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RESEARCH PAPER

Comprehensive profiling and characterization of quassinoids from the seeds of *Brucea javanica* via segment and exposure strategy coupled with modified mass defect filter

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Abstract Quassinoids, the predominant constituents in the seeds of *Brucea javanica* (BJ), have gained an increasing interest over the past decades since the discovery of their extensive biological activities. In the present study, a method based on the segment and exposure strategy coupled with two mass spectrometer data acquisition techniques was firstly developed and validated for comprehensive profiling of quassinoids in the seeds of BJ via high-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (HPLC-QTOF/MS). The segment and exposure strategy could significantly improve the detection efficiency for trace quassinoids in the seeds of BJ. Furthermore, the five-point screening approach based on modified mass defect filter (MDF) and the visual isotopic ion technique could rapidly screen the precursor ions of interest. The fragmentation behavior of quassinoids was systematically investigated, and a total of 148 quassinoids including 86 potentially new ones were unambiguously or tentatively identified in the seeds of BJ. Collectively, our results demonstrate that the integrated strategy reported in this study has considerable potential for rapid screening of natural compounds especially for the trace ones in herbal medicines.

Ting Tan and Chang-Jiang-Sheng Lai contributed equally to this work.

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Introduction

Brucea javanica (BJ, Simaroubaceae family), a medicinal evergreen shrub, is widely distributed in Southeast Asia and Northern Australia. The seeds of BJ were used in traditional Chinese medicine and are currently recorded in *Pharmacopoeia of the People's Republic of China* (2010 edition) for treatment of various ailments including inflammation, cancer, amebic dysentery, and malaria [1]. Previous studies indicated that BJ is an abundant source of quassinoids, which are reported to possess extensive biological activities, such as potential anthelmintic [2, 3], antitumor [4, 5], cytotoxic [6–9], and anti-inflammatory [10] activities. To date, more than 104 quassinoids have been isolated and identified in genus *Brucea*, including 77 from BJ, by phytochemical studies [11–13]. However, there is no report on comprehensive profiling of the quassinoids in the seeds of BJ. In order to further understand the pharmaceutical effects and control the overall quality of the crude herbs, it was urgent to develop a sensitive and reliable method to characterize the quassinoids in BJ.

In the last decades, high-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (HPLC-QTOF/MS) has been successfully applied to the on-line characterization of natural products. However, the number of analytes detected is usually limited, mainly due to the MS signals' suppression of the trace analytes, as well as the small injection volume [14]. In LC/MS analysis, the segment and exposure strategy could avoid the detector saturation as the major or moderate compounds were gradually removed under the set segment. With gradient increase of the sample loading, this on-line

method could effectively profile the trace analytes in complex mixtures.

The exploration of the potential precursor ion plays an important role in nontargeted analysis of natural products. To date, several modified mass defect filter (MDF) strategies have been developed for investigating the detailed distributed spaces of precursor ions [15]. Recently, our group has proposed a modified MDF with few borders, which we termed "five-point screening" approach, and it could be conveniently used for nontargeted and comprehensive analysis of saponins in extracts of *Panax notoginseng* [16]. Moreover, in mass spectrometric analysis of natural products, the isotopic distribution approach could obtain the visually distinctive features of the selected precursor for further verification.

Here, we propose an approach based on the segment and exposure, five-point screening, and visual isotopic ion techniques for comprehensive characterization of the quassinoids in the seeds of BJ via HPLC-QTOF/MS. The segment and exposure strategy was used to gradually remove the major or moderate compounds for detection of the minor quassinoids. The five-point screening based on modified MDF coupled with the visual isotopic ion technique was employed to screen and validate the precursor ions of quassinoids.

Materials and methods

Chemicals and reagents

Four reference standards, including bruceine D, brusatol, bruceine A, and bruceantin, were purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing, China). The purity of bruceine D (>98 %), brusatol (>98 %), bruceine A (>92 %), and bruceantin (>95 %) was determined by HPLC-DAD. The seeds of BJ were collected from Qinzhou City, Guangxi Province, China, in April 2014 and identified by Prof. Ping Li from the Department of Pharmacognosy in China Pharmaceutical University, and have been deposited in the State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing, China.

