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ORIGINAL PAPER

Simultaneous identification and quantification of pentacyclic triterpenoids and phenolic compounds from the leaves of *Boswellia serrata* using LC–MS/MS tandem mass spectrometry

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Abstract

Boswellia serrata (B. serrata) is an important medicinal plant widely used as dietary supplements to provide a support for osteoarthritic and inflammatory diseases. The occurrence of triterpenes in leaves of *B. serrata* is very little or none. Therefore, the qualitative and quantitative determination of phytoconstituents (triterpenes and phenolics) present in the leaves of *B. serrata* is very much needed. The aim of this study was to develop an easy, rapid, efficient and simultaneous liquid chromatography–mass spectrometry (LC–MS/MS) method for the identification and quantification of the compounds present in the leaves extract of *B. serrata*. The purification of ethyl acetate extracts of *B. serrata* was performed by solid phase extraction method, followed by HPLC-ESI-MS/MS analysis. Chromatographic parameters of the analytical method included negative electrospray ionization (ESI^-) with a flow of 0.5 mL/min in gradient mode consisting of acetonitrile (A) and water (B) containing 0.1% formic acid, at 20 °C. Total 19 compounds (13 triterpenes and 6 phenolic compounds) were separated, and simultaneously quantified using a validated LC–MS/MS method with high accuracy and sensitivity. Good linearity was obtained with $r^2 > 0.973$ in the calibration range. The overall recoveries were in a range between 95.78 and 100.2% with relative standard deviations (RSD) below 5% for the entire procedure of matrix spiking experiments. Overall, there was no ion suppression from the matrix. The quantification data showed that the total amount of triterpenes and phenolic compounds in the leaves of *B. serrata* ethyl acetate extract samples ranged from 14.54 to 102.14 mg/g and 2.14 to 93.12 mg/g of dry extract, respectively. This work provides, for the first time, a chromatographic fingerprinting analysis on the leaves of *B. serrata*. A rapid, efficient, and simultaneous liquid chromatography–mass spectrometry (LC–MS/MS) method was developed and used for the both identification and quantification of triterpenes and phenolic compounds in the leaves extracts of *B. serrata*. The method established in this work can be used as quality-control method for other market formulations or dietary supplements containing leaf extract of *B. serrata*.

Keywords *Boswellia serrata* · Leaf extract · LC-ESI-MS/MS · Triterpenes · Phenolic compounds

Introduction

Herbal remedies are increasingly used by patients with chronic diseases such as arthritis, osteoporosis, inflammatory bowel diseases (IBD), etc. [1, 2]. One such herbal supplement widely used by dietary supplement manufacturers to treat ulcerative colitis is *Boswellia serrata* (*B. serrata*) [3]. The therapeutic value of dried resinous gum derived from stem bark of the *B. serrata* tree, which grows in hilly areas of India, has been known since antiquity [4]. The oleo-gum resin from *B. serrata* is a traditional Ayurvedic remedy for inflammatory diseases also known Indian frankincense. For many years, natural products from *Boswellia* have been used in the prevention and cure of many serious diseases, including cancers [5]. *B. serrata* extracts (BSE) have shown anti-inflammatory, hepatoprotective, antitumor, anti-HIV, antimicrobial, anti-fungal, anti-ulcer, gastroprotective, hypoglycemic, and antihyperlipidemic activity etc. and are also reported in the literature [6–8]. The phytochemical content of *B. serrata* oleo-gum resin mainly reported from the stem

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and bark exudates of this plant. The gum oleoresin consists of 20–60% triterpenes, 5–10% essential oils, polysaccharides, etc. [9–11].

The plant leaves are used by Indian traditional medicine as an anti-inflammatory and analgesic agent to relieve arthritic pain, kidney stones, urinary tract and back pain [12, 13]. These pharmacological effects might be associated mainly with pentacyclic triterpenoids in the leaves. There are a very few or no reports described chemical constituents in the leaves of this plant, and somehow it is still unnoticed. Undoubtedly, it is very important to determine the bioactive compounds in the leaves of this plant. Therefore, a reliable and robust methods for separation, identification and quantitative analysis are required. There are many publications dealing with the determination of phenolic and terpenes compounds, but most of the methods are based on reverse-phase high-performance liquid chromatography (HPLC) techniques. LC–MS/MS is a technique that has important advantages over other chromatographic methods [14]. It requires minimal purification steps of multiple constituents, less solvents, and it allows the analysis of different samples and different analytes simultaneously, which translates into cost-effective method. ESI–MS provides the masses of analytes through their pseudo molecular ions and their identification through fragmented ions. The aim of this study was to develop an analytical method for simultaneous determination and quantification of triterpenes and phenolic compounds from leave extract of *B. serrata* using LC–MS/MS.

Experimental

Chemicals and reagents

Acetonitrile, formic acid, ammonium acetate, and acetic acid were HPLC grade, purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagent grade methanol, and ethyl acetate were obtained from Merck (Darmstadt, Germany). Ultra-pure water (Milli-Q, Millipore, USA) was used to prepare all the solutions. Analytical standards of serjanic acid, 11-keto-β-boswellic acid, maslinic acid, 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA), 3-*O*-acetyl-11-hydroxy-β-boswellic acid (from chem face, Chiana), madecassic acid, β-boswellic acid (β-BA), asiatic acid, alphitolic acid, β-Amyrin, and lupeol, corosolic acid, quinic acid, chlorogenic acid, rosmarinic acid, fisetin, quercetin, and myricetin were purchased from Sigma- Aldrich (St. Louis, MO, USA). The purity of all standards was > 95%.

Preparation of standard solution

The stock solution was prepared to contain 100 μg/mL^{-1} of each analyte, diluted in methanol 95% (v/v in water), named diluent solution (DS). Working standard solutions were prepared to contain 10 μg/mL^{-1} of each analyte, by diluting stock solution. All solutions were kept protected from light and stored at 4 °C.

Plant material and extraction method

Leaves of *B. serrata* Roxb. were collected from different regions of India. Authentication and identification of the specimens were done by the taxonomist. A voucher specimen (VSN/31/2042 & 2043) for leaves of *B. serrata* were deposited in the herbarium of the University laboratory. For the preparation of methanolic extract, 200 g of leaves was oven-dried at 50 °C, grounded to a coarse powder and kept in air-tight desiccators. The powdered leaves material (10.0 g) was drenched in MeOH (0.5 L) for 48 h and, thereafter, sonicated at 30% amplitude (pulser 5 s on/off) for 45 min, then again for 30 min at pulser 3 s on/off cycle. The methanolic extract was centrifuged for 10 min at 10,000 rpm. The supernatant was collected, filtered and concentrated in a rotary evaporator at 40 °C (Buchi Rotavapor R-200, Tokyo, Japan). This methanolic extract was further separated using organic (100 mL ethyl acetate) and aqueous (100 mL double distilled water). The ethyl acetate portion was dried under reduced pressure in a rotary evaporator at 40 °C. This extract was dissolved in HPLC grade methanol (10 mL), filtered via a Millipore filter (0.45 mm) and kept in a refrigerator until analysis.

Colorimetric method for triterpenoids analysis

Total triterpenoid was determined as described by Xiang et al. (2001) [15], with minor modifications. In brief, the stock solutions of 0.1 to 1 mg/mL were prepared by dissolving oleanolic acid (OA) in ethanol. The standard curve of OA was obtained by transferring 100, 200, 400, 600, and 800 μL from the stock solution (100 μg.mL^{-1}) to test tubes. 100 μL of the OA solution and 100 μL of perchloric acid ($HClO_4$) were heated at 60 °C for 10 min. 100 μL of 8% *w/v* vanillin in acetic acid were then added and the solutions were shaken and left for 2 min. 1000 μL of acetic acid (99.7%) was added to both quench the reaction and to dilute the sample. The resulting solutions were maintained for 20 min at room temperature. The same procedure was conducted after adding 200 μL of ethyl acetate leaf extract sample to the test tubes to determine the triterpenoid present in the extracts. A blank solution was similarly prepared and the absorbances were determined at 548 nm using a dual beam UV–Vis Genesys 10 spectrophotometer (Thermo Scientific, USA) equipped with 10 mm matched quartz cells.

