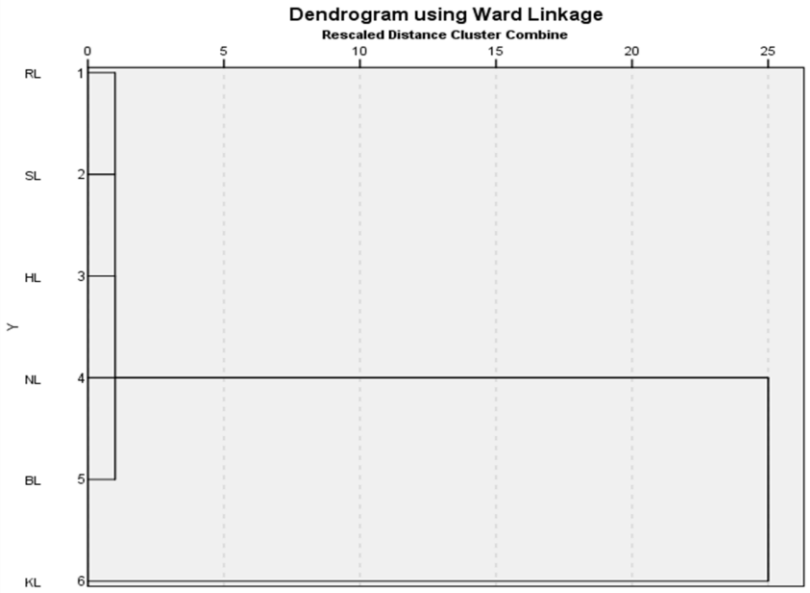

FIGURE 3 Dendogram of hierarchical cluster analysis for Leaf samples of *Oroxylum indicum* tested from six different locations. Abscissa indicates the squared Euclidean distances and the ordinate expresses the samples

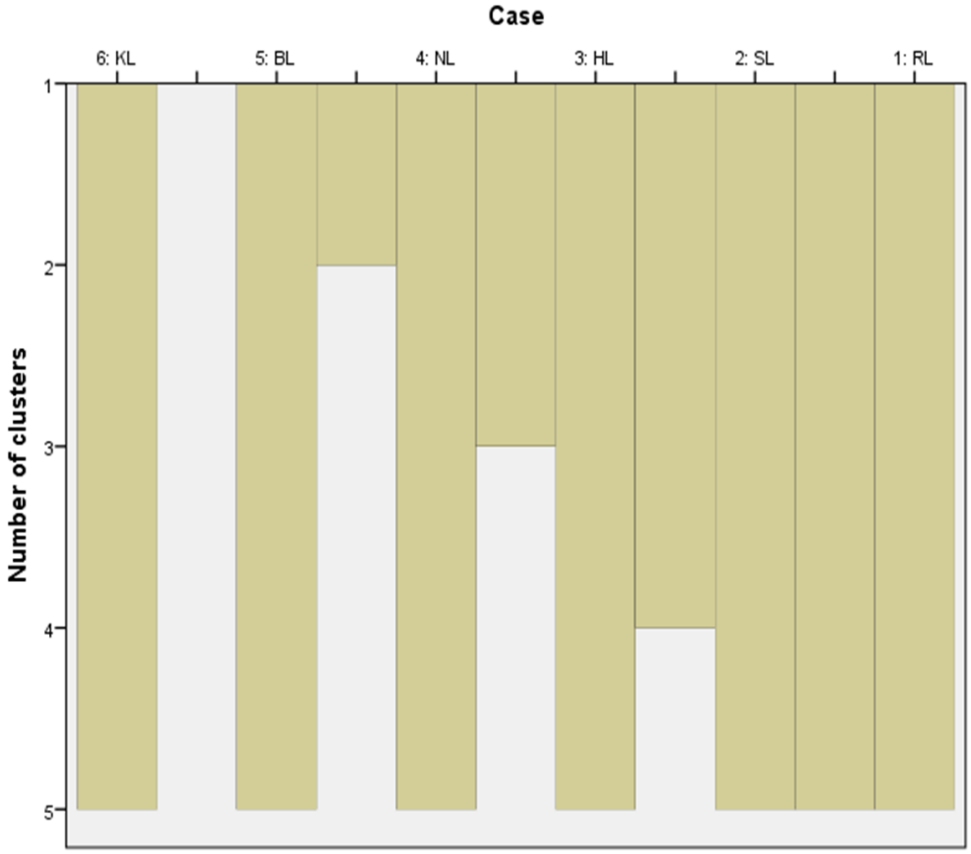

FIGURE 4 Icicles for the leaf samples of *Oroxylum indicum* from varied locations

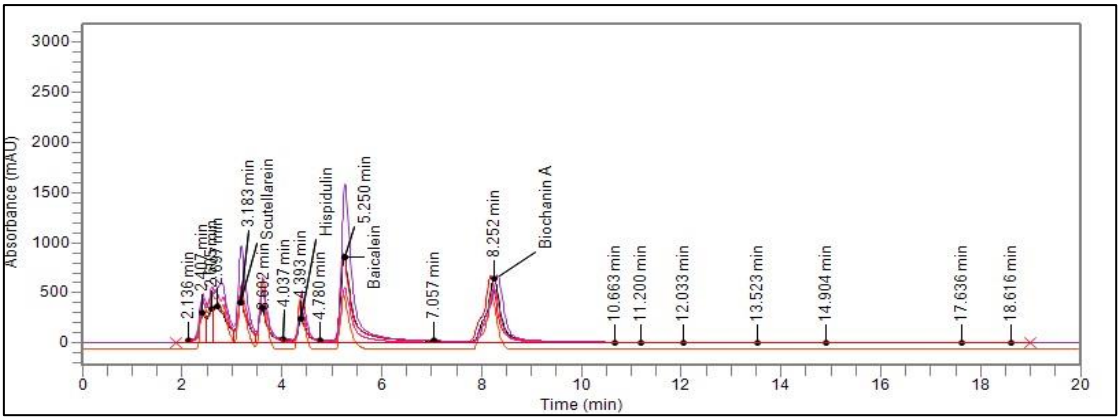

FIGURE 5 Stacked plot of characteristic HPLC fingerprint of *Oroxylum indicum*

Conclusion

The HPLC method validation showed reasonable RSD (<2%, as standardized by Indian Pharmacopoeia) for LOQ, LOD, recovery and repeatability tests. Reproducibility, precision and sensitivity of the method proves it as a powerful tool which can be applied to the holistic quality control of *O. indicum.* The chromatographic fingerprints showed a small diversity of chemical constituents in different samples of *O. indicum* from varied locations. Additionally, HCA method clustered the samples into two classes which are in accordance with the fact that it belongs to a monotypic genus.

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