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Fast identification of anticancer constituents in Forsythiae Fructus based on metabolomics approaches

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ABSTRACT

An herb commonly contains hundreds of constituents. Identification of bioactive compound(s) in each herb using conventional approaches is usually inefficient and eco-unfriendly. In this study, we aimed to fast identify anticancer compounds in Forsythiae Fructus using UPLC/MS-based metabolomics analysis. We firstly fractionated Forsythiae Fructus crude extracts with organic solvents of different polarity, then the chemical profile of each fraction was analyzed by UPLC/Q-TOF/MS, and the anticancer activity profiles of all fractions were determined by MTT assay. Next, orthogonal projections to latent structures discriminant analysis (OPLS-DA) was applied to discriminate fractions with different anticancer activity to determine the compound(s) that contributes most to the anticancer activity. Betulinic acid was then identified to be the most potent anticancer compound in Forsythiae Fructus. Its predicted anticancer activity was confirmed by MTT assay. Taken together, our results demonstrated that the present integrated metabolomics strategy could be used for fast identification of anticancer compound(s) in herb extracts or other complex mixtures of chemicals.

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1. Introduction

As a major public health problem worldwide, cancer is the top leading cause of death in China and the second leading cause of death in the United States. [1,2] Since ancient times, natural products have been the main source of many drugs displaying various medicinal properties, particularly in the case of anticancer drugs. It is estimated that over 50% of anticancer drugs used in modern therapeutics originally came from natural products [3]. Therefore, natural products serve as valuable source for novel therapeutic drug discovery. In recent decades, to develop novel anticancer drugs using conventional approaches is still a difficult challenge. Chinese medicine has proven its medicinal efficacy and safety on treating and preventing diseases through thousands of years' practice in Asian. Therefore, exploring new therapeutic compounds or medicinal applications from existing long-prescribed traditional Chinese medicine (TCM) reservoir is considered to be of lower risks and higher cost-effective than using conventional approaches.

Forsythiae Fructus, the dry fruit of *Forsythia suspensa*, is widely used as heat-clearing and detoxicating drug in traditional Chinese medicinal formulas for over 4000 years. In TCM, Forsythiae Fructus is commonly used to treat boils, carbuncles, mumps, tonsillitis, urinary tract infections, allergic rashes, colds or flu-like symptoms (e.g. fever, chills, and headache), etc. Forsythiae Fructus is one of essential herbs in TCM formulas used for treating various cancers (e.g. advanced pancreatic cancer, stomach cancer, liver cancer, etc.) [4–9]. Our previous study also reported that Forsythiae Fructus aqueous extract (FAE) exhibited antitumor activity against melanoma both *in vitro* and *in vivo* [10].

Because of the complexity of constituents in herbs, purification and identification of bioactive compounds by conventional approaches usually require time-consuming multi-step procedures. Therefore, new strategies and approaches for anticancer compounds screening are highly required. In order to develop a more efficient and time-saving method to identify the effective compound(s) from the herbs, we apply metabolomics-based approaches with bioactivity-guided multivariate data analysis for the fast identification of the effective compound(s) directly.

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Metabolomics is a comprehensive qualitative and quantitative analysis of all metabolites that present in an organism, which could be used to explore the complex mixture of plant extract [11–13]. Metabolomics employed with high-resolution mass spectroscopy or nuclear magnetic resonance (NMR) and bioinformatics tools could find out the correlation between two different data sets, such as metabolomics and bioactivity results, and quickly identify the discrepant compounds [14,15].

Multivariate data analysis is widely used in metabolomics to determine disease-related biomarkers, efficacy, toxicity and mechanisms of therapeutic drugs [16]. In recent years, multivariate data analysis has been proven to be unprecedented powerful tool for determining bioactive constituents in a complex system (e.g. Chinese medicine and combinatorial chemistry) without requirement of isolating and evaluating compounds one by one [17–20]. Therefore, the efficiency of screening anticancer constituents in herbs using multivariate data analysis approaches could be much higher than that using conventional methods.

Large data set (e.g. metabolomics profile in body fluid and chemical profile in an herb) contains high-dimensional information which is too complex to be easily analyzed and interpreted. Principal component analysis (PCA) is a mathematical procedure that uses an orthogonal transformation to convert a set of high-dimensional data to a lower-dimensional picture from the most informative viewpoint, which greatly facilitates the analysis and interpretation of complex data. OPLS-DA is a regression extension of PCA by introducing class information to maximize the separation between groups of observations, and to determine which variables contribute to the separation.

In present study, Forsythiae Fructus was primarily extracted using water and ethanol, respectively. The aqueous extract and ethanol extract were subjected to further fractionation using solvents with different polarity (e.g. petroleum ether, ethyl acetate, and *n*-butyl alcohol). Components in each subfraction were separated and analyzed by UPLC/Q-TOF MS. The *in vitro* anticancer activity of each subfraction was measured by MTT assay. The chemical signals of UPLC/Q-TOF MS were extracted, filtered, aligned, and normalized by Markerview software, and introduced to SIMCA-P 13.0 software for multivariate data analysis. OPLS-DA was applied to enhance the discrimination of fractions with different anticancer activities and determine which compound(s) contribute most to the anticancer activity. Betulinic acid in Forsythiae Fructus was predicted to have high anticancer potential. Meanwhile, pharmacological experiments confirmed the anticancer activity of betulinic acid. Our data indicate that the multivariate data analysis-based metabolomics approaches could be applied for effective and fast screening of anticancer compounds in herbs.

2. Materials and methods

2.1. Chemicals and reagents

Forsythoside A, forsythoside B, forsythoside E, phillyrin, rutin, ursolic acid, quercetin, astragalin, oleanolic acid, chlorogenic acid, betulinic acid, isoquercetin were purchased from Sichuan Weikeqi Biological Technology Co.,Ltd. (Chengdu, Sichuan, China). Phillygenin and forsythoside I were purchased from Shanghai Winherb Medical Technology Co.,Ltd. (Shanghai, China). Pinoresinol and (+)-pinoresinol-4-*O*-β-D-glucoside were purchased from Shanghai Tauto Biotech Co.,Ltd. (Shanghai, China) and Chengdu Must Biotech Co.,Ltd (Chengdu, Sichuan, China), respectively. HPLC-grade acetonitrile (CH_3CN) and formic acid were obtained from Merck KGaA (Darmstadt, Germany). HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified by the Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagent were of analytical grade.

2.2. Extraction and fractionation of components in Forsythiae Fructus

Dried Forsythiae Fructus was purchased from Guangdong Province Chinese Herbal Pieces Co., Ltd (Foshan, Guangdong, China). The voucher specimens of Forsythiae Fructus were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China. Ethanol extract was prepared by adding 100 g of dried herbal powder to 1000 mL 90% ethanol to a boiler and simmering at 60 °C for 30 min. The dried herbal powder was extracted twice with ethanol. After cooling down, the upper ethanol layer was filtered and concentrated by rotary evaporator under reduced pressure at 40 °C. Then, the extract was heated on a heat plate to remove ethanol. Followed, the weight of the ethanol extract was recorded and the ethanol concentrate was re-dissolved by ethanol. After that, the re-dissolved solution was successively fractionated by normal-phase silica gel column chromatography eluting with petroleum ether (PE), and ethyl acetate (EA). The fractions were concentrated by rotary evaporator. Thus, four fractions, i.e. EE, PEEE, EAEE and EtEE, were obtained. Similarly, aqueous extracts were prepared by simmering at 80 °C for 60 min. After cooling down, the upper aqueous layer was filtered and extensively concentrated by a rotary evaporator. Followed, the aqueous extract was dried by vacuum freeze dryer. Then, the weight of the aqueous extract was recorded and the aqueous concentrate was re-dissolved by water. After that, the aqueous extract was successively extracted with PE, EA, and *n*-butyl alcohol (NB) to generate the respective fractions. The fractions were concentrated by rotary evaporator. Thus, five fractions, i.e. AE, PEAE, EAAE, NBAE and WAE, were obtained.

2.3. Cell culture

Murine melanoma B16-F10 cell line was used for the assessment of cell viability, which was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). According to the guideline of Cell Bank, cells were cultured in basic medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every other day.

2.4. Assessment of cell viability

Cell viability was determined by MTT colorimetric assay as described in our previous paper [10]. Cells were plated in 96-well plates in corresponding complete medium and allowed to attach overnight. Then the medium was removed and replaced with fresh medium with series concentrations of fractions or compounds. At different time points (24, 48 and 72 h) after exposure to fractions, the number of viable cells in each well was evaluated by adding MTT solution. The spectrophotometrical absorbance was measured at the wavelength of 570 nm by a SpectraMax® M5 microplate reader (Molecular devices, Sunnyvale, CA). The half maximal inhibitory concentration (IC_{50}) is calculated by nonlinear regression of XY analyses using GraphPad Prism software.

2.5. Liquid chromatography-mass spectrometry (LC–MS) analysis

The comprehensive chemical profile in each fraction was analyzed by UPLC/Q-TOF MS on a Waters ACQUITYTM ultra performance liquid chromatography coupled to a SYNAPT G2-Si high-definition mass spectrometer (Waters Corp., Milford, MA, USA) operated using negative (ESI-) ion mode. Five μl aliquot

of each fraction sample with the concentration of 1 mg/ml was injected into an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) maintained at 45 °C. The mobile phase consisted of a linear gradient system of 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B), 0–3 min, 10% B; 3–15 min, 10–20% B; 15–18 min, 20%–100% B; 18–20 min, 100% B. The flow-rate was 0.4 mL/min. Source temperature of mass spectrometry was set at 120 °C with a cone gas flow of 10 L/h. Meanwhile, the desolvation gas temperature was 450 °C with gas flow of 900 L/h. The capillary voltage was set to 2.4 kV (ESI-), sampling cone voltage was set to 40 V. The extraction cone voltage was 4.0 V, the TOF acquisition rate was 0.1 s/scan. MS/MS data were collected for all the ions observed in the preceding MS scan. In order to ensure the accuracy and reproducibility of Q-TOF MS, the leucine enkephalin calibrant solution at the concentration of 200 ng/ml was used as the lock mass in negative ion mode (m/z 554.2615). A full scan mass range from m/z 50 to m/z 1000 was scanned.

2.6. Data processing and multivariate data analysis

Progenesis QI software (Waters Corporation, MA, USA) was used for LC–MS peak extracting, filtering and alignment. The intensity of each peak was then normalized with respect to the total ion intensity of each chromatogram. Thus, a three-dimensional data matrix composing of retention time, m/z value, normalized peak area and bioactivity index was generated and introduced to SIMCA-P software (Umetrics AB, Umea, Sweden) for multivariate pattern recognition analysis, including PLS and OPLS analysis. OPLS, a supervised multivariate statistical method, was employed to sharpen an already established weak separation between the groups of observations, and to reveal the chemicals that mostly contribute to the separation of fractions with different anticancer activities. R^2 and Q^2 (cum) are used for model evaluation, and values of these parameters close to 1.0 indicate a good fitness for the constructed model. From the S-plot of OPLS, the clustering information and ions with potential bioactivity were acquired. Significantly changed potential markers between active and inactive groups were chosen according to the VIP (Variable Importance for the Projection) values which was based on their contribution to the variation and correlation within the data set. To support the potential bioactivity compound identification and further understanding of this study, PubChem (http://ncbi.nim.nih.gov), MassBank (http://www.massbank.jp), METLIN (http://metlin.scripps.edu), and Respect for Phytochemicals (http://spectra.psc.riken.jp/) were queried.

2.7. Cell cycle analysis

Flow cytometry analysis was performed to determine the effect of betulinic acid on cell cycle arrest of B16-F10 cells. Cells were treated with indicated concentration of betulinic acid for 24 h. Then cells were collected and fixed with ice-cold 70% ethanol at −20 °C overnight. After that, cells were procured again by centrifugation, followed by PBS washes. Finally, cells were incubated with propidium iodide (PI) solution for 30 min at room temperature. After staining, the cell cycle distribution was analyzed using flow cytometry (BD Accuri™ C6 Plus, BD Biosciences, San Jose, CA, USA). The results were analyzed by FlowJo software.

2.8. Determination of cellular reactive oxygen species (ROS) generation

B16-F10 murine melanoma cells were plated in 25 mm diameter cell culture dishes at a density of 2×10^6 and incubated in a humidified incubator for 24 h. Then cells were treated by different concentration of betulinic acid for 4 h. After drug treatment, cells were washed with PBS. The culture medium was replaced with FBS-free medium containing 2.5 μM H_2DCF-DA probe (Sigma-Aldrich, St Louis, MO, USA) and incubated for 30 min to assess the ROS levels. *t*-BHP treatment was used as a positive control. For quantitative analysis, the cells were dissociated by trypsin, re-suspended in PBS and analyzed by flow cytometry. The mean fluorescence intensity of 10,000 analyzed cells of each group was measured for the total ROS generation. For image taking, after staining with H_2DCF-DA probe, images were captured using IN Cell Analyzer 2000 (GE Healthcare, Piscataway, NJ, USA).

2.9. Determination of mitochondrial membrane potential (MMP)

JC-1 was used to determine the MMP. B16-F10 murine melanoma cells were seeded in 25 mm diameter cell culture dishes and in 96-well plate at a density of 2×10^6 and 4×10^4/well, respectively. After drug treatment, cells were washed with PBS. The culture medium was replaced with phenol red-free medium containing 2.5 μg/ml JC-1 probe and the cells were incubated for 15 min at 37 °C. For quantitative analysis, cells in dishes were collected and fluorescence intensity was detected by flow cytometry. MMP was calculated as ratio between red and green fluorescence. For image taking, after staining with JC-1 probe, images were captured using IN Cell Analyzer 2000.

2.10. Annexin V/PI assay

Cell apoptosis was determined by Annexin V/PI labeling. B16-F10 murine melanoma cells were seeded in 25 mm diameter cell culture dishes at a density of 2×10^6. After 24 h drug treatment, cells were processed according to the manufacturer's protocol. The early and late apoptotic cells were detected using flow cytometry.

2.11. Determination of caspase-3 and −9 activity in B16-F10 cells

Caspase activity assay kits were purchased from Beyotime Institute of Biotechnology (Nantong, Jiangsu, China). B16-F10 murine melanoma cells were seeded in 100 mm diameter cell culture dishes at a density of 1×10^7. Caspase-3 and −9 activity were determined after 24 h drug treatment according to the manufacturer's protocol.

2.12. Statistical analysis

Each experiment was repeated at least three times. All results were presented as mean ± SD. Student's *t*-test was used for between-group comparison and One-way ANOVA analysis with Tukey post hoc was performed to compare differences in variables of different treatment groups. Statistical significance was accepted at the level of $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5.0 software (La Jolla, CA, USA).

3. Results and discussion

In last decades, increasing attention has been paid to natural products, especially TCM herbs, in novel drug discovery. However, to screen bioactive natural compounds is still challenging work for researchers due to the complex composition of TCM herbs. Metabolomics provides a global view of metabolites in an organism and the relevant approaches can be used to assess the chemical profile of herb extract. Particularly, metabolomics combined with bioactivity-guided multivariate data analysis is an effective tool for the identification of active compounds in the plant [21,22]. Recently, Li et al. [23] employed an integrated strategy combining LC–MS-based multivariate data analysis with bioactivity-guided fractionation method in the fast discovery of neuroprotective

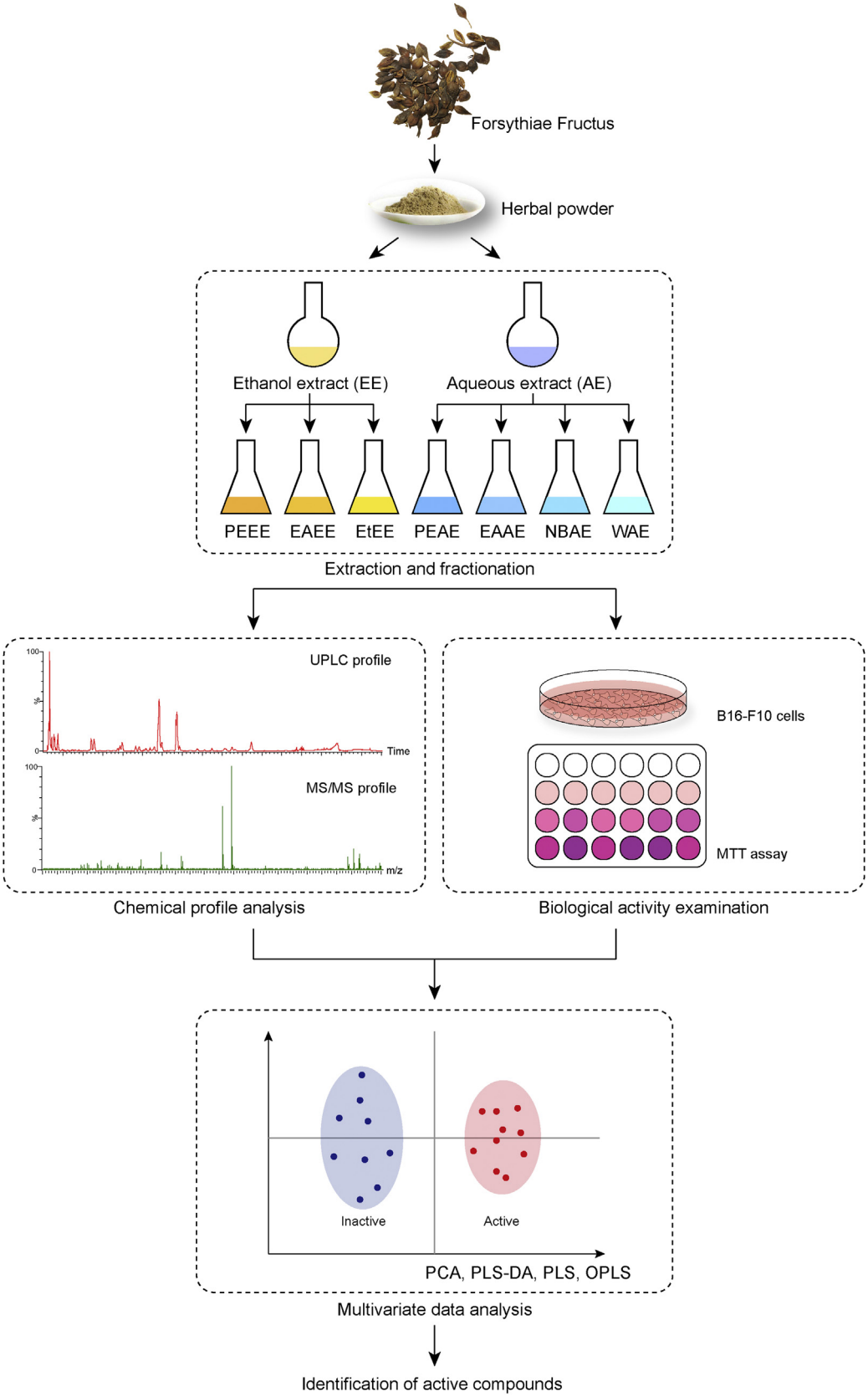

Fig. 1. Schematic diagram of the discovery of potential anticancer constituent(s) in Forsythiae Fructus.

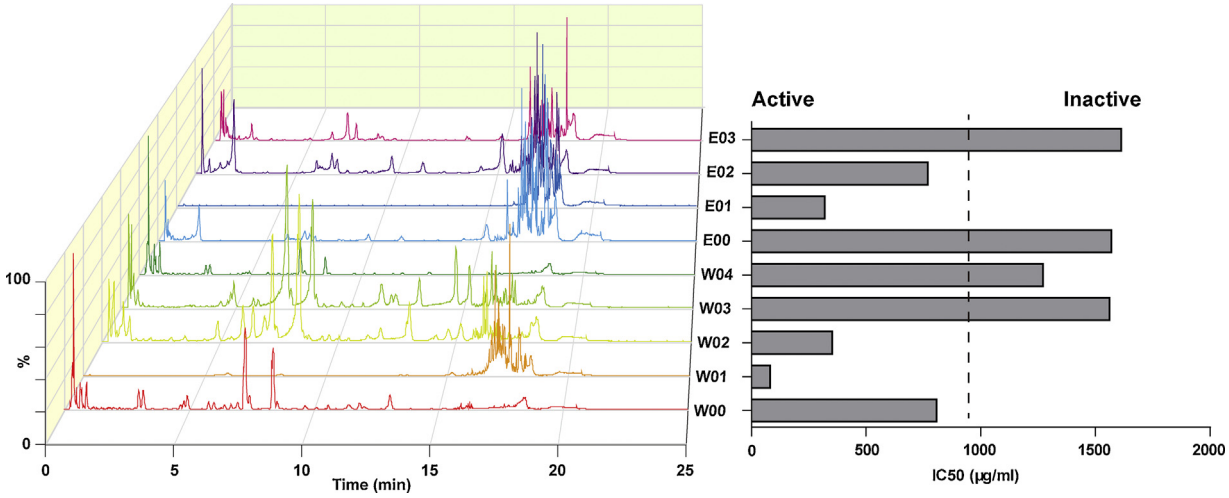

Fig. 2. Representative UPLC/Q-TOF MS and bioassay profiling for Forsythiae Fructus extract fractions. Each of the 9 Forsythiae Fructus fraction samples was subjected to UPLC/Q-TOF MS analysis in negative mode. The base peak chromatograms within the first 25 min are displayed in the left-hand panel. Meanwhile, The IC_{50} of different extract fractions from Forsythiae Fructus on B16-F10 cells was obtained by MTT assay. The IC_{50} value of 24 h treatment was used as the index of the potency of their anticancer activity, which is shown in the right-hand panel. E01 (PEEE), E02 (EAEE), W00 (AE), W01 (PEAE) and W02 (EAAE) were classified in the active group. E00 (EE), E03 (EtEE), W03 (NBAE), W04 (WAE) were classified in the inactive group.

agents in herbs. They efficiently identified that oxyphylla A, a compound derived from the fruit *Alpinia oxyphylla*, was a potent neuroprotective agent against Parkinson's disease.

The aim of this study was to employ UPLC/MS-based metabolomics and multivariate data analysis method in fast identification of potential anticancer constituent(s) in Forsythiae Fructus. According to our previous study, the extract of Forsythiae Fructus could inhibit cell proliferation of B16-F10 melanoma cells and prolong the survival time of tumor-bearing mice, demonstrating its potential anticancer effects on B16-F10 melanoma in vitro and in vivo [10]. In present study, we aim to further identify the anticancer constituent(s) of Forsythiae Fructus. As depicted in Fig. 1, we firstly extracted Forsythiae Fructus by 90% ethanol and water, and got the ethanol extract and aqueous extract respectively. Then, we further separated the ethanol extract with three solvents of distinct polarities to generate the respective PE, EA, and EtOH fractions. Similarly, we separated the aqueous extract with four solvents to generate the respective PE, EA, NB and water fractions. Thus, 9 fractions of Forsythiae Fructus were obtained. Each fraction was subjected to comprehensive chemical profile analysis using UPLC/Q-TOF MS in negative ESI mode (Fig. 2). The anticancer activity of each fraction was evaluated by MTT assay (Table S1, Fig. 2, Fig. S1). Multivariate data analysis was used to correlate the chemical profiling and biological data to predict the candidate compounds. The top five ions were isolated and characterized. The original molecule of the compound was identified combining with *m/z* of the ions and the MS/MS information. The anticancer activity of the identified compounds were further examined.

3.1. *Fractions of Forsythiae Fructus exhibited different inhibitory effect on the proliferation of B16-F10 cells* in vitro

In this study, MTT assay was used to test the anticancer activity of all 9 fractions in B16-F10 cells. The cells were treated with a series of concentrations of 9 fractions for 24, 48 or 72 h, respectively. As expected, these fractions exhibited different levels of anticancer activity against B16-F10 cells (Fig. S1). IC_{50} value was used as the index of the potency of their anticancer activity. The IC_{50} value of each fraction was calculated and summarized in Table S1. Overall, aqueous extract showed stronger inhibitory effect than ethanol extract, and PE extract showed higher anticancer activity than other solvent extracts. All 9 fractions were classified into two groups according to the IC_{50} value. Fractions PEEE, EAEE, AE, PEAE and EAAE were classified in the high anticancer activity group, whereas fractions EE, EtEE, NBAE and WAE were placed in the low bioactivity group (Fig. 2).

Then, we employed OPLS-DA with Pareto scaling to separate all subfractions into two blocks and obtain better discrimination between the active and inactive groups. As shown in Fig. 3, OPLS-DA revealed a significant separation between the active and inactive groups. These results indicated that the compounds at the upper–right corner of S-plot played essential roles in the anticancer effect on melanoma cells. By using the metabolomics platform, 5 potential targeted ions of characteristic components with top VIP values were defined as variables with greater bioactivity, whereas 5 potential targeted ions of characteristic components with top VIP values were defined as variables with lower bioactivity.

3.2. *Validation of UPLC/Q–TOF MS method*

To evaluate the reproducibility and stability of the UPLC/Q-TOF MS method, QC sample was prepared by mixing the aliquots of each batch of Forsythiae Fructus extract fractions in a single sample vial, and analyzed three times at the beginning of each run to ensure that the system was properly equilibrated. QC sample was also analyzed once every five tested samples to further assess the stability of the method. PCA of the MS data can show the differences among all samples, but no grouping of active and inactive samples. Therefore, PCA was firstly performed to investigate general interrelation among the samples, including QC samples. As shown in Fig. S2A, 84.07% of the variables had relative standard deviation (RSD) less than 20% among the 546 ions acquired from QC samples in ESI negative ion mode, suggesting that the analytical platform was robust with excellent reproducibility and stability. In addition, the reproducibility and stability of the system were also evaluated by overall amount of variability of QC samples using PCA. As shown in Fig. S2B, the cluster of QC samples presented in a tight manner in PCA score plots of all samples analyzed, indicating that the UPLC/Q-TOF MS method had excellent stability and reproducibility.

3.3. *Multivariate data analysis*

The supervised multivariate data analysis, such as PLS-DA or OPLS-DA can classify samples into different groups according to

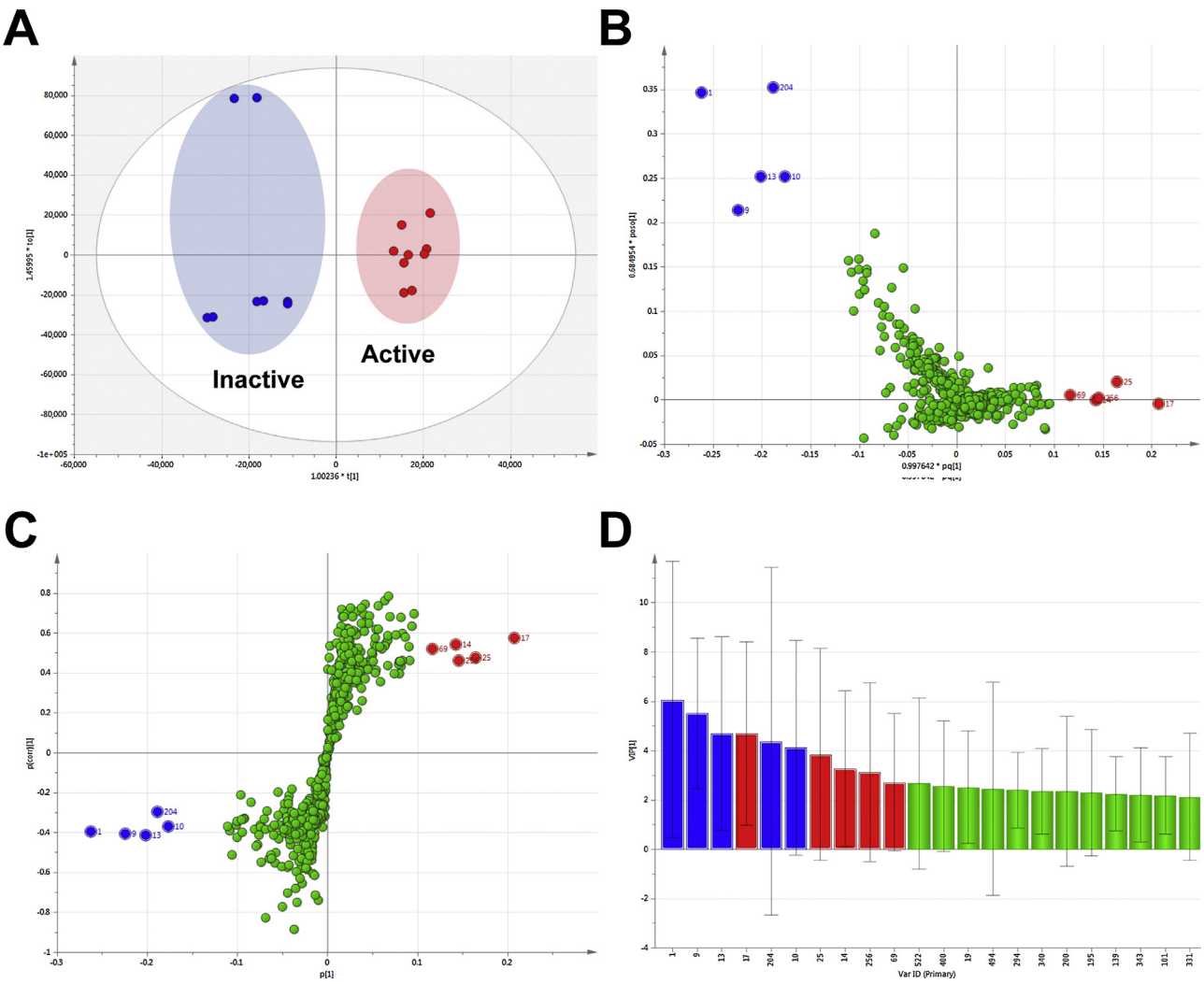

Fig. 3. OPLS score plot based on the global chemical profiling of Forsythiae Fructus extract fractions. (A) OPLS score plot of inactive group with high IC_{50} value (blue dot) and active group with lower IC_{50} value (red dot). (B) OPLS loading plot of Forsythiae Fructus extract fractions detected in negative ion mode, with fitting and predictive performance (3 latent variables, R2X = 0.921, R2Y = 0.933, Q2 = 0.755), which displays the weighting each variable carried along the first principal component. (C) S-plot of OPLS, the 10 variables with top VIP value were highlighted with red filled circle and blue filled circle. (D) VIP plot, which summarizes the importance of the variables both to explain X and to correlate to Y. The 10 variables with top VIP value were highlighted with red filled circle and blue filled circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the bioactivity of samples [22]. In particular, the S-plot generated by OPLS-DA after data standardized using Pareto-scaling can be used to screen biomarkers. Since Pareto scaling is commonly used to reduce the influence of intense peaks while emphasizing weaker peaks that may have more biological relevance, the corresponding loadings of intense peaks will be reduced and loadings from weak peaks will be increased due to Pareto scaling [24]. The signals related to the activity can be separated from the large amount of variable ions via OPLS-DA. As shown in Fig. 3, the fractions with greater bioactivity can be clearly separated from those with lower bioactivity, and 10 variables were identified to be mostly responsible for the separation. The ions at the end of the S-shaped curve were considered to be characteristic components that made the greatest contribution to distinguish active group and inactive group. The 5 red dots at the upper–right corner of S-plot represented the ions with higher MS intensity in active group, which were considered to be characteristic components with greater bioactivity. On the contrary, the blue dots at the bottom-left corner of S-plot represented the ions with higher MS intensity in inactive group, which were considered to be characteristic components with lower bioactivity.

Table 1
The top 5 potential targeted ions of characteristic components with greater bioactivity.

No.	Var ID	Ret. Time	*m/z*	VIP[1]	p[1]
1	17	16.984	333.2069	4.69109	0.207456
2	25	17.583	347.2228	3.84639	0.164649
3	14	17.134	319.2274	3.27356	0.145792
4	256	17.975	455.3529	3.12733	0.142846
5	69	17.291	317.2118	2.71456	0.116554

3.4. Identification of the targeted ions

The variables with high VIP value (Fig. 3C, Tables 1 and 2) were considered as potential compounds that contribute to the discrimination of anticancer activity of Forsythiae Fructus fractions. Molecular mass was determined within a reasonable degree of measurement error (< 2 ppm) using Q-TOF MS, and the potential element composition and degree of saturation of the compounds were obtained. The identity of the compounds was predicted by comparing the accurate MS and MS/MS fragments of our measurement with that in on-line databases. Due to the complexity of small

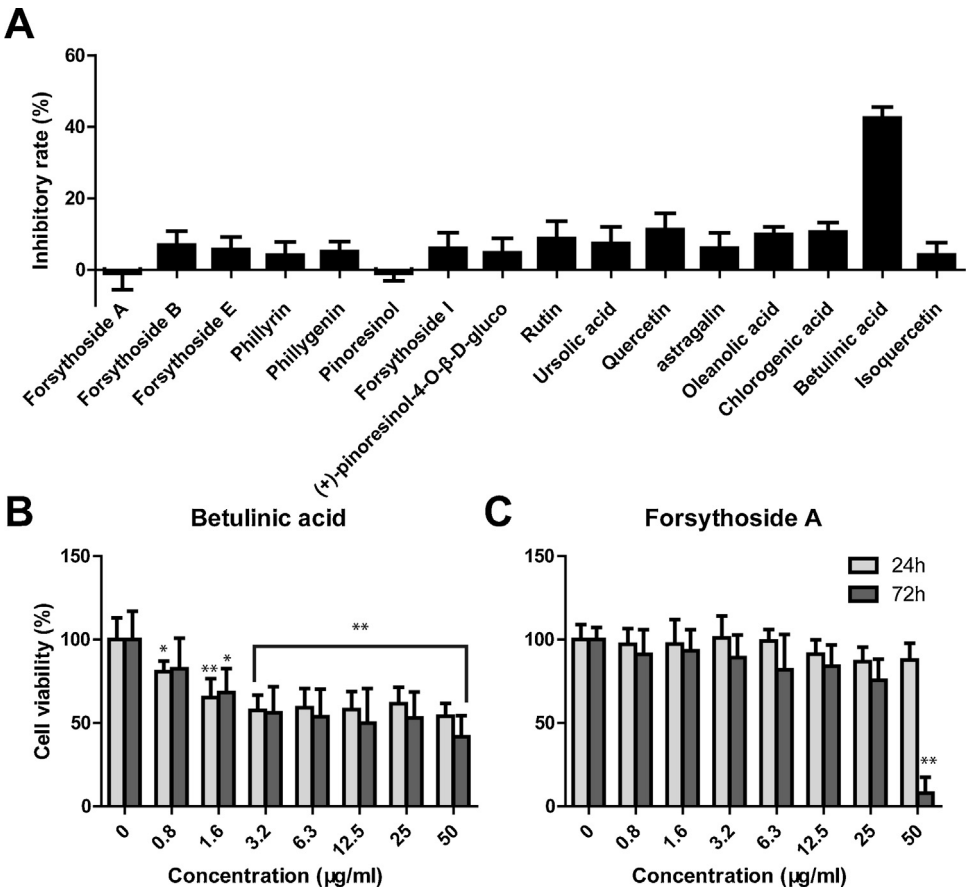

Fig. 4. The anticancer activity of the representative compounds in Forsythia suspensa. (A) Inhibitory rate of the representative compounds in Forsythia suspensa. B16-F10 cells were treated by the representative compounds for 24 h at the concentration of 3.2 μg/ml, respectively ($n = 6$). MTT was used to test the cell viability of B16-F10 cells treated by betulinic acid (B) and forsythoside A (C) for 24 h and 72 h. Data represent means ± SD. $^{**}p < 0.01$, $^{*}p < 0.05$.

Table 2
The top 5 potential targeted ions of characteristic components with lower bioactivity.

No.	Var ID	Ret. Time	*m/z*	VIP[1]	p[1]
1	1	0.405	249.0079	6.07316	−0.262409
2	9	7.247	623.1979	5.51215	−0.224384
3	13	0.405	235.0283	4.70147	−0.201658
4	204	18.439	116.9284	4.37632	−0.188354
5	10	0.926	703.1554	4.12009	−0.176387

molecules in plants and databases, we restrict the comparison with the known compounds found in *Forsythia* species.

MS analysis of Var 9 revealed an *m/z* value of 623.1976 for $[M-H]^-$ in the negative ion mode, which indicated that this compound had an empirical molecular formula of $C_{29}H_{36}O_{15}$. MS analysis of chemical marker Var 256 revealed an *m/z* value of 455.3529 for $[M-H]^-$ in the negative ion mode, which suggested that its empirical molecular formula was $C_{30}H_{48}O_3$. Through comparing their MS data with those compounds isolated from *Forsythia* species, and searching the online databases, the potential targeted variables Var 9 and Var 256 were identified as forsythoside A and betulinic acid, respectively (Table 3). Moreover, these compounds were further identified by comparison with the authentic standards.

3.5. Experimental verification of the anticancer activity of betulinic acid

In order to validate the prediction of multivariate data analysis, we examined the cell viability of the representative compounds from *Forsythia suspensa* by MTT assay. Betulinic acid exhibited the strongest anticancer activity at the concentration of 3.2 μg/ml, whereas forsythoside A had no activity at the same concentration (Fig. 4). These results were consistent with the prediction from multivariate data analysis. Flow cytometry analysis was performed to examine the effect of betulinic acid on cell cycle of B16-F10 cells. As shown in Fig. 5A and S3A, treatment with betulinic acid led to obvious changes in cell cycle distribution. Betulinic acid could significantly increase the proportion of cells in G1 phase and reduced cell numbers in S phase and G2/M phase. These results indicated that betulinic acid induced a cell cycle arrest at G1 phase in B16-F10 cells. B16-F10 murine melanoma cells were exposed to a series of concentrations of betulinic acid for 4 h. ROS generation was determined by flow cytometry. As shown in Fig. 5B and S3B, betulinic acid treatment significantly induced ROS generation in B16-F10 cells in a dose-dependent manner. These results suggested that the inhibition of B16-F10 cell proliferation could be via, at least partially, ROS generation induced by betulinic acid treatment.We next determined whether the treatment of betulinic acid could affect MMP and mitochondria function by JC-1 staining. Red fluorescence of the J-aggregate form of JC-1 indicates intact mitochondria, whereas green fluorescence shows a monomeric form of JC-1 which is due to the breakdown of the mitochondrial membrane potential. As shown in Fig. 5C and S3C. The red fluorescence (J-aggregate) intensity was diminished significantly whereas the green fluorescence (monomer) intensity was enhanced after betulinic acid treatment. Betulinic acid treatment induced more than 30% decrease in the ratio of red to green fluorescence. These results suggested that betulinic acid could decrease the MMP and induce mitochondrial

Table 3
Identification of potential targeted ions that contribute to the discrimination.

Var ID	Ret. Time	*m/z* Determined	*m/z* Calculated	MS Error(ppm)	Ion Form	Molecular weight	Molecular Formula	Metabolite identification
256	17.975	455.3529	455.3525	0.4047	[M-H]	456.69	$C_{30}H_{48}O_3$	Betulinic acid*
9	7.247	623.1979	623.1976	0.2995	[M-H]	624.59	$C_{29}H_{36}O_{15}$	Forsythoside A*

* Metabolites identified by comparison with authentic standards.

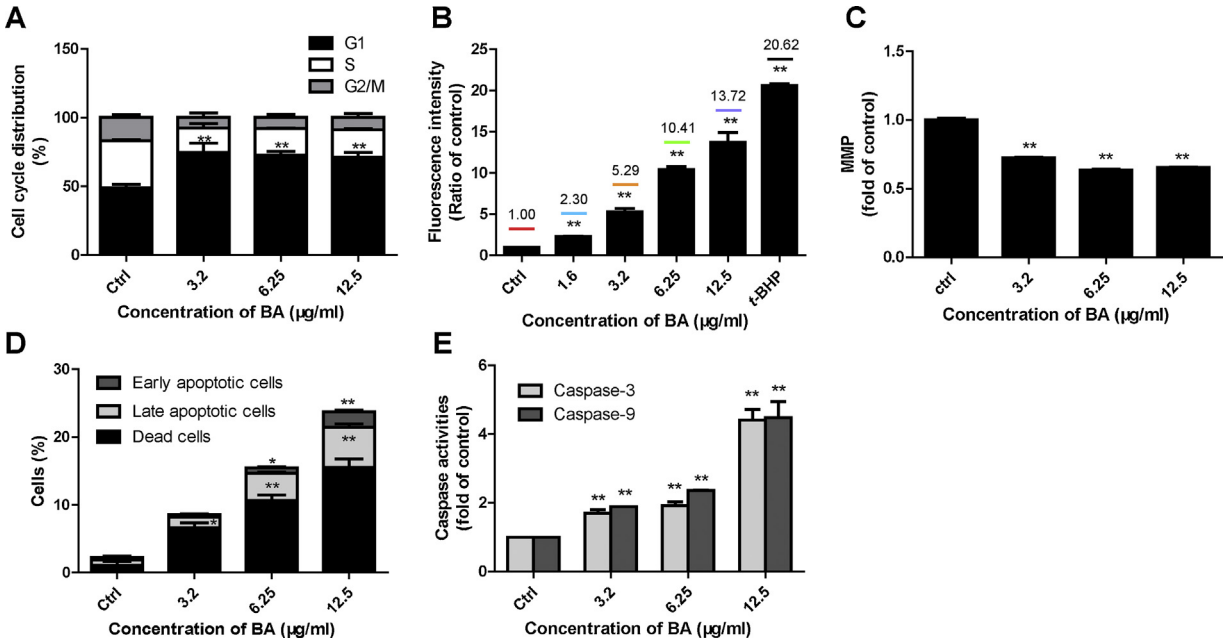

Fig. 5. The anticancer effect of betulinic acid on B16-F10 cells. (A) The effect of betulinic acid on cell cycle arrest. After exposure of B16-F10 cells with indicated concentration (3.2–12.5 μg/ml) of betulinic acid for 24 h, the proportion of cells at different cell cycle phases was determined by flow cytometry analysis. (B) ROS generation in B16-F10 cells after betulinic acid treatment. B16-F10 murine melanoma cells were exposed to inducated concentrations of betulinic acid for 4 h. ROS generation was determined by flow cytometry. t-BHP treatment was used as a positive control. (C) Betulinic acid decreased the mitochondrial membrane potential of B16-F10 cells. B16-F10 cells were treated with betulinic acid for 24 h followed by incubation with JC-1 probe. The fluorescence intensity was detected by flow cytometry. MMP was calculated as ratio of red fluorescence to green fluorescence. (D) Quantification of apoptosis by flow cytometry. Q1: Dead cells, annexin V-FITC−/PI+, Q2: Late apoptotic cells, annexin V-FITC+/PI+, Q3: Early apoptotic cells, annexin V-FITC+/PI−, Q4: Live cells, annexin V-FITC-/PI-. (E) Effects of betulinic acid on the activation of Caspase-3 and −9 in B16-F10 cells. Data were expressed as mean ± SEM of three independent experiments. Significant difference was performed by one-way ANOVA. $^{**}p < 0.01$, $^{*}p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

depolarization in B16-F10 cells. To test whether betulinic acid treatment could induce apoptosis in B16-F10 cells, we performed Annexin V/PI staining assay using flow cytometry. As shown in Fig. S3D, significant difference was shown in the representative scatter plots of cells treated by a series concentration of betulinic acid. The percentage of early/late apoptotic cells and death cells was summarized in Fig. 5D. In the control group, the proportion of early and late apoptotic cells was 1.19%. After 24 h treatment with 3.2–12.5 μg/ml betulinic acid, the ratio of early and late apoptotic cells was significantly increased by up to 8.21%. Moreover, the proportion of total death cells was increased from 2.24% to 23.7%, which was consistent with the cell inhibitory effect of betulinic acid. Activation of caspases is a hallmark of apoptosis (Miura et al., 1993). We then determine the status of caspases to confirm the induction of apoptosis by betulinic acid. As shown in Fig. 5E, betulinic acid treatment caused remarkable increase of caspase-3 and −9 proteolytic activities in B16-F10 cells in a dose-dependent manner.

It was reported that forsythoside A was the main active substance in *Forsythia suspensa*, which showed antioxidant and anti-inflammatory activities [25–27]. The antitumor effect of Forsythiae Fructus extract has been investigated on several cell lines and mouse models. Forsythoside A is generally considered to be the major antitumor component owing to its high content in *Forsythia suspensa* [10,28]. However, forsythoside A only showed inhibitory effect on cell proliferation at concentration of 50 μg/ml after 72 h treatment. Notably, forsythoside A was identified at the bottom-left corner of S-plot in Fig. 3C, which is a component with lower bioactivity, indicated that forsythoside A at certain concentration range might play a negative role in the anticancer activity of Forsythiae Fructus extract.

Both multivariate data analysis (Fig. 3) and *in vitro* assay (Fig. 4A) indicated that betulinic acid was mostly responsible for the anticancer activity of Forsythiae Fructus extract. Betulinic acid (3b, hydroxