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FULL ARTICLE

Phytochemical profiling of the stem bark of *Betula utilis* from different geographical regions of India using UHPLC-ESI-MS/MS

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Abstract

A simple, rapid, sensitive, and reliable ultra-high-performance liquid chromatography-hybrid linear ion trap triple quadrupole mass spectrometry method was developed and validated for simultaneous determination of 10 bioactive compounds in stem bark of *Betula utilis* grown in high altitude of Himalaya, India. The objective of the study is to develop and validate ultra-high-performance liquid chromatography-hybrid linear ion trap triple quadrupole mass spectrometry for investigation of geographical variations of triterpenoids, phenolics, and flavonoids contents in stem bark of *B. utilis*. The validated method was successfully applied to investigate geographical variations of triterpenoids, phenolics, and flavonoids in stem bark of *B. utilis*. The contents of betulinic acid and oleanolic acid were detected higher among selected analytes. The present variation study reveals great importance for the application and overall assessment of *B. utilis*.

KEYWORDS
Betula utilis, quantitation, tandem mass spectrometry, triterpenoids, UHPLC

1 | INTRODUCTION

Betula utilis D. Don (family Betulaceae) is long lived for more than 400 years old and is commonly known as Himalayan birch or *bhojpatra* or *Bhurja* in Sanskrit. It is universally known for its importance as a medicinal plant.[1,2] *Betula utilis* often grows at high altitudes in the Himalayas, mainly on slopes with unstable soils or under high snow pressure. The stem bark of *B. utilis* is substantially used in Ayurveda and Unani system of medicine for treatment of various diseases and ailments, that is, skin disinfectant, wound healing, bronchitis, leprosy, convulsions, and diseases related to ear and blood.[3–6] There have been reports that pharmacologically it has antimicrobial,[7] antihyperglycemic,[8] anticancer, antioxidant, and anti HIV activities.[9–11]

The bark of birch tree has been the subject of scientific research and industry in the modern world and attracted high interest due to the presence of triterpenoids, that is, betulinic acid and oleanolic acid, which were amply known for their anticancer activities. Betulinic acid is a novel anticancer drug that induces apoptosis. Hence, it differs from classical anticancer agents.[12,13] Other classes of compounds reported from *B. utilis* species are phenolics and flavonoids.[14,15]

In the earlier reports, high-performance thin-layer chromatography[16] was used for the quantification of components in *B. utilis*, which exhibited low sensitivity and low resolution. Hence, in the present study ultra-high-performance liquid chromatography (UHPLC) is being used for progressive output. UHPLC-triple quadrupole/ linear ion trap (QqQ_{LIT}) mass spectrometer provides

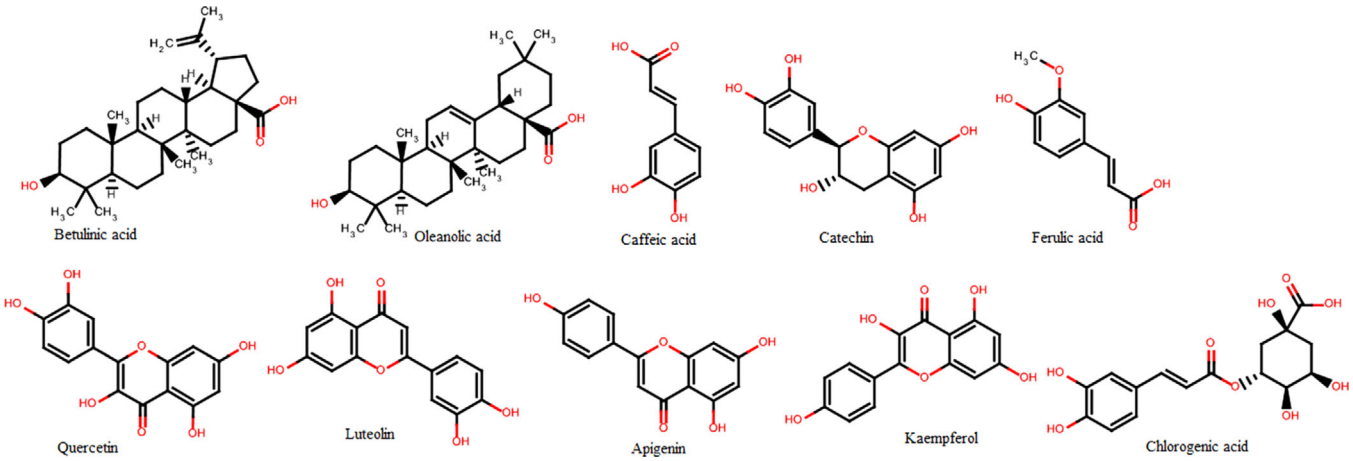

FIGURE 1 Chemical structure of 10 reference analytes

an ease to detect the analytes available in ultra-low concentration owing to its particular and higher grade sensitivity. Multiple reaction-monitoring (MRM) technique is preferable in the study due to its precision, sensitivity, and concurrent quantitation of chosen analytes in highly compounded matrices.

Betula plant, owing to its therapeutic characteristics, is a subject of considerable interest, and therefore, emphasis on the development of newer formulations is under process. Despite everything, as far as we are aware, no report of quantitative inspection of the selected molecules by using UHPLC-QqQ$_{LIT}$ techniques in *B. utilis* stem barks is available. The present study involves investigating a method engaging MRM scans procedure for quantification of various components in negative ionization mode using UHPLC-QqQ$_{LIT}$ tandem mass spectrometric technique, which permits the concurrent quantification of triterpenoids, phenolics, and flavonoids in stem barks of *B. utilis*.

2 | MATERIALS AND METHOD

2.1 | Reagents, chemicals, and materials

The stem barks of the Himalayan Silver Birch from four locations (27 accessions), that is, Jammu and Kashmir, Himachal Pradesh, Sikkim, and Uttarakhand were collected during August-September 2014. The plant taxonomists at CSIR-National Botanical Research Institute, Lucknow, India have authenticated the samples. The details are available as Supporting Information Materials in Table S1.

The analytical reference standards betulinic acid, apigenin, kaempferol, ferulic acid, caffeic acid, chlorogenic acid, catechin, and oleanolic acid of purity percentage ≥96% were purchased from Sigma Aldrich Ltd. Analytical reference standards of quercetin and luteolin (purity percentage ≥ 95%) were purchased from Extrasyntheses (Genay, France).

Solvents for mobile phase like methanol (LCMS grade), acetonitrile, and formic acid (analytical grade) were purchased from Fluka, Sigma-Aldrich (St. Louis, MO). Ultra-high-purity water used as mobile phase was prepared using a Milli-Q water purification system (Millipore Corporation, Bed- ford, MA). Syringe filters (0.22 µm) were purchased from Millipore (Billerica, MA, USA). The chemical structures of these standards are shown in Figure 1.

2.2 | Extraction and sample preparation

The dried bark of the plant (10.0 g) were milled into powder and sieved through a 40-mesh sieve and extracted with methanol (1 L) in an extractor (a stoppered container) and kept at room temperature for 36 hours until the soluble matter has dissolved. Further, the extract was filtered by using Whatman filter paper and solvent evaporated under reduced pressure to obtain completely dry extract using rotatory evaporator (Buchi Rotavapor-R2, Flawil, Switzerland) at 45°C. Each sample was processed in triplicates and extractive yield has been given in Table S1. Dried residues (1 mg) from each sample were precisely measured and solvated in 1 mL of methanol. Ultrasonicator (Bandelin SONOREX, Berlin) was used for thorough mixing. The mixed solutions were further filtered via syringe filter of 0.22 µm dia (Millex-GV, PVDF; Merck Millipore, Darmstadt, Germany). The filtrates were utilized to compose final working concentrations by using methanol as a diluent. Internal standards (50 µL) were added in final working solution for spike study, vortexed for 30 seconds for proper mixing. Finally, for analysis, only 5 µL aliquot was found sufficient for injection into the UHPLC-MS/MS system.

2.3 | Preparation of standard solution

A mixed standard stock solution was made ready in methanol carrying triterpenoid (betulinic acid, oleanolic acid), phenolics (ferulic acid, caffeic acid, and chlorogenic acid), and flavonoids (quercetin, kaempferol, apigenin, catechin, and luteolin). Further, working standards solutions ranges from 0.5 to 1000 ng mL^{-1} were prepared from mixed

standards after dilution with methanol and utilized for making calibration curve plot. In the present study, curcumin at a final concentration of 40 ng mL^{-1} (40 µL of internal standards mixture of 1000 ng mL^{-1} of curcumin in methanol) was used as an internal standard; in the negative mode, it was spiked to each concentration solution and mixed perfectly. The prepared stock of standards as well as the working solutions was stored at −20°C until further use and additionally, prior to injection, they were vortexed properly.

2.4 | Instrumentation and analytical conditions

Acquity UHPLC was used as an instrument that incorporated with a binary pump and an autosampler (Waters, Milford, MA) along with an injector loop of 10 µL capacity. Acquity BEH C18 (2.1 mm × 50 mm, 1.7 µm; Waters, Milford, MA) analytical column at a column temperature of 30°C was used for compound separation. The mobile phase was prepared by using 0.1% (v/v) formic acid water (A) and acetonitrile (B) and kept at 0.4 mL min^{-1} flow rate. The programming for the sample run was followed in a gradient manner, which started with the linear increase of solvent B from 5% to 77% B over 0.8 min, followed by hold of 77% B for 2 min, then up to 90% B over 2.5 min, again hold of 90% B up to 3.5 min, return to the initial condition over 2.5 min with a sample injection volume of 5 µL.

The current experiment was carried out in the UHPLC system, which was coupled to triple-quadrupole linear ion trap mass spectrometer (API 4000 QTRAP™ MS/MS system from AB Sciex, Concord, ON, Canada) equipped with electrospray (Turbo V™) ion source operated in negative ionization mode. The parameters for negative ionization mode were optimized as follows: the turbo spray temperature 550°C, ion spray voltage was −4200 V; nebulizer gas (gas 1), 20 psi; heater gas (gas 2), 20 psi; collision gas, medium; the curtain gas (CUR) was kept at 20 psi. The mass spectrometric conditions were optimized by infusing 70 ng mL^{-1} solutions of the analytes dissolved in methanol at 10 µL min^{-1} using a Harvard 22 syringe pump (Harvard Apparatus, South Natick, MA). Further, regarding quantification through MRM in case of every compound, product ions with highest abundance from the precursor ion have been chosen. Analyst 1.5.1 software package (AB Sciex) was used for instrument control and data acquisition and the spectra covered the range from *m/z* 100 to 1000 during full-scan ESI-MS analysis.

3 | RESULTS AND DISCUSSION

3.1 | Optimization of chromatographic and MS/MS conditions

The most important chromatographic factors such as flow rate, stationary, and mobile phase that influence the analysis in a critical way were considered appropriately for the separation of molecules. In this study, complete separation of close analytes is not required for MS/MS detection. Different combinations of solvents such as methanol-water, acetonitrile-water, methanol-formic acid aqueous solution, and acetonitrile-formic acid aqueous solution were examined to finalize the mobile phase but acetonitrile-formic acid aqueous solution was found most suitable for the satisfactory separation and good resolution of analytes. An acquity UPLC BEH C18 column of dimensions 2.1 mm × 50 mm, 1.7 µm (Waters, Milford, MA) was selected for analysis as it was found appropriate as compared to other tested columns. Formic acid was found more effective regarding ionization of compounds in the negative ESI mode. Various concentrations of formic acid with water (0.05%, 0.1%, and 0.2%) were evaluated but finally formic acid of 0.1% concentration was selected after screening. The present analysis was performed by gradient programming using two mobile phases, that is, acetonitrile and 0.1% formic acid in water at a flow rate of 0.4 mL/min at 30°C column temperature exhibited in separation of all the analytes in 6 min chromatographic run time.

All the MS parameters for the analytes such as declustering potential, collision energy, entrance potential, and cell exit potential including product and precursor ion were optimized by flow injection analysis in negative ESI mode. Further on the basis of the retention behavior, MS fragmentation pattern as $[M\text{-}H\text{-}COO]^-$, $[M\text{-}H\text{-}COO\text{-}CH_3]^-$, and $[M\text{-}CO\text{-}H_2O]$ as well as the information such as quasimolecular ions $[M\text{-}H]^-$ of the analytes afterward comparing them against standards and literature study helped in the chemical structures characterization.[17–19] MRM parameters were optimized to identify the specific, most abundant, and stable MRM transition for each analyte shown in Supporting Information Materials and Table S2. MRM extracted ion chromatogram of all the compounds is shown in Figure 2.

3.2 | Analytical method validation

The developed method (UHPLC-MRM) for quantitative analysis of analytes in the present study was validated as per the guidelines of international conference on harmonization (ICH, Q2R1) by limit of quantitations (LOQs) and limit of detections (LODs), linearity, precision, recovery, and solution stability.

3.2.1 | Linearity, LOD, and LOQ

For the calibration curve, construction different working concentrations were prepared by using the stock solution after dilution with methanol. Linearity of the method was determined by plotting the area ratio of analytes/IS against the known concentration of analytes. The least squares regression analysis ($1/x^2$) technique was used for calibration curves construction. The LOD and LOQ were measured with S/N of 3 and 10, respectively, as criteria. The results are listed in Table 1. All the calibration curves produced good linearity with correlation coefficients (r^2) from 0.9989 to 0.9999 within the test ranges. The LODs for each analyte varied from 0.09 to 5.12 ng mL^{-1} and LOQs from 0.35 to 6.05 ng $mL.^{-1}$

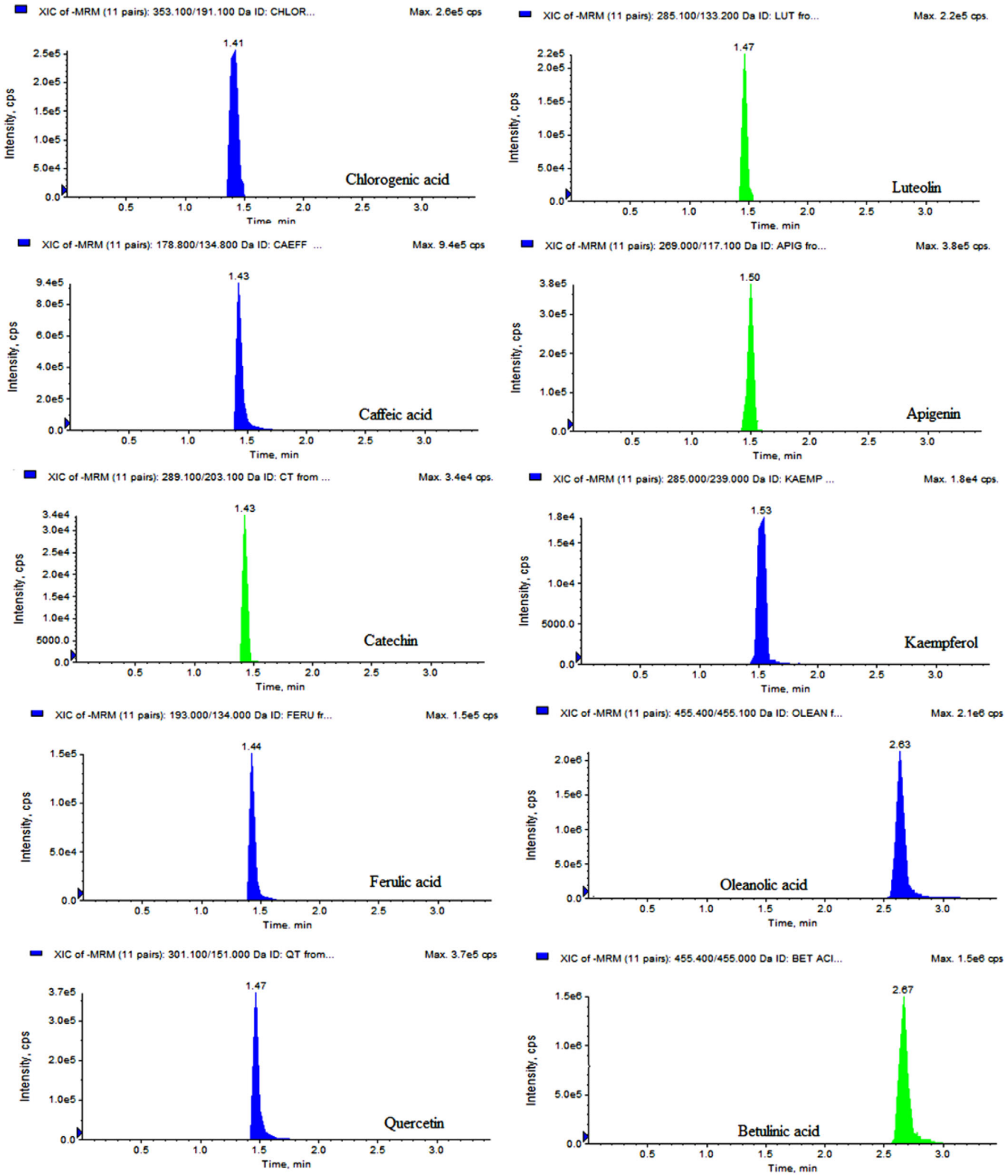

FIGURE 2 UHPLC-MRM extracted ion chromatogram of analytes

TABLE 1 Regression equations, correlation coefficients (r^2), linearity ranges, limit of detection (LOD), limit of quantitation (LOQ), precision, stability, and recovery for 10 reference analytes

Analytes	Regression equation	r^2	Linear range, ng/mL	LOD, ng/mL	LOQ, ng/mL	Precision RSD (%)		Stability RSD (n = 5)	Recovery RSD (%)
						Intraday (n = 6)	Interday (n = 6)		
Chlorogenic acid	y = 976.6x + 539.8	0.9995	1-100	0.12	0.66	1.03	2.26	1.43	0.92
Caffeic acid	y = 2215.6x + 3146	0.9998	0.5-100	0.09	0.24	1.67	1.11	1.32	2.22
Ferulic acid	y = 379.8x – 566.5	0.9999	10-250	3.34	4.12	1.53	2.55	2.19	3.62
Quercetin	y = 956.7x – 879.5	0.9995	1-100	0.30	0.67	0.89	1.06	0.84	0.54
Luteolin	y = 340.7x + 1213	0.9989	10-500	2.14	3.73	0.22	0.61	2.15	1.42
Kaempferol	y = 61.31x + 832.7	0.9998	1-250	0.13	0.39	1.82	0.93	1.13	1.54
Catechin	y = 76.32x - 1016	0.9996	25-500	5.12	6.05	1.55	3.36	3.15	1.24
Apigenin	y = 1048.7x + 3114	0.9993	1-250	0.13	0.64	1.62	2.13	1.05	2.46
Betulinic acid	y = = 5155.4x - 5239	0.9993	0.5-100	0.10	0.35	1.26	1.21	2.33	1.67
Oleanolic acid	y = 6792.3x + 5685	0.9998	1-100	0.15	0.73	0.92	2.11	2.29	2.02

3.2.2 | Precision, stability, and recovery

Intra- and interday variations of the developed method were evaluated by determination of 10 analytes in six replicates on a single day and by duplicating the experiments over a period of three successive days. Relative standard deviation (RSD) was used for precision determination, which was less than 3.36%. Stability of sample solutions was checked by replicate injections at 0, 2, 4, 8, 12, and 24 hours, which was stored at room temperature. The stability RSD% value of 10 analytes was ≤3.15%. Recovery test of the analytical standards into the samples was performed by spiking three different concentration levels (high, middle, and low) to evaluate the accuracy. At each level, three replicates were performed. The developed analytical method showed overall recovery in the range of 98.15-101.33% (RSD ≤ 3.62%) with good accuracy for all the analytes (Table 1).

3.3 | UHPLC-ESI-MS/MS characterization of compounds

3.3.1 | Triterpenoids

Betulinic acid characterized having parent molecular ion 455.400 $(M-H^-)$ found product ion at *m/z* 438 and 411 for loss of OH and CO_2H, respectively, and oleanolic acid detected as parent ion *m/z* value 455.800 and product ions at *m/z* 392 (after successive loss of OH, CO, and H_2O).

3.3.2 | Phenolics

Chlorogenic acid was characterized based on its *m/z* value at 353.100 $(M-H)^-$ and it was further supported by the product ion at *m/z* 191.000 (M-163; loss of $C_6H_5O_2$, $3H_2O$), whereas in case of caffeic acid *m/z* 178.800 and product ion at 134.800 (M-44; loss of CO_2). In case of ferulic acid, parent ion *m/z* was detected as 193.00 and product ion at *m/z* 134.000 (M-59; loss of CH_3 and CO_2).

3.3.3 | Flavonoids

Quercetin was identified on the basis of its molecular ion at *m/z* 301.100 and product ion at *m/z* 151.000 (M-150; loss of $C_7H_20_4$). kaempferol parent ion at *m/z* 285.000 $(M-H)^-$, and its precursor ion at *m/z* 239.000 (M-46; loss of CH_2O_2), apigenin parent ion at *m/z* 269.000, whereas product ion at *m/z* 117.100, catechin parent ion identified at *m/z* 289.100 $(M-H)^-$, and further precursor ion at *m/z* 203.000 due to cleavage of A ring of flavon-3-ol), and parent ion of luteolin found at *m/z* value of 285.100 and product ion at *m/z* 133.200.

3.4 | Quantitative analysis in *B. utilis*

The developed UHPLC-ESI-MS/MS method was applied to quantify 10 bioactive compounds in stem barks of *B. utilis*, which were collected from different geographical locations. The present study showed remarkable differences in the quantity of terpenoids, phenolics, and flavonoids during quantitative analysis of 27 accessions. Betulinic acid and oleanolic acid were detected most abundant in all the stem bark samples of *B. utilis* among all the quantified analytes as listed in Table 2.

Triterpenoids betulinic acid (24.2 mg g^{-1}) and oleanolic acid (22.2 mg g^{-1}) were present in highest amount in stem barks collected from Golaba, Kullu, Himachal Pradesh, which clearly showed its characteristic pattern. Similarly, stem bark collected from the same place gave higher content of all the selected flavonoids also such as quercetin (0.116 mg g^{-1}), luteolin (1.320 mg g^{-1}), kaempferol (0.439 mg g^{-1}), apigenin (1.17 mg g^{-1}), and catechin (0.720 mg g^{-1}). In the case of phenolics, higher contents of caffeic acid (0.063 mg g^{-1}) and chlorogenic acid

TABLE 2 Contents (mg g^{-1}) of 10 reference analyte

Samples	Betulinic acid	Oleanolic acid	Caffeic acid	Catechin	Chlorogenic acid	Ferulic acid	Apigenin	Kaempferol	Luteolin	Quercetin
NBMP-1	7.64	9.42	0.063	0.44	0.30	0.046	0.017	0.014	0.013	0.046
NBMP-2	16.8	18.4	0.017	0.58	0.25	0.037	0.028	0.028	–	0.051
NBMP-3	23.3	25.8	0.045	0.32	0.06	0.042	0.018	0.057	–	0.022
NBMP-4	14.2	14.7	0.023	0.26	0.10	0.039	–	–	–	0.042
NBMP-5	9.91	10.8	0.045	0.51	0.16	0.041	–	–	–	–
NBMP-6	17.9	17.7	0.033	0.29	0.26	0.065	–	–	–	–
NBMP-7	12.4	12.3	0.051	0.57	0.21	0.046	–	–	–	0.038
NBMP-8	6.29	6.84	0.020	0.56	0.42	0.043	0.0198	–	–	0.027
NBMP-9	18.9	18.2	0.030	0.19	0.05	0.040	–	–	–	–
NBMP-10	6.26	6.3	–	0.60	0.32	0.042	–	–	0.014	0.036
NBMP-11	5.94	6.26	0.029	0.51	0.15	0.038	–	0.019	–	0.058
NBMP-12	3.33	3.87	–	0.41	0.10	0.047	–	0.044	–	0.042
NBMP-13	8.01	7.72	0.015	0.28	0.12	0.079	–	0.054	–	0.037
NBMP-14	14.9	15.5	0.013	0.31	0.09	0.043	0.049	–	–	–
NBMP-15	13.9	14.2	0.018	0.33	0.25	0.063	–	–	–	–
NBMP-16	8.07	7.9	0.029	0.49	0.20	0.046	–	–	–	0.040
NBMP-17	6.63	6.63	0.012	0.35	0.14	0.046	–	–	–	0.043
NBMP-18	13.2	12.9	0.015	0.43	0.18	0.111	1.17	0.439	1.320	0.116
NBMP-19	14.4	13.3	–	0.43	0.14	0.047	0.017	0.048	–	0.048
NBMP-20	6.05	5.18	0.010	0.28	0.16	0.045	–	0.009	–	0.040
NBMP-21	4.22	3.83	–	0.47	0.34	0.054	0.024	0.046	–	0.042
NBMP-22	24.2	22.2	0.031	0.11	0.04	0.217	–	0.046	–	–
NBMP-23	1.32	1.21	–	0.72	0.16	0.036	0.020	0.066	–	0.041
NBMP-24	0.09	–	0.046	0.50	0.04	0.154	0.019	0	0.133	0.071
NBMP-25	19.0	16.5	0.055	0.25	0.15	0.124	0.021	0.0172	0.094	0.115
NBMP-26	11.5	12.1	–	0.35	0.13	0.044	–	–	–	–
NBMP-27	18.3	17.5	0.033	0.37	0.04	0.110	–	–	–	0.068

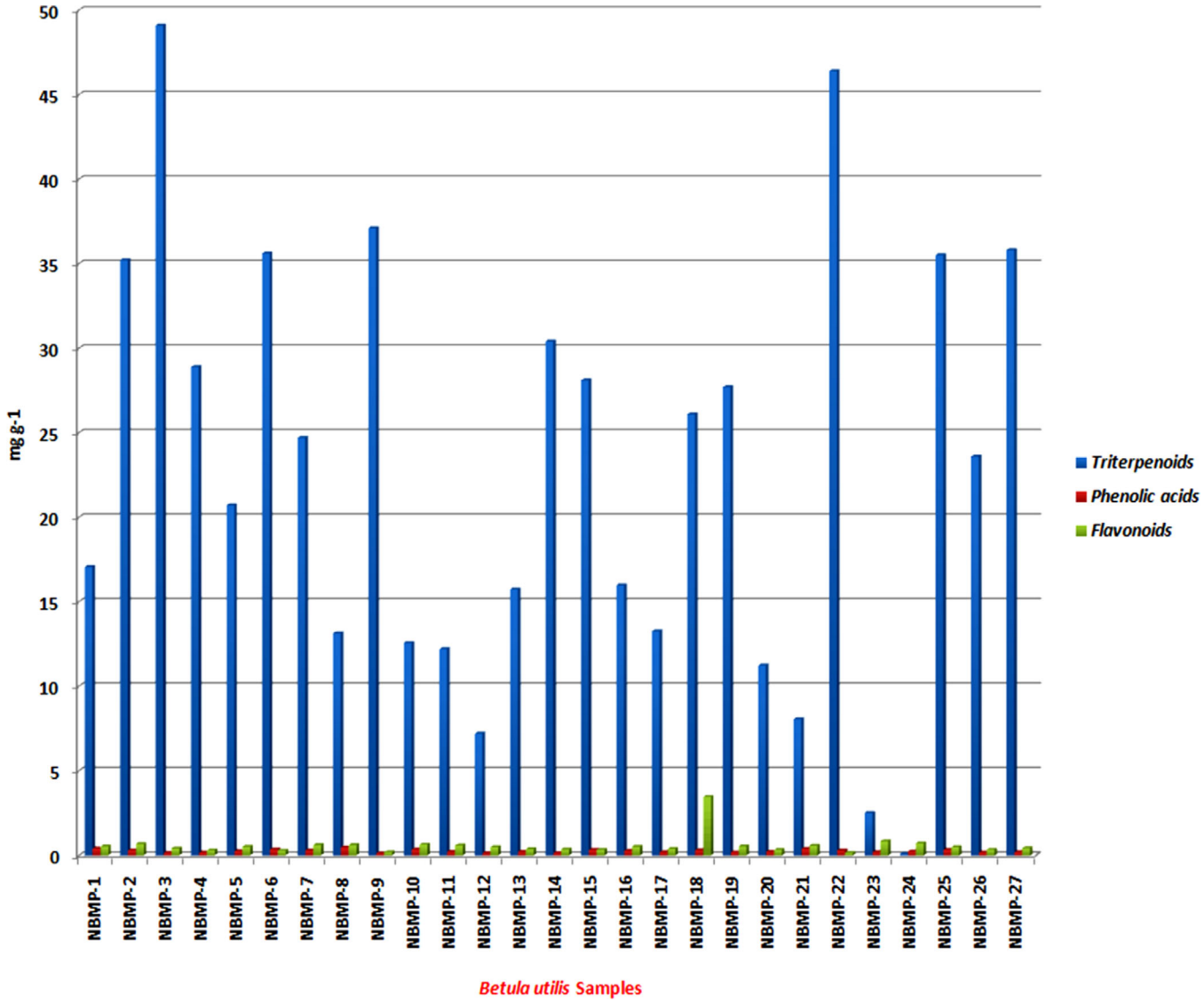

FIGURE 3 Graphical representation of geographical variation of reference analytes

(0.417 mg g^{-1}) were obtained in the samples collected from Khilenmarg, Gulmarg, and Jammu & Kashmir, but higher content of ferulic acid (0.217 mg g^{-1}) was determined in the samples from Golaba, Kullu, Himachal Pradesh.

Further, graphical representation of these observations is shown in Figure 3, which clearly explains the chemical variations among bioactive compounds due to different geographical locations, altitudes, and time of collection. Active components proportion of the bioactive analytes are undoubtedly responsible for the therapeutic medicinal properties of the plant and its effectivity and hence any alteration in their quantity may lead to the straight variations. Therefore, selection and quantification of active chemical markers is important in order to enhance the desired pharmacological effect on the basis of their content. The overall study exhibited that this method has played a significant role in comprehensive evaluation of 10 selected compounds, which could be used in future as the major markers for quality control of *B. utilis*. This comparative variation study is of great importance for the quality control of *B. utilis*.

4 | CONCLUSIONS

A sensitive, accurate, and reliable UHPLC-ESI-MS/MS method has been developed and validated for the simultaneous determination of triterpenoids (betulinic acid and oleanolic acid), phenolics (caffeic acid, ferulic acid, and chlorogenic acid), and flavonoids (quercetin, kaempferol, apigenin, catechin, and luteolin) from stem bark of *B. utilis*. The analytical results demonstrated strongly preferable performance in terms of linearity, intra- and interday precision, and accuracy as compared to previously reported methods. The quantitative result revealed significant variation of selected bioactive compounds from different geographical locations. The contents of betulinic acid

(24.2 mg g^{-1}) and oleanolic acid (22.2 mg g^{-1}) were detected highest in the accessions, which were collections from Himachal Pradesh. This study also showed that the collection of *B. utilis* from Himachal Pradesh might be the best place to obtain the higher amount of selected active phytoconstituents. This study would prove beneficial to the establishment of quality standards for overall assessment of *B. utilis*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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