Thin-layer chromatography (TLC)

Using micro-capillary pipettes, 10 µL of ethyl acetate extracts (10 mg/mL) of the leaves of *B. serrata* were applied on normal phase silica gel thin-layer plates (Kieselgel 60 F254, aluminium backed, Merck, Germany) and on reversed-phase thin-layer plates (RP-18 F254s, Merck, Germany) to detect compounds of a wide range of polarities. Toluene: ethyl acetate: formic acid (5:4:1, *v:v:v*) was used as an eluent for NP-TLC, while methanol: water: acetic acid (7:2:1) was used for RP-TLC. The development distance was 10 cm. To detect and identify various types of compounds (terpenes, phenolics, alkaloids, and flavonoids), TLC plates were developed with Vanillin-H_2SO_4, dragendorff reagent, aluminum chloride and Natural Products reagents [16]. The plates were observed in UV-light at 260 and 360 nm.

Clean up of ethyl acetate extract using solid phase extraction (SPE) procedure

SolE_XC18 reversed-phase cartridges, Cat# N0074410 (Thermo Scientific., USA) were used for solid phase extraction in order to purify and enrich terpenoids/phenolics and for separation of sugars and other interfering matrix compounds. All experiments were performed at a constant temperature of 25 ± 1 °C. The flow rate was 1 mL/min. The SPE cartridges was deprotonated before use by passing 2 mL of 2.5% (*v/v*) ammonia in methanol through the SP cartridge and next washing with methanol (3 mL) and water (3 mL). The extract was purified as follows: the cartridges were conditioned with 2 mL of methanol and next 2 mL of the methanol/water solution (80:20 v/v) and then 1 mL of the ethyl acetate sample of the at a concentration of 1 mg/mL was applied on the SPE column. 3 mL of the washing solution (methanol: water 80:20 (v/v)) was passed through the column and next the samples were eluted with 2 mL of 2.5% ammonia in methanol. The eluates were evaporated to dryness in a stream of N_2. The residues were dissolved in 2 mL of methanol-H_2O (4:1, v/v), and then, 0.5 mL was filtered using Millipore membranes (0.22 µm) and transferred to an auto-sampler vial for LC/ESI–MS/MS analysis.

Identification and quantification of triterpenes and phenolic compounds by LC/ESI-MS/MS

A Shimadzu LC20AD HPLC system (Kyoto, Japan) was used for the HPLC runs. The system equipped with two pumps, a vacuum degasser, an auto-sampler (SIL HTc, Kyoto, Japan), a controller module, and LC20AD series 1100 DAD detector. Chromatographic separation was performed on Waters Symmetry Shield RP 18 column (75 × 4.6 mm, 3.5 µm, Waters, Massachusetts, Ireland) at 20 °C temperature and the flow rate was 0.5 mL/min. The mobile phase was consisted of 100% acetonitrile (A) and water (B), both containing 0.1% formic acid, for gradient elution. The gradient program was performed in the following manner: 25% A at 0–1 min, 50% A at 2–15 min, 80% A at 16–30 min, and 90% A at 31–35 min. After this, 100% B was used for 5 min at 35–40 min. Wavelengths of 210, 254, 260, 280, 320 and 360 nm were used for detection. The data were compared to standard compounds following gradient was employed for the separation. Mass spectrometric detection was conducted on a QTRAP 4500 (AB SCIEX, Foster City, CA, USA) coupled with an electrospray ionization (ESI) source, and equipped with Analyst software (version 1.6.2) for data processing. The analytical method adopted the MRM mode. Mass analysis of compounds was performed using negative ion mode. The spray voltage was set to 5000 V. The heated ESI temperature was set 550 °C. MS conditions were set as follows: source voltage—3.0 kV; cone voltage—40.0 V; desolvation temperature—550 °C; capillary temperature—350 °C; nebulizing gas flow rate—6.0 L/min; sheath gas (N_2) pressure—40 psi; Aux gas (N_2) pressure—60 psi. Nitrogen Ion Source Gas GS1 and GS2 set at 30 and 60 psi. The collision assisted dissociation (CAD) was used at pressure 6 psi. Collision-induced dissociation (CID-MS^n) was applied to induce fragmentation of the molecular ions, and their fragments were analyzed using tandem mass spectrometry. Helium was used as collision gas at 0.8 m Torr. Collision energies of 15 and 30 eV were used to investigate neutral loss and product ions and scanning was performed using a mass range from 50 to 1000 m/*z*. Data from the literature, and authentic standard samples were used for the structural identifications of triterpenes. Quantitative analyses were performed by Multiple Reaction Monitoring (MRM) by monitoring the fragmentation of quasi-molecular ions for standard compounds and internal standard (IS, Ursolic acid, MRM transition $[M\text{-}H]^-$ 456.7 → 310.4) (Table 1). In this study, an internal standard, ursolic acid (UA) was added during sample preparation and analyzed at the same time as the sample. The ratio of analyte signal in the sample / internal standard was calculated. A linear equation ($y = mx + b$) was obtained. Concentration of the analyte was calculated by solving for x when y was the ratio of analyte signal/internal standard signal in the unknown sample.

Method validation

Developed LC/ESI–MS/MS analysis method was validated in terms of Limit of Detections (LOD), Limit of Quantifications (LOQ), linearity, precision and accuracy as per ICH Q2 R1 Guidelines [17]. Quantification was performed by external calibration curves with pure standards dissolved in methanol. LOD and LOQ for all compounds were calculated utilizing the S/N ratio methods based on the determination of the peak to peak noise. LOD and LOQ were therefore

Table 1 Molecular mass and fragmentation pattern of compounds identified in the ethyl acetate extracts of *Boswellia serrata* leaves

Peak	R_t (min)	*m/z*		Identified Compound	Molecular Formula	Molecular weight
		MS of $[M-H]^-$	MS/MS of $[M-H]^-$			
1	3.8	191.2	93.2, 85.4, 49.6	Quinic acid	$C_7H_{12}O_6$	192.2
2	6.9	499.7	467.3, 437.4, 409.6	Serjanic acid	$C_{31}H_{48}O_5$	500.7
3	11.4	469.1	453.7, 437.2, 373.4	11-keto-β-boswellic Acid (KBA)	$C_{30}H_{46}O_4$	470.7
4	12.2	471.7	445.3, 425.7, 393.1	Maslinic acid)	$C_{30}H_{48}O_4$	472.7
5	13.6	511.7	451.2, 361	3-*O*-acetyl-11-keto-β-B boswellic Acid (AKBA)	$C_{32}H_{48}O_5$	512.7
6	16.5	441.7	411.5, 347.4, 309.6	β-boswellic alcohol	$C_{30}H_{50}O_2$	442.7
7	17.2	513.7	452.4, 407.6, 358.7, 322.3	3-*O*-acetyl-11-hydroxy-β-boswellic acid	$C_{32}H_{50}O_5$	514.7
8	18.3	503.7	435.2, 407.7, 359.4	Madecassic acid	$C_{30}H_{48}O_6$	504.7
9	18.8	455.7	437.2, 409.4, 377.5	β-boswellic acid (β-BA)	$C_{30}H_{48}O_3$	456.7
10	19.6	487.6	441.2, 409.2, 391.6	Asiatic acid	$C_{30}H_{48}O_5$	488.6
11	21.3	471.6	405.1, 369.2	Alphitolic acid	$C_{30}H_{48}O_4$	472.6
12	23.4	425.7	396.6, 307.4, 205.7	β-amyrin	$C_{30}H_{50}O$	426.7
13	24.5	285.2	161.2, 135.6	Fisetin	$C_{15}H_{10}O_6$	286.2
14	25.7	353.3	191.3, 162.6	Chlorogenic acid	$C_{16}H_{18}O_9$	354.3
15	27.2	317.2	179.3, 137.6	Myricetin	$C_{16}H_{18}O_8$	318.2
16	28.6	301.2	179.3, 151.4, 121.7	Quercetin	$C_{15}H_{10}O_7$	302.2
17	29.4	467.7	408.3,358.2, 336.8, 189.5, 73.6	Lupeol	$C_{30}H_{48}O_4$	468.7
18	30.8	359.3	161.3, 133.7, 82.4	Rosmarinic acid	$C_{18}H_{16}O_8$	360.3
19	32.3	471.5	423.2, 405.6, 393.5	Corosilic acid	$C_{30}H_{48}O_4$	472.5

calculated as the concentrations producing a recognizable peak with a signal-to-noise ratio of 3:1 and 10:1, respectively. Linearity of the developed method was determined by plotting calibration curves with different concentrations of analytes versus peak area. From calibration, data regression equation and correlation coefficients were calculated via regression analysis. Precision of method was evaluated in terms of intra- and inter-day precision by analyzing the samples for a week by following the same extraction procedure in triplicate manner. Further, the samples were subjected to accuracy study. Accuracy was determined by analyzing samples to which reference analytes were added at three different concentrations followed by extraction and analysis. The recovery was determined using the following equation: Recovery (%) = 100 × [analyte peak area (sample spiked before extraction)—analyte peak area (sample)]/[analyte peak area (sample spiked after extraction)–analyte peak area (samples)]. Briefly, the ME was determined using the following equation: ME (%) = 100 × [analyte peak area (sample spiked after extraction)—analyte peak area (sample)]/average analyte peak area (internal standard) [18]. In these terms, a ME close to 100% depicts no ion suppression. The accuracy was determined as the relative mean error (RME) between the concentration of the analyte in the spiked biological sample and the theoretical concentration. ME (%) = [average analyte concentration (sample spiked)—mean analyte concentration (sample)—theoretical concentration]/theoretical concentration. Stability studies of standards in methanol, acetonitrile, and mobile phase were conducted at three QC levels under different storage conditions: at room temperature for 24 h (bench top), at − 70 °C for 60 days (long term), after three freeze–thaw cycles, and for 32 h at 4 °C in an auto-sampler tray. The accuracy was expressed as the relative error (RE), while the precision was evaluated with the relative standard deviations (RSD). Sample stability was confirmed based on the stability analysis results where the values for accuracy (± 15%) and precision (± 15%) found were within the acceptable limits [19].

Results

Phytochemical screening of ethyl acetate extracts of *Boswellia serrata* leaves

Thin-layer chromatograms of the extracts of the leaves of *Boswellia serrata showed* no reaction with Dragendorff reagent, indicated that the extracts were devoid of alkaloids. Pink to deep purple color was developed upon spraying with vanillin-H_2SO_4, which suggested the presence of triterpenoids and phenolic compounds. The TLC plates revealed the presence of flavonoids when sprayed with aluminium

trichloride ($AlCl_3$) and Natural Product reagent (NPR). The color changes were from quenching fluorescence to yellow, orange or blue color, that was typical for flavonoids acids and/or other phenolic acids.

Identification of pentacyclic triterpenes and phenolic compounds

Multiple Reaction Monitoring (MRM) was performed by monitoring the fragmentation of quasi-molecular ions for standard compounds (Table 1). Diode Array Detector (DAD) was also used at 210 nm for β-BA and β-amyrin, 254 nm for flavonoids, 260 nm for AKBA, and 280 nm for serjanic acid. It is commonly demonstrated that the LC system coupled with MS is an excellent analytical method for simultaneously identifying complicated metabolites owing to the product ions produced from the fragmentation of a selected precursor ion. Therefore, compound profiles in the leaves of *B. serrata* ethyl acetate extract were tentatively investigated by LC-ESI- MS/MS through the full scan negative ESI mode. In the current research, a complete chromatographic separation of various compounds, including major and minor peaks, was reached within 40 min (Fig. 1). Altogether nineteen (19) compounds with retention times (R_t)

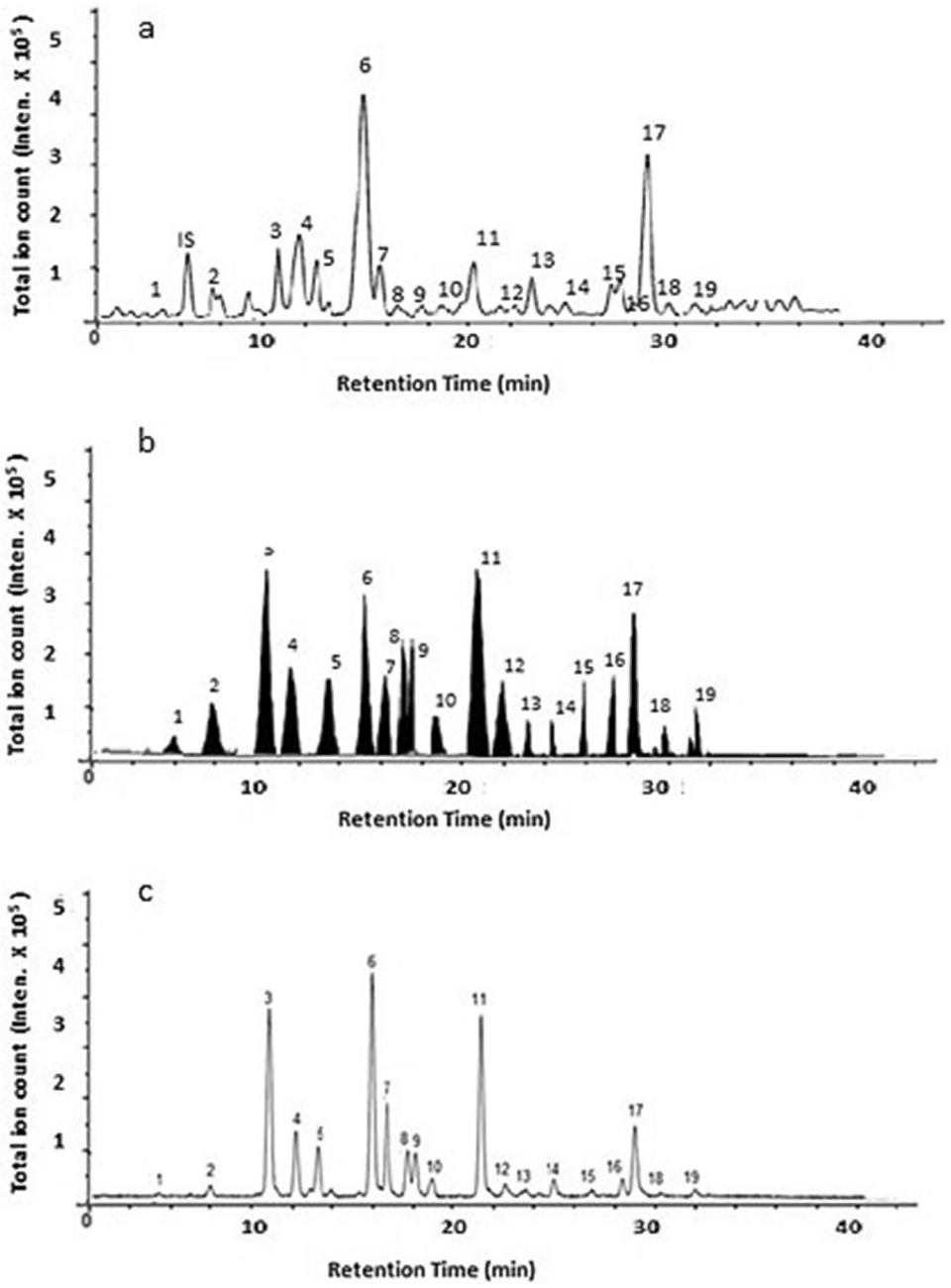

Fig. 1 LC–MS total ion chromatograms (TIC) of ethyl acetate extracts of *Boswellia serrata* leaves (**a**), reconstructed TIC chromatograms of the extract (**b**) and aligned with the standard mixtures of reference compounds (**c**) quinic acid (1), serjanic acid (2), 11-keto-β-boswellic acid (3), maslinic acid (4), 3-*O*-acetyl-11-keto-β-boswellic acid (5), 3α-hydroxy-24- hydroxymethyl -urs-12-ene-24-ol (β- boswellic alcohol) (6), 3-*O*-acetyl-11-hydroxy-β-boswellic acid (7), madecassic acid (9), β-boswellic acid (9), asiatic acid (10), alphitolic acid (11), β-amyrin (12), lupeol (13), fisetin (14), chlorogenic acid (15), myricetin (16), quercetin (17), rosmarinic acid (18), and corosilic acid (19)

between 3.8 and 32.3 min could be identified using a library of standard compounds. Ethyl acetate extract was subjected to LC–MS/MS advanced analysis for identification of the major compounds. Table 1 reports the molecular mass and the fragmentation pattern of the compounds identified in the ethyl acetate extracts, whereas Fig. 2 shows the chemical structure of the identified compounds triterpenoids. The identification of these compounds was achieved by comparison with the chromatograms of pure standards compounds and their reported mass spectrometry fragmentation pattern (Fig. 1 and Table 1). Figure 3 shows the representative MRM ion chromatograms of 19 compounds and ursolic acid (UA) as an internal standard. No matrix signals interfere with the quantitation of the identified compound because of the high selectivity of MRM detection. All 19 compounds were characterized by comparing the obtained molecular (precursor) ions and fragmentation patterns (*i.e.,* product ions) from LC–MS/MS data (Fig. 4) and the data from the reported literature (Table 1) [14, 20]. Two (β-boswellic alcohol, 3-*O*-Acetyl-11-hydroxy-β-boswellic acid) of them were newly found in the leaves of *B. serrata* and reported here for the first time. Thirteen (13) pentacyclic triterpenes were identified such as serjanic acid (Rt—6.9), 11-keto-β-boswellic acid (KBA, Rt—11.4), maslinic acid (Rt—12.2), 3-*O*-acetyl-11-keto-β-boswellic acid (AKBA, Rt—13.6), 3α-hydroxy-24- hydroxymethyl -urs-12-ene-24-ol (β-boswellic alcohol) (Rt—16.5), 3-*O*-acetyl-11-hydroxy-β-boswellic acid (Rt—17.2), madecassic acid (Rt—18.3), β-boswellic acid (β-BA) (Rt—18.8), asiatic acid (Rt—19.6), alphitolic acid (Rt—21.3), β-amyrin (Rt—23.4), Lupeol (Rt—29.4), and corosilic acid (Rt—32.3). Six (6) phenolic compounds (flavonoids) were also identified, which were quinic acid (Rt—3.8), fisetin (Rt—24.5), chlorogenic acid (Rt—25.7), myricetin (Rt—27.2), quercetin (Rt—28.6), and rosmarinic acid (Rt—30.8). MS/MS combined with collision-induced dissociation has been found to enable the accurate identification of specific flavonoids and triterpenes in complex extracts with many eluting peaks. MS/MS was used as the method of choice for the identification of compounds in the extract of *B. serrata* leaves.

LC–MS/MS method validation for the quantification

A reverse-phase C18 column (75 × 4.6 mm, 3.5 µm) was tested with different solvents, additives, flow rates and temperatures for its capacity to resolve the 19 compounds of interest within a 40 min run time. The best performing method was able to resolve all compounds using a gradient of acetonitrile, while formic acid remained at 0.1%. The initial conditions were 25% acetonitrile for 1 min, and then the acetonitrile was linearly increased to 80% for 30 min, which permitted the elution of all the triterpenes and phenolic compounds (Table 1, Fig. 1). The selectivity was determined once the efficient chromatographic separation was achieved, with no co-eluted peaks. The comparison with the standard solution was used for identification (Fig. 1). The compounds of interest in ethyl acetate extract of *B. serrata* leaves, showed Rt corresponding to the respective authentic standard compound. All compounds were displayed in MS spectra with an $[M–H]^-$ ion to their corresponding to the corresponding fragment ions (Table 2, Fig. 4).

The mean linear equations computed by least square regression analysis for all identified compounds, where y is the peak area ratio of the analyte/IS and x the concentration of the analyte, with correlation coefficient value (r^2) (Table 2). Linearity was observed for a wide range (1–1000 ng/mL), over a six-point linear calibration curve covering the expected range for each analyte in the ethyl acetate extract (EAE), with an adequate coefficient ($r^2 > 0.973$). The residual coefficient was also evaluated and the results were acceptable. The intra-day accuracy and precision (% CV) observed for the CSs ranged from 90.6% to 103.7% and 0.94% to 3.6%, respectively, for all analytes. Similarly, for inter-day experiments, the accuracy and precision varied from 93.2% to 101.5% and 1.5% to 3.9% for all analytes (data not shown). Precision was determined by repeatability and reported as *r*elative standard deviation (RSD). RSD% was less than 5%. The LLOQ from the standard curves for each compound was 1.0 ng/mL at a signal-to-noise ratio (S/N) of ≥ 10. The matrix effect (ME) and the recovery efficiency (RE) were investigated for each compound from the ethyl acetate fractions (Table 2). The matrix effect was within the range of 97.64–100.60%, indicating that no significant matrix effect was observed for this method. The percent recovery range was from 95.78 to 100.2% for all the analytes. Overall, there was no ion suppression from the matrix. Thus, the method was considered robust enough to be included in the Quality Control Analysis.

Quantification of pentacyclic triterpenes and phenolic compounds

For both qualitative and quantitative purpose, an accurate, raid and efficient method on a mass spectrometer equipped with a triple quadrupole analyzer was developed for the analysis of ethyl acetate extracts from *B. serrata* leaves. In order to monitor the mentioned compounds by MRM, the specific fragmentation reactions were selected (Table 2). Nineteen compounds including quinic acid, serjanic acid, 11-keto-β-boswellic acid, maslinic acid, 3-*O*-acetyl-11-keto-β-boswellic acid, 3α-hydroxy-24-hydroxymethyl -urs-12-ene-24-ol (β-boswellic alcohol), 3-*O*-acetyl-11-hydroxy-β-boswellic acid, madecassic acid, β-boswellic acid, asiatic acid, alphitolic acid, β-amyrin, lupeol, fisetin, chlorogenic acid, myricetin, quercetin, rosmarinic acid, and corosilic acid were monitored by the transition from the specific deprotonated molecular ions

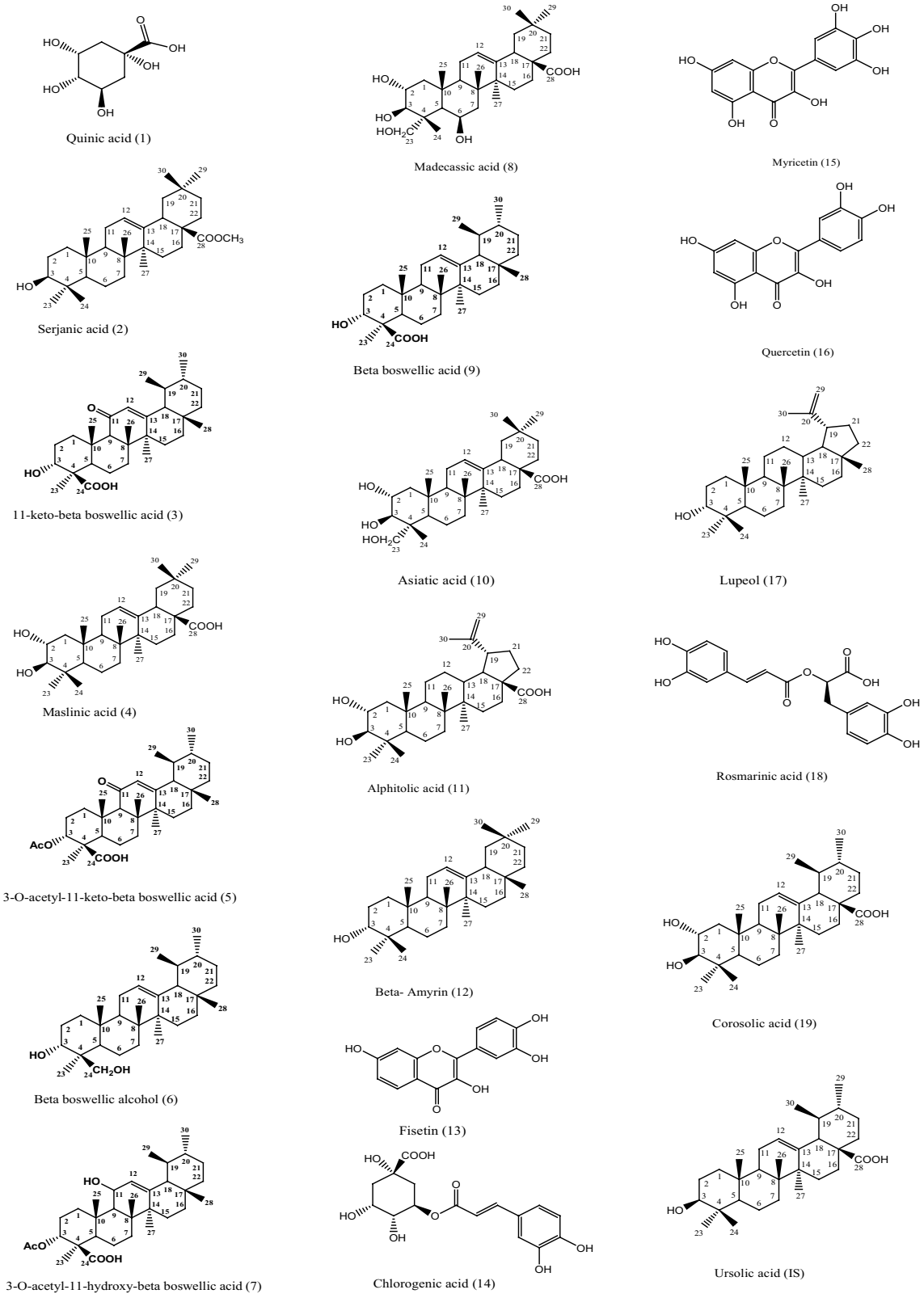

Fig. 2 Chemical structure of the triterpenes and phenolic compounds identified in *Boswellia serrata* leaves. Numbers correspond to compounds listed in Table 1

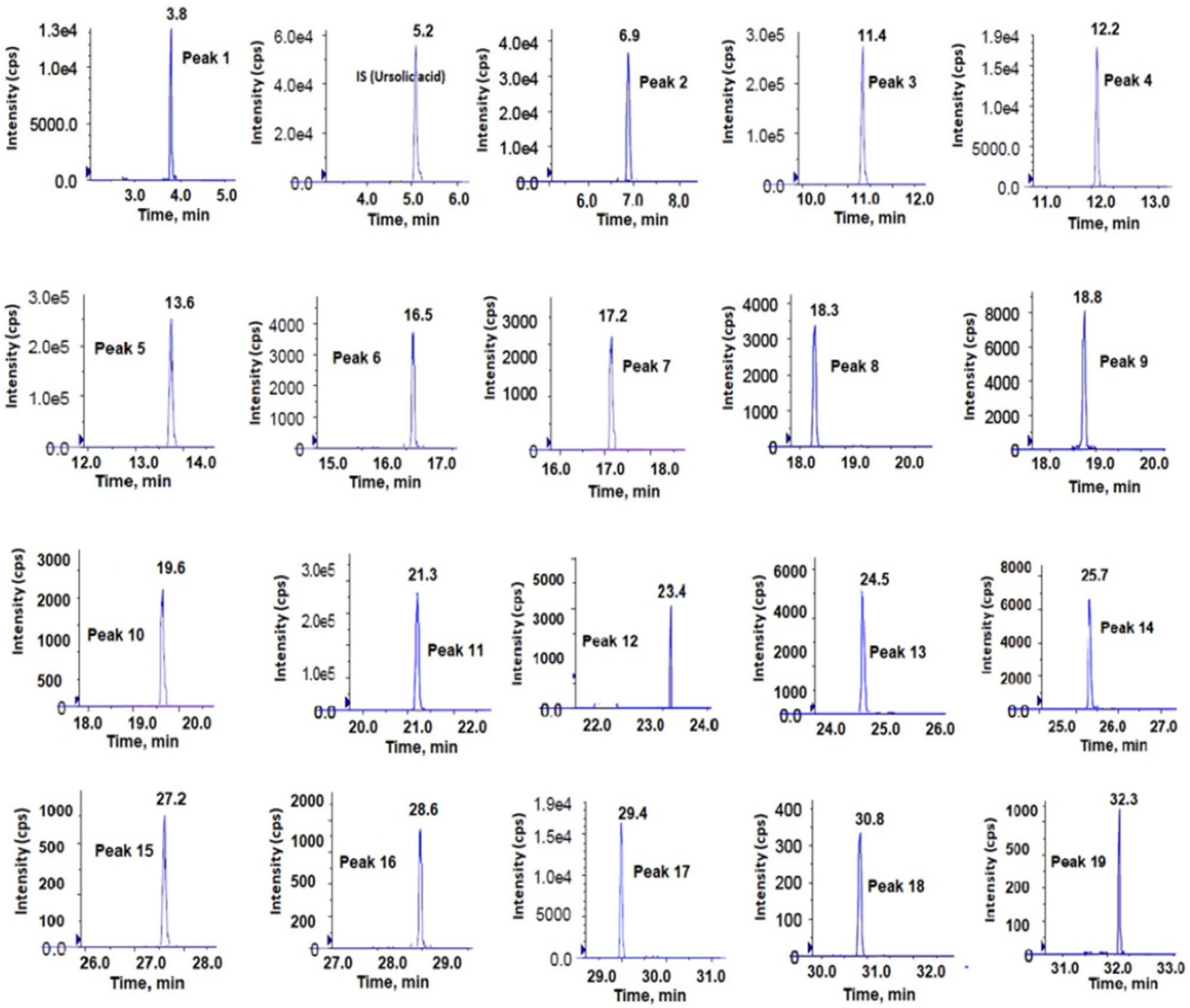

Fig. 3 Representative MRM ion chromatograms of 19 compounds and internal standard, ursolic acid (UA). In each case, the peak number corresponds to molecule listed in Table 1

$[M\text{-}H]^-$ to the corresponding fragment ions (Fig. 4). Table 1 shows molecular ions, and fragments observed in MS/MS for ethyl acetate extracts of *B. serrata* leaves. Table 2 shows for the quantitative determination of all triterpenes and phenolic compounds. The evaluation showed that the total amount of triterpenes and phenolic compounds in the leaves of *B. serrata* ethyl acetate extract samples ranged from 14.54 to 102.14 mg/g and 2.14 to 93.12 mg/g dry extract, respectively.

Discussion

Pentacyclic triterpene saponins and phenolic compounds are known complex molecules, which were isolated from ethyl acetate extracts of *T. brownii* stem bark. These compounds are known to have good anti-inflammatory, anti-arthritis [21, 22], anti-HIV, anti-diabetes, anti-Alzheimer's, and antifungal activity [23]. Considering the enormous bioactive potential of this plant gum resin extract, it is known that substances can show more than one effect. Therefore, the aim of the present study was to develop a validated LC–MS/MS method for simultaneous identification and quantification of the compounds in the leaves of *B. serrata* ethyl acetate extract. In this work, negative ionization mode for all compounds and the internal standard, ursolic acid were evaluated in MS method optimization experiment. It was found that the response was better in the negative ionization mode than that in the positive ionization mode. The electrospray ionization (ESI) of the analytes and IS (UA, ursolic acid) was conducted in negative ionization mode using 5.0 ng/

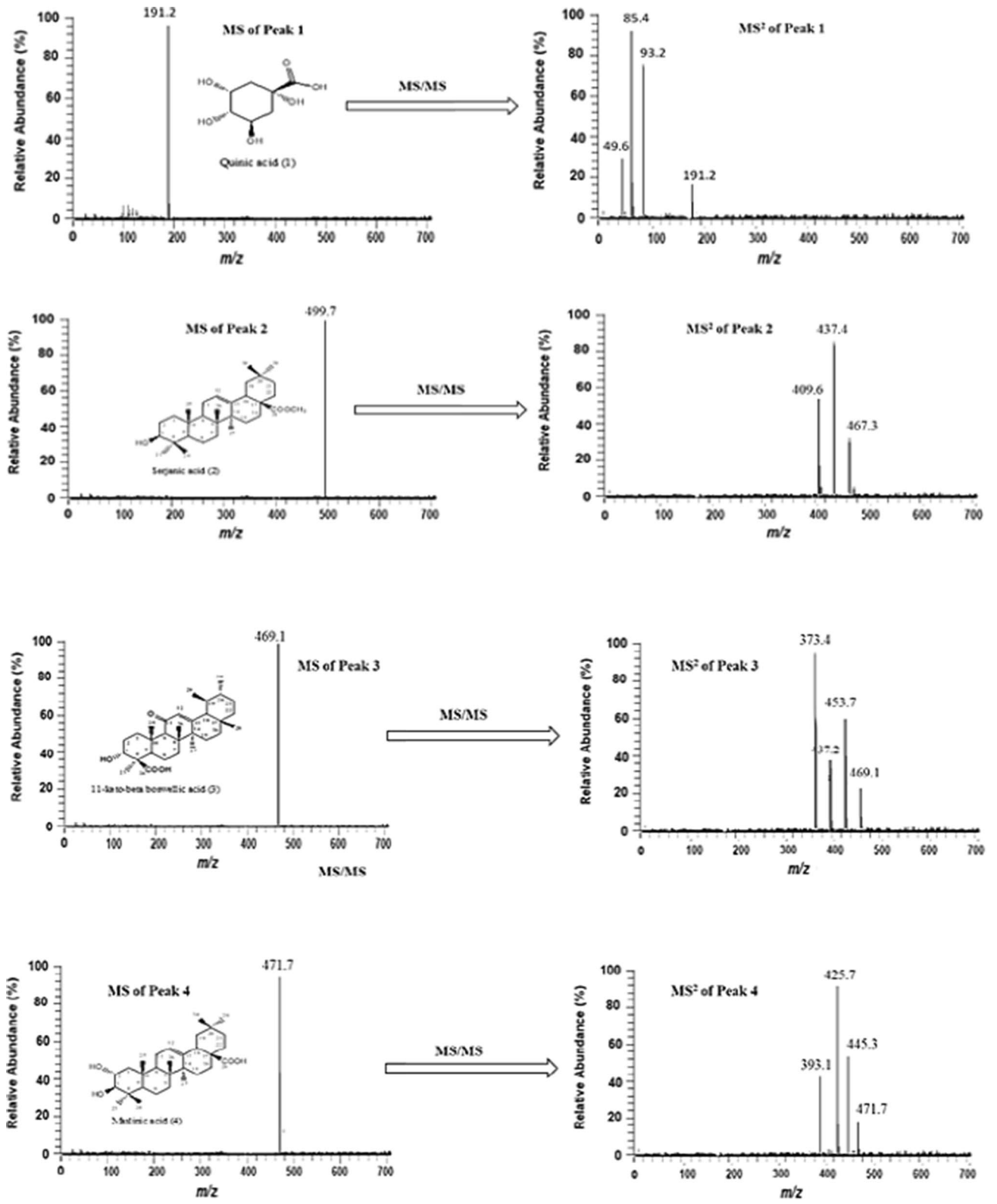

Fig. 4 MS/MS spectra (MS^2) and fragmentation pattern of identified compounds. In each case, upper left peak number corresponds to molecule listed in Table 1

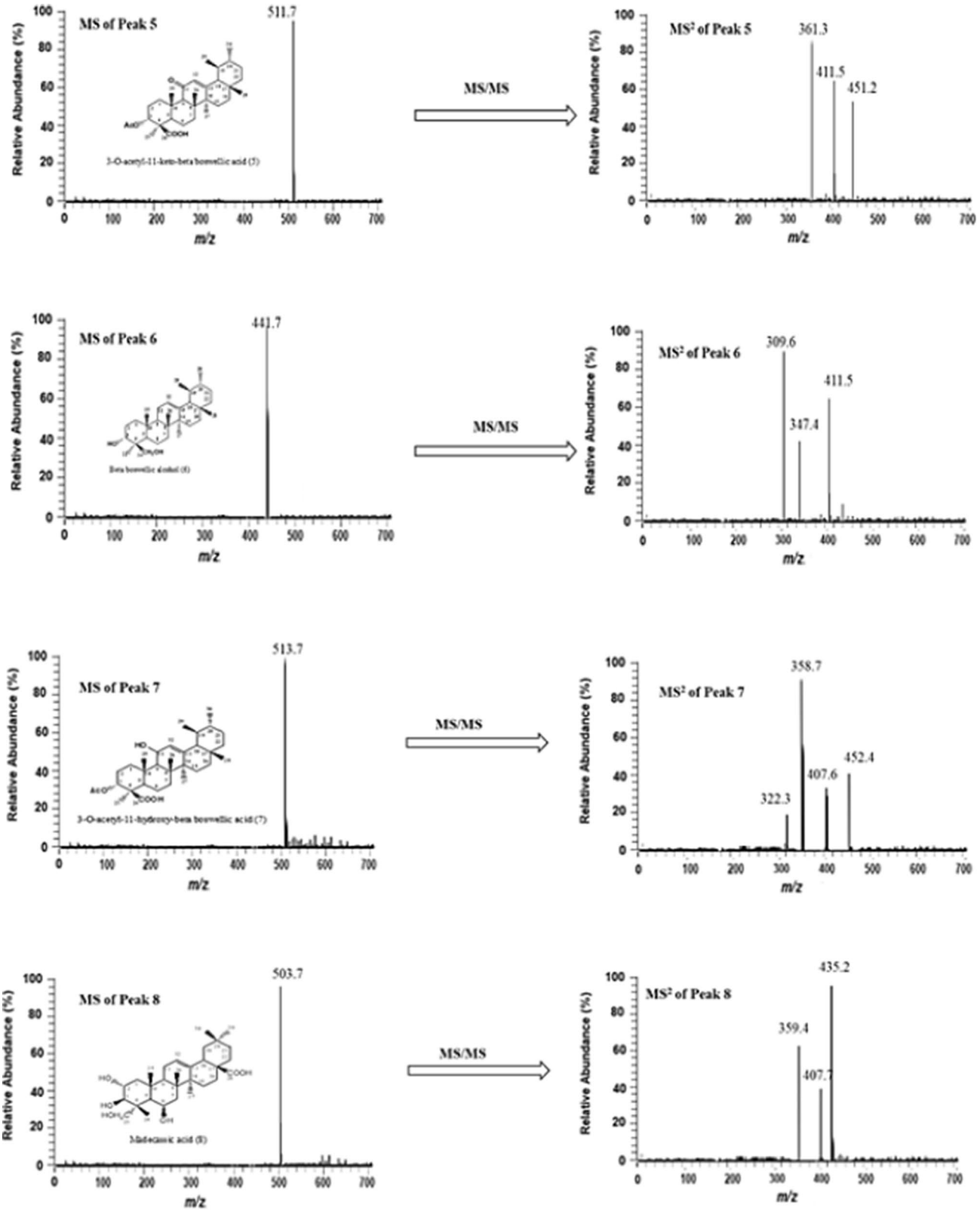

Fig. 4 (continued)

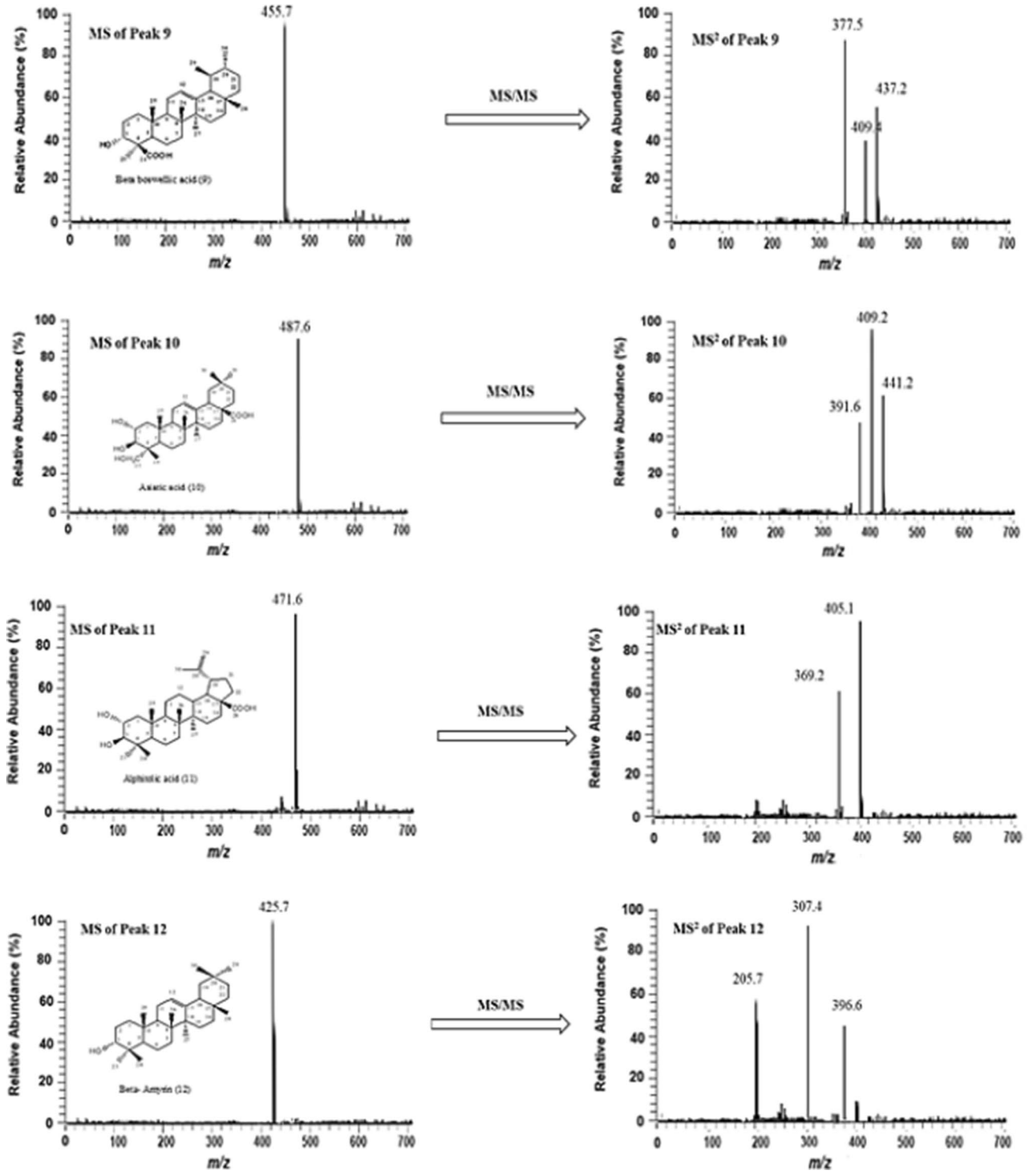

Fig. 4 (continued)

mL tuning solution as the analytes. The analytes and IS gave predominant singly charged deprotonated precursor $[M\text{-}H]^-$ ions. The most abundant and consistent product ions in Q3 mass spectra of the analytes are shown in Table 2. For ursolic acid (IS), the most stable and reproducible product ion was observed at *m/z* 310.4. The dwell time of 200 ms was enough and no cross-talk was observed among the MRMs of the analyte and IS, ursolic acid, shown in Fig. 3. In this study, $SolE_XC18$ reversed-phase cartridges were used for solid phase extraction (SPE) for sample clean-up. SPE

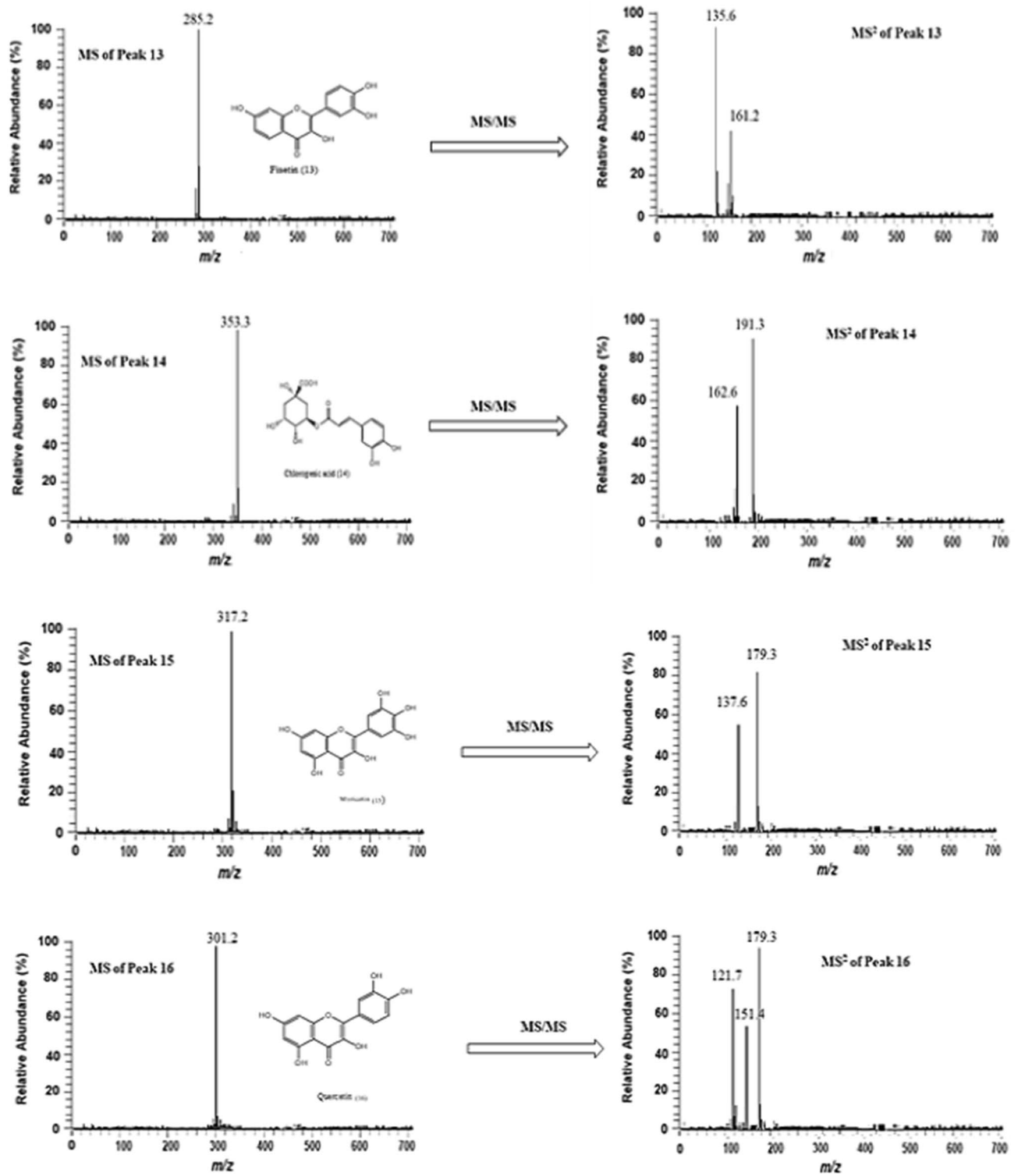

Fig. 4 (continued)

cartridges showed easier handling as an acidic raw extract can be applied directly on the cartridges, avoiding time-consuming and error-prone pH adjustment to neutral or alkaline pH conditions.

The different mobile phases with a gradient elution were tried including acetonitrile (0.1% formic acid), acetonitrile and 10 mM ammonium acetate (plus 0.1% formic acid), methanol (0.1% formic acid), and the mixture of methanol

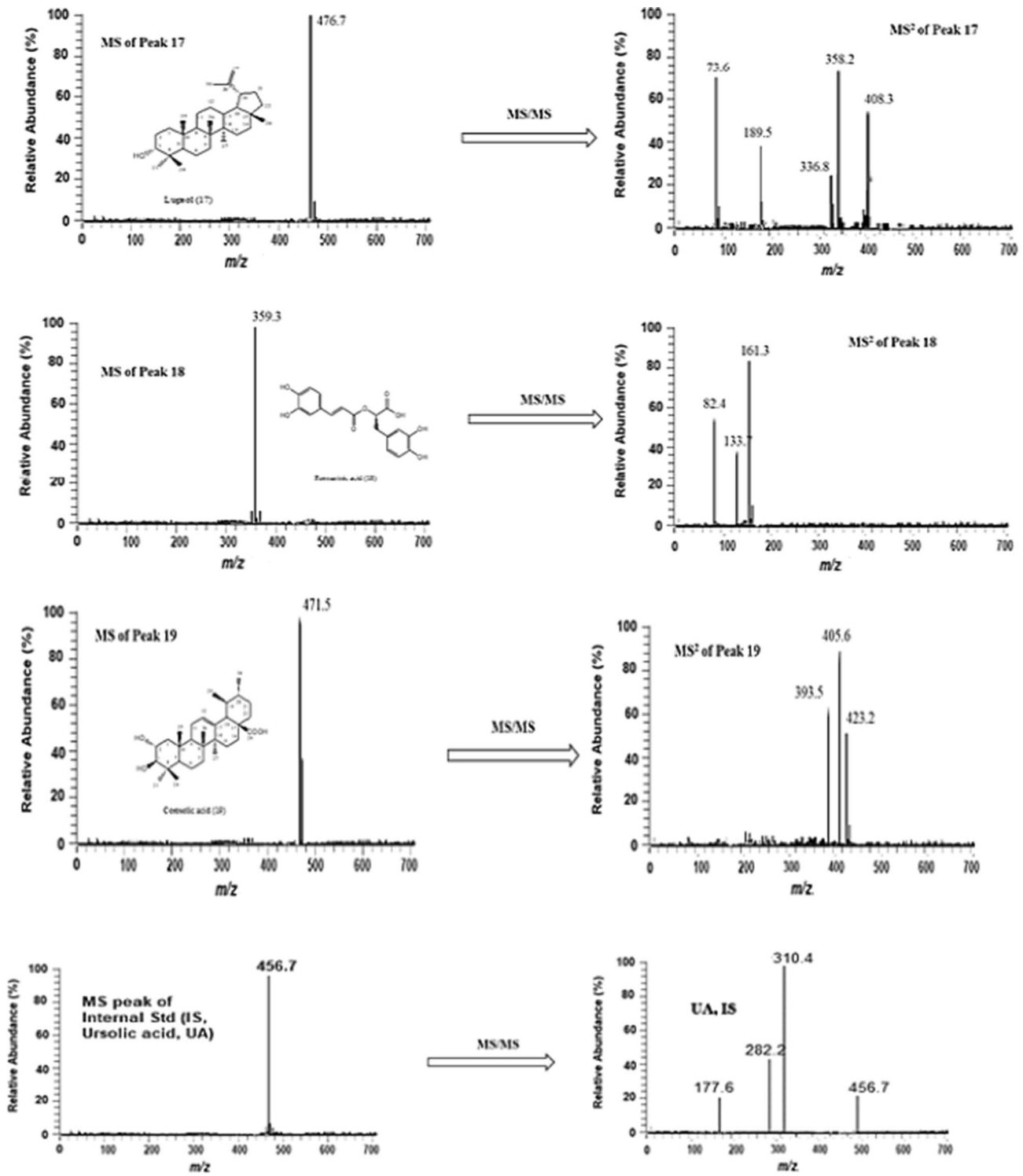

Fig. 4 (continued)

and 10 mM ammonium acetate (0.1% formic acid). The choice of mobile phase was a crucial factor in achieving fine chromatographic behaviour and appropriate ionization. The best peak shape and ionization were achieved adapting 0.1% formic acid buffer. Acetonitrile provided higher sensitivity and sharp peaks shapes as compared to methanol. Another important observation was that higher proportion (> 70%) of organic diluents was necessary for optimum resolution

Table 2 Validation and quantitative determination of the identified compounds in the ethyl acetate extracts from *Boswellia serrata* leaves by Liquid Chromatography-coupled to Electro Spray Ionization and tandem Mass Spectrometry (LC-ESI-MS/MS), by using calibration curves from pure standards

Compound	MRM transition	Regression equation	R_2	Linearity (ng/mL)	LOD/LOQ (ng/mL)	RSD%	Matrix effect (ME)	Recovery (%)	Amount in the ethyl acetate extract (mg/g dry extract)
Quinic acid	191.2→85.4	$y = 314.42x – 534.27$	0.993	1-1000	1.00/2.10	2.68	97.64 ± 3.85	95.78	62.04 ± 0.04
Serjanic acid	499.7→437.4	$y = 1084.38x + 14753.76$	0.973	1-1000	1.00/3.40	4.15	98.26 ± 2.17	98.20	14.54 ± 0.16
11-Keto-β-Boswellic Acid (KBA)	469.1→373.4	$y = 378.17x + 3171.57$	0.987	1-1000	1.00/1.10	2.05	98.88 ± 2.38	97.75	54.54 ± 4.47
Maslinic acid	471.7→425.7	$y = 482.37x + 10628.26$	0.995	1-1000	1.00/2.00	1.75	99.77 ± 1.05	99.20	49.58 ± 3.51
3-O-Acetyl-11-Keto-β-Boswellic Acid (AKBA)	511.7→361.3	$y = 349.84x + 4328.24$	0.993	1-1000	1.00/1.15	3.20	100.60 ± 0.52	97.99	68.74 ± 0.56
β-Boswellic Alcohol	441.7→309.6	$y = 364.48x + 1147.21$	0.999	1-1000	1.00/2.05	2.36	97.94 ± 3.22	98.00	53.26 ± 5.27
3-O-Acetyl-11-hydroxy-β-Boswellic Acid	513.7→358.7	$y = 98.34x + 1621.33$	0.989	1-1000	1.00/1.15	1.80	96.79 ± 4.27	98.25	102.14 ± 0.62
Madecassic acid	503.7→435.2	$y = 423.64x + 3278.20$	0.985	1-1000	1.00/2.80	3.10	99.56 ± 6.20	96.69	94.22 ± 0.20
β-Boswellic Acid (β-BA)	455.7→377.5	$y = 832.42x – 1021.5$	0.998	1-1000	1.00/2.55	6.35	98.47 ± 2.35	100.20	29.37 ± 0.14
Asiatic acid	487.6→409.2	$y = 643.46x – 621.10$	0.978	1-1000	1.00/1.75	2.65	99.34 ± 0.68	99.00	22.18 ± 0.10
Alphitolic acid	471.6→405.1	$y = 133.62x + 1132.65$	0.989	1-1000	1.00/2.10	1.60	98.00 ± 3.12	98.20	62.14 ± 4.36
β-Amyrin	425.7→307.4	$y = 555.30x + 123.34$	0.999	1-1000	1.00/2.01	3.17	97.69 ± 2.88	97.00	23.04 ± 0.77
Fisetin	285.2→135.6	$y = 1714.42x + 4034.43$	0.990	1-1000	1.00/2.25	2.68	98.78 ± 2.13	96.75	4.21 ± 0.07
Chlorogenic acid	353.3→191.3	$y = 267.38x + 575.41$	0.989	1-1000	1.00/2.65	1.46	99.69 ± 2.90	98.45	27.31 ± 0.20
Myricetin	317.2→179.3	$y = 154.42x – 495.7$	0.978	1-1000	1.00/2.15	3.48	98.84 ± 1.96	98.00	93.12 ± 0.03
Quercetin	301.2→121.7	$y = 732.42x – 121.5$	0.998	1-1000	1.00/2.00	2.37	99.10 ± 1.36	99.10	26.22 ± 0.73
Lupeol	467.7→358.2	$y = 322.37x + 142.35$	0.999	1-1000	1.00/3.05	3.59	98.44 ± 2.58	99.25	44.54 ± 1.42
Rosmarinic acid	359.3→161.3	$y = 714.42x – 14653.42$	0.998	1-1000	1.00/2.27	2.50	99.26 ± 1.35	98.05	2.14 ± 0.03
Corosilic acid	471.5→405.6	$y = 622.76x – 5587.38$	0.99	1-1000	1.00/1.25	2.10	99.48 ± 2.10	99.50	5.87 ± 0.04

of these compounds. The best chromatographic conditions were achieved on RP 18 column (75 × 4.6 mm, 3.5 µm) with adequate response, resolution, symmetric peak shape, baseline separation within 40.0 min. The LC conditions were optimized to ensure appropriate peak shapes and complete separation of all analytes and IS (UA) from matrix components so as to minimize matrix effects while keeping the total analysis not more than 40 min.

It is known that matrix effects could induce poor results in LC–MS/MS analyses. Therefore, a good internal standard

(IS) mimics the analytes during the preparation of samples and compensates the sample loss during the preparation process. It is necessary to use the stable labelled isotopes of the analyte as an IS is recommended for bioanalytical assays on LC–MS/MS to increase assay precision and limit variable recovery between analyte and the IS. In the present study due to non-availability of deuterated boswellic acid to use it as an IS, several structurally similar compounds with the analytes (such as oleonic acid, and ursolic acid) were evaluated to find out a suitable IS. Finally, ursolic acid (UA) was found to be the best for present purpose based on the chromatographic elution, ionization and reproducible and good extraction efficiency. The acceptable limit for both intra- and inter-day accuracy and precision was ± 15% of the nominal values for all. In this method, both intra- and inter-day accuracy and precision were well within the limit, indicating that the developed method was precise and accurate for all analytes. Detection by tandem mass spectrometry in multiple reaction monitoring mode (MRM) enabled sensitive quantification with limits of detection between 1 ng/mL. Recoveries between 95.78 and 100.2% confirmed that the method allows precise quantification of the analytes in complex matrices. Authors believe that the reported LC-MS-MS method for the simultaneous identification and quantification of pentacyclic triterpenes and phenolic compounds with little or no modifications can be extended to other pre-clinical species and human plasma matrix.

Conclusions

This is the first report on the method development and validation of a high-throughput LC–MS/MS method using MRM scan survey for the simultaneous separation and quantification of pentacyclic triterpenes and phenolic compounds in the ethyl acetate extracts of leaves of *Boswellia serrata*. A total of 19 compounds were separated, and simultaneously quantified with high accuracy and sensitivity. Two (β-boswellic alcohol, 3-*O*-Acetyl-11-hydroxy-β-boswellic acid) of them were newly found in the leaves of *Boswellia serrata* and reported here for the first time. The chromatographic fingerprinting analysis has attracted great attention, can be used to elucidate the diversity of phytochemicals (triterpenes and phenolics) from any plant extracts.

Author contributions MKT: conceptualisation and designing of the study, review and editing; AB: reviewing draft manuscript and editing; DT: visualization, formatting the manuscript, reviewing and language editing; TS and SJ: designing the experiment, experiment performed, methodology, validation, data analysis; SM and TS: data interpretation and writing original draft, MKT and SJ: project supervision and administration. All authors have read and agreed to the published version of the manuscript.

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Data availability Not applicable.

Declarations

Conflict of interest Authors MKT, AB, and DT were employed by Trivedi Global, Inc. Author TS was employed by Sai Life Sciences. Authors SM and SJ were employed by Trivedi Science Research Laboratory Pvt. Ltd.

Institutional review board statement Not applicable.

Informed consent Not applicable.

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