HPLC-grade acetonitrile and formic acid were purchased from Merck (Merck, Germany). Analytical grade anhydrous ethanol was obtained from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). Ultrapure deionized water (18 MΩ cm^{-1}) was supplied by a Millipore Milli-Q water system (Milford, MA, USA).

Sample preparation

The seeds of BJ were powdered and sieved through a no. 60 mesh. The dried seed powder (2 g) was soaked in petroleum ether (3×30 mL, 24 h) to give defatted seeds, which were then extracted with 95 % ethanol (3×30 mL, 3 h) under reflux. After removal of the solvent under vacuum, the viscous concentrate obtained was suspended in water (30 mL) and then partitioned with petroleum ether (90 mL). The water-solution was evaporated under vacuum to afford the extracts (107 mg). The sample and the four standards were prepared in methanol at a final concentration of 25 and 1 mg mL^{-1}, respectively. These solutions were centrifuged at 12,000 rpm for 10 min prior to analysis.

HPLC-QTOF/MS conditions

Nontargeted analysis was performed on an Agilent 1260 series liquid chromatograph system (Agilent Technologies, Germany) coupled with a 6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) via a dual electrospray ionization (ESI) interface (HPLC-ESI-QTOF/MS). Sample separation was achieved on an Agilent Eclipse XDB-C_{18} column (250 mm×4.6 mm, 5 μm) with a flow rate of 1 mL/min in split mode at 25 °C. The flow rate was split at the column outlet to allow 50 % eluent to flow into the mass spectrometer. The mobile phase was composed of water (A) and acetonitrile (B). The gradient elution program was set at 10 % B from 0 to 10.0 min, 10–37 % B from 10.0 to 60.0 min, 37–55 % B from 60.0 to 84.0 min, 55–100 % B from 84.0 to 92.0 min, and 100 % B for 8 min to fully elute the less polar compounds. The equilibration time was 8 min.

All quassinoids were determined by using the total ion current chromatograms in the positive-ion ESI-MS mode in the mass range *m/z* 100–1000. The other operating parameters were optimized as follows: drying gas (N_2) flow rate 12.0 L min^{-1}, pressure of the nebulizing gas 45 psig, temperature of the drying gas 350 °C, capillary voltage 3500 V, and fragment voltage 120 V. 0.5 and 3 μL (25 mg mL^{-1} crude medicine) were used to obtain the auto MS/MS spectra for the relatively high-abundance compounds. As for the low-abundance one, 10 μL of the 25 mg mL^{-1} seeds of BJ extract was used. The collision energy was set at 10 and 30 V for these different molecular weights or structural compounds (Fig. S1).

Method validation

The nontargeted approach for detection and identification of quassinoids in BJ is summarized in schematic form in Fig. 1. It mainly includes three steps: (1) the segment and exposure strategy was firstly applied to improve the detection capability by removing the major or moderate compounds under the set segment; (2) the modified MDF and the visual isotopic ion technique were subsequently used to screen the potential precursor ions of quassinoids; and (3) the precursor ions of quassinoids were selected to generate more fragmental ions at the targeted MS/MS detection mode. According to the characteristic fragmental ions and the published data, the

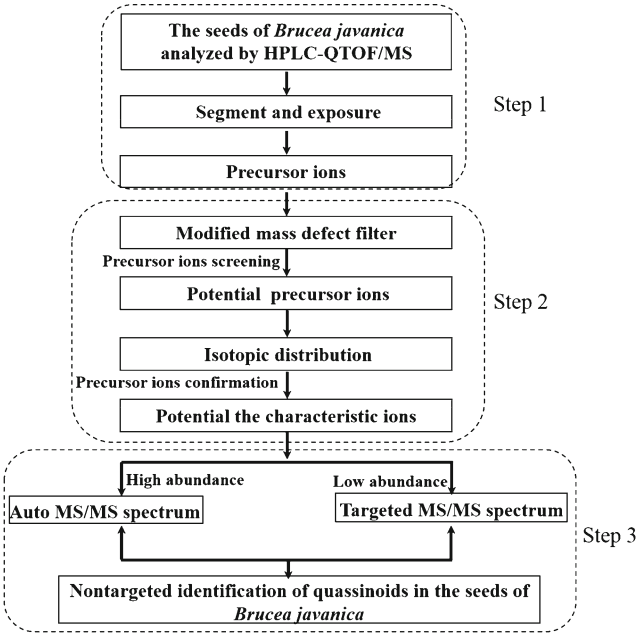

Fig. 1 Schematic diagram of quassinoid screening in the seeds of BJ using the segment and exposure strategy coupled with modified mass defect filter

profiling of quassinoids was comprehensively illustrated by the proposed integrated method.

The low-concentration sample was used as a control without setting series of segments in this study. To avoid the detection of high-abundance quassinoids and certain classes of compounds eluted in solvent peaks, the series of the segments were designed at 0–1.9, 17.9–18.3, 43.3–44.0, 47.5–48.0, 51.6–52.3, and 88.0–94.0 min for the 3 μL samples (25 mg mL^{-1}), respectively. Moreover, the additional time segment at 0–5.0, 8.6–9.2, 13.5–15.0, 30.2–30.8, 35.8–36.4, 58.6–59.3, 63.0–63.8, and 72.0–72.6 min was set to remove the mass signals of certain compounds with moderate content and then to profile the trace quassinoids in 10 μL samples (25 mg mL^{-1}).

In MDF calculations, most of the adducted groups contribute positively to the mass shifts of quassinoids. Compared with the classical MDF, the five-point screening based on modified MDF could increase the screening efficiency owing to the assistance from the integer mass [16]. In this study, the mass distributed space of quassinoids was established by the endpoint (point *a* represented the smallest molecular weight compound), the lower border (line *bc*), and the upper border (line *de*). Under the set detected mass range, the aglycone of quassinoids containing the smallest or the largest decimal mass was linked with the corresponding substituted groups to produce the lower border and upper border, respectively [16].

The reference standards of bruceine D, brusatol, bruceine A, and bruceantin were selected to verify the accuracy of the characteristic isotopic distribution strategy. When the peak heights were above 1.0×10^3 counts and the charge equaled +1 for the selected compound ions, they were exported as .xls documents by the function of the molecular feature extractor from the data-processing software. The obtained data are listed in Table S1.

Data analysis

The MS data were processed by the MassHunter Workstation software (Version B.02.00 Qualitative Analysis, Agilent Technologies). The mass error of the predicted chemical formula should be less than 10.00 ppm, and some characteristic ions should be required for structure elucidation. The detected compounds were annotated as novel if there were no hits of the recently reported studies on seeds of BJ or absence of records in the ChemSpider (http://www.chemspider.com/) and Scifinder (https://scifinder.cas.org/scifinder/).

Results and discussion

With the proposed segment and exposure strategy, the extracts of BJ seeds were directly analyzed by HPLC-QTOF/MS in positive mode to profile the quassinoids. Without sample pretreatment or utilization of two-dimensional chromatography, trace quassinoids in the seeds of BJ could be monitored and identified. Moreover, the developed five-point screening and the isotopic distribution approach could rapidly pick out the potential precursor ions of quassinoids, and their structures could be elucidated based on the MS/MS spectra. In the present study, a total of 148 quassinoids including 86 potentially new ones were characterized in the seeds of BJ, and 15 quassinoid compounds were firstly reported in BJ (Table S2).

The improvement of detection capability by segment and exposure strategy

By gradually removing the major and moderate compounds, the segment and exposure strategy could significantly enhance the detection efficiency when the sample loading amount was increased 20-fold (Fig. 2). When the injected amount of samples was 3 μL (25 mg mL^{-1}), the polar compounds and some nonpolar unknown compounds were firstly excluded to be detected in the chromatogram. When the sample loading amount was further increased, more trace quassinoid compounds could be detected under the set segment.

Screening of the quassinoid ions by five-point screening approach

The five-point screening approach based on the knowledge/experiment rules could more comprehensively cover with the candidate compound ions in a planar region (Fig. S2). One point in the validated spaces

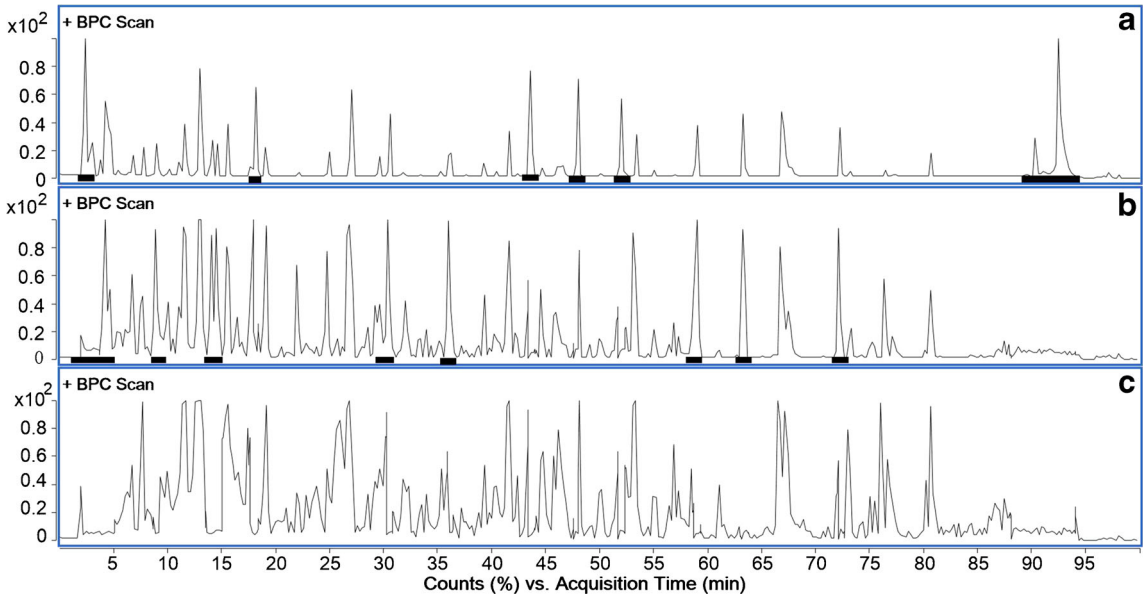

Fig. 2 The base peak chromatogram (BPC) of the seeds of BJ extract at 0.5 μL (**a**), 3 μL (**b**), and 10 μL (**c**) of crude medicine by the segment and exposure strategy

represents a candidate precursor ion. The ion ($C_{19}H_{23}O_7$) with the smallest mass was selected and defined as point *a* (*m/z* 363.1444). The ion ($C_{19}H_{25}O_9$) substituted by the 3× O (oxygen) in their skeleton or side chain was defined as point *b* (*m/z* 457.1346), and the correspondingly produced formula further substituted by 1×Glc ($C_6H_{10}O_5$), 1×M ($C_{10}H_{14}O_3$) group, 1×N ($C_5H_8O_2$) group, and 1×2/3 (C_2H_2O) group was defined as point *c* (*m/z* 943.3447). The ion ($C_{19}H_{23}O_7$) substituted by 1×M ($C_{10}H_{14}O_3$) group, 1×N ($C_5H_8O_2$) group, and 1×2/3 (C_2H_2O) group or 1×Glc ($C_6H_{10}O_5$) and 6×H (hydrogen) was defined as point *d* (*m/z* 855.4014) and point *e* (*m/z* 531.2442), respectively. By setting the point with the smallest molecular weight and the low, upper border, the five points construct the distributed space of quassinoids.

To operate more efficiently, we used the "IF" function of the Microsoft Excel platform to rapidly screen out the potential ions in the .xls documents. Every selected ion should be distributed in the region established by five line segments: $y \geq -0.0001x + 0.1822$ ($363 < x < 457$); $y \geq 0.0004x$

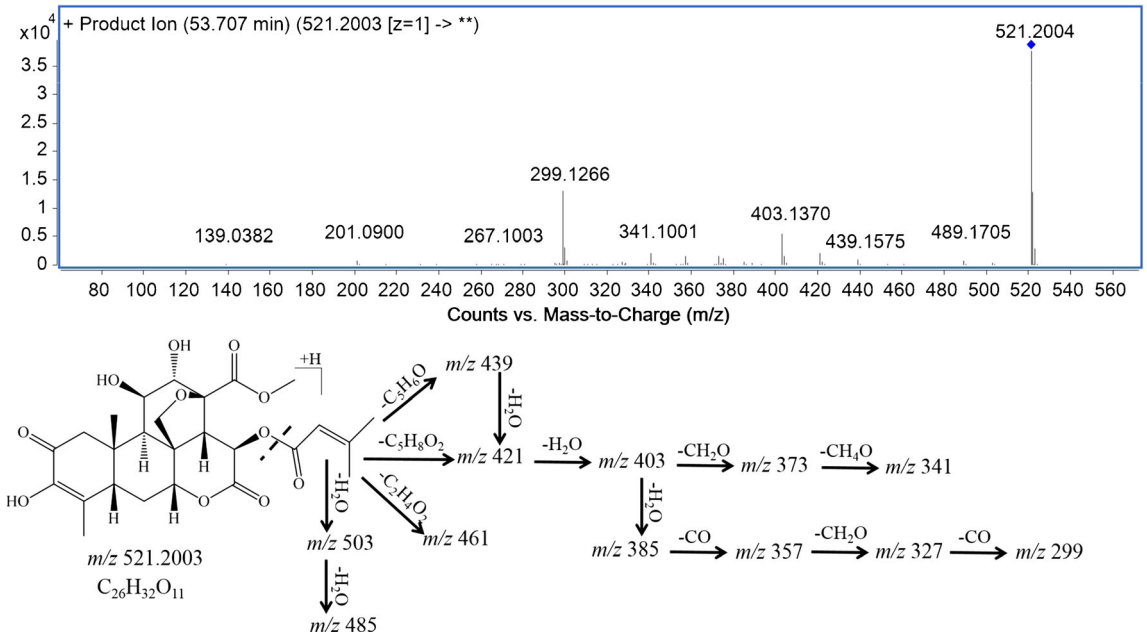

Fig. 3 The MS/MS spectrum of brusatol at positive mode

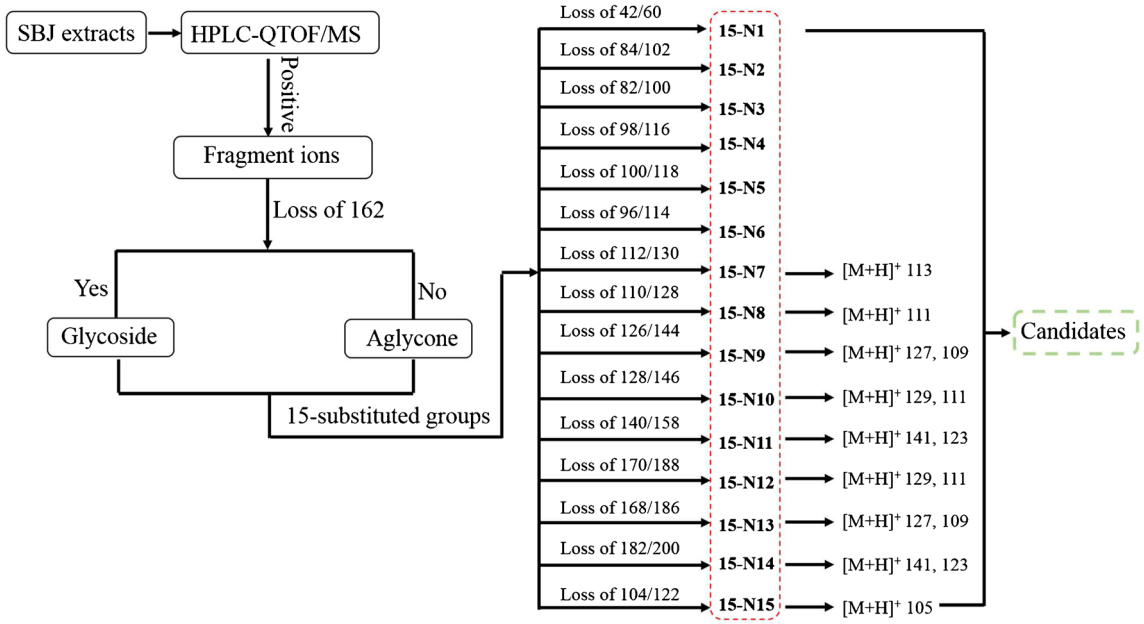

Fig. 4 A diagram for rapid classification and identification of quassinoids by HPLC-QTOF/MS

-0.063 ($457 \le x < 943$); $y \le -0.0006x + 0.9523$ ($855 \le x < 943$); $y \le 0.0005x - 0.0134$ ($531 < x \le 855$); and $y \le 0.0006x - 0.0712$ ($363 \le x \le 531$) (Fig. S2b), where y and x were the decimal mass and the integer mass, respectively. Moreover, all selected ions allowed 0.01 Da mass errors compared with the border of the established distributed place.

In this study, more detailed parameters set operation could see the following example. Point *b* represented the molecule containing the smallest decimal mass. The substituted groups containing an oxygen atom (the smallest negative contribution) or the Glc group (the largest positive contribution), the m group (the second largest positive contribution), and the acyl group (the smallest positive

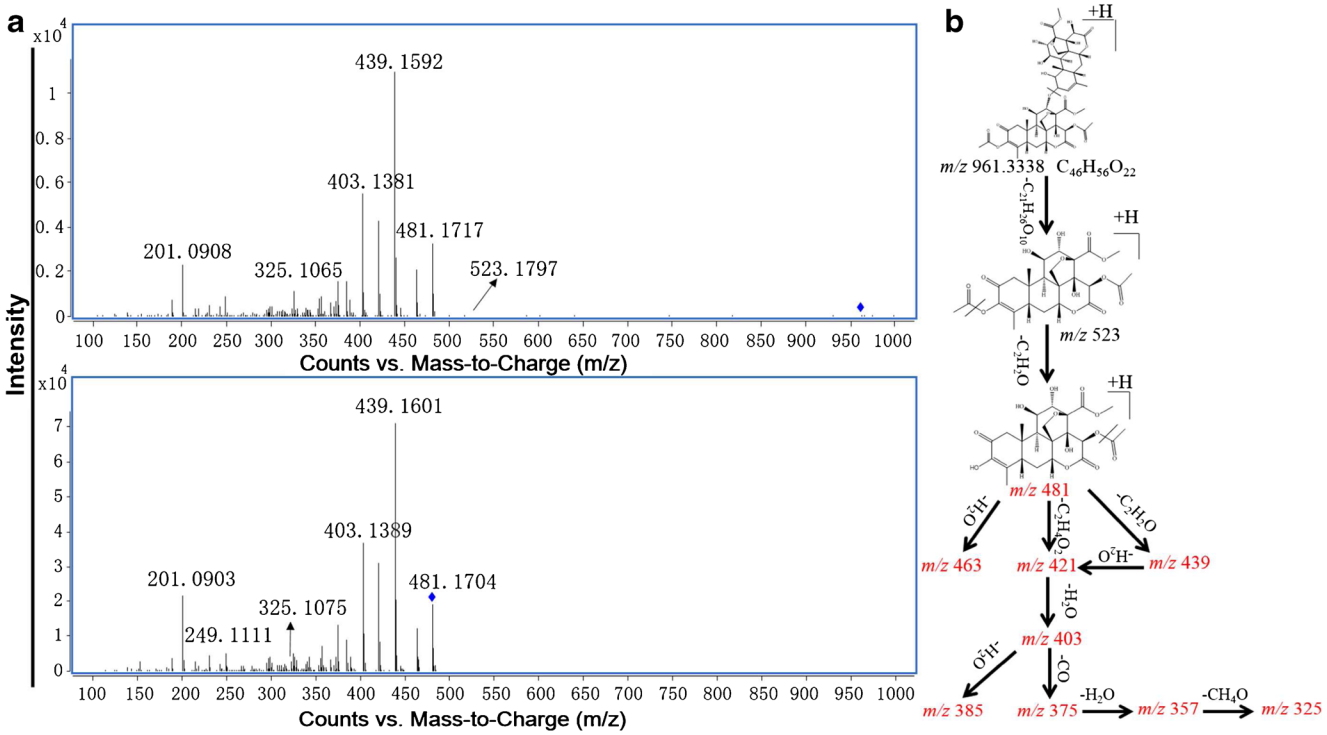

Fig. 5 The MS/MS spectrum of a quassinoid dipolymer at positive mode

contribution) were picked out and their corresponding numbers were set to screen the candidate ions with the accurate mass below 1000 Da (Fig. S2). The number of m groups was set less than two for quassinoids, which were consistent with the reported compounds in previous studies. Point *c* could not contain more than one Glc group. Therefore, the ion at *m/z* 457.1346 ($C_{20}H_{25}O_{12}$, point *b*) linked with the small positive contribution substituted groups (1×Glc, 1×M, 1×N, and 1×2/3 groups) could establish point *c*. Similarly, the line established by points *d* and *e* presented the upper border for the ions containing the largest decimal mass under corresponding integer masses. Thus, the five-point screening approach could effectively remove more putative spaces compared with the classical methods.

The isotopic distribution for verifying the precursor ions

In the present study, the isotopic distribution approach was subsequently employed to screen the quassinoid precursor ions of interest. The mass spectra of bruceine D, brusatol, bruceine A, and bruceantin presented when more positive substituted moieties were linked, the corresponding relative abundance of the intensity for the two isotopic ions would increase (Figs. S3 and S4). Moreover, the isotopic distribution characteristics for quassinoids were consistent with the results simulated by software (Fig. S5). The presence of two more visual isotopic ions could be applied in further excluding the interference ions.

Structural elucidation of quassinoids

The chemical structures of reported quassinoids in seeds of BJ have been shown in Fig. S6 and Fig. S7. The LC-positive-ESI-QTOF/MS fragmentation of four standard quassinoids has been shown in Fig. S8–Fig. S10 and Fig. 3. The compound brusatol (t_R=53.471 min, *m/z* 521.2026, mass error=−1.66 ppm) produced the precursor ion *m/z* 521 in the positive mode (Fig. 3), which generated three main product ions by successive neutral loss of $C_2H_4O_2$ (60 Da), C_5H_6O (84 Da), and $C_5H_8O_2$ (102 Da), indicating that it contained two groups, M1 and N3. The compound bruceine A (t_R=58.878 min, *m/z* 523.2187, mass error=−2.51 ppm) produced the precursor ion *m/z* 523 in the positive mode (Fig. S8), which generated three main product ions by successive neutral loss of $C_2H_4O_2$ (60 Da), C_5H_8O (84 Da), and $C_5H_{10}O_2$ (102 Da), indicating that it contained two groups, M1 and N2. There is a major ion at *m/z* 439, which is a characteristic of quassinoids and corresponds to the 15-group of quassinoids. The compound bruceantin (t_R=67.851 min, *m/z* 549.2337, mass error=−1.21 ppm) produced the precursor ion *m/z* 549 in the positive mode (Fig. S9), which generated three main product ions by successive neutral loss of $C_2H_4O_2$ (60 Da), $C_7H_{10}O$ (110 Da), and $C_7H_{10}O_2$ (128 Da), indicating that it contained two groups, M1 and N8. N1–N14 groups are easily eliminated via neutral diluted ketone. A diagram for rapid classification and identification of quassinoids is shown in Fig. 4. Compound 1 (t_R= 30.62 min, *m/z* 961.3338, mass error=−0.21 ppm) produced the precursor ion *m/z* 961 in the positive mode (Fig. 5), then generated two main product ions by successive neutral loss of $C_{21}H_{26}O_{10}$ (438 Da) and C_2H_2O (42 Da), suggesting that this compound is a dipolymer. The major product ion was *m/z* 481 ($[M+H–C_{21}H_{28}O_{10}–C_2H_2O]^+$), indicating that one of the dipolymers is bruceine B isomer (480 Da). The fragmentation pathway of being prone to lose H_2O and C_2H_4O (44 Da) was consistent with that of bruceine B isomer (Fig. 5). Compound 1 was identified as a quassinoid dipolymer.

Based on the modified MDF, the developed five-point screening approach provided a new data acquisition approach for effective and nontargeted discovery of the analytes of interest from TCMs. The special isotopic feature (containing three more isotopic ions) was a visual tool to validate and confirm the screened quassinoids ions. Herein, the developed integrated strategy was a universal tool and it could be used in analyzing the complex mixtures from the plant medicine and their processed products in vivo or in vitro. Notably, the detailed linkages for the oligosaccharide chains and the position of double bond for quassinoids should be further studied under the assistance of the powerful tool with labeling techniques in positive-mode mass spectrometry analyses.

Conclusion

In the present study, we proposed an integrated strategy by combining the segment mode and MS data acquisition methods to rapidly characterize the quassinoids in BJ seed extract via HPLC-QTOF/MS analysis. The segment and exposure strategy could significantly improve the detection efficiency and profile the trace quassinoids, and the five-point screening approach coupled with the isotopic distribution could effectively screen and validate the precursor ions. Consequently, a total of 148 quassinoids including 86 potential new ones were characterized in the seeds of BJ. The results of the present study may be helpful for further elucidation of the health-promoting potential of BJ.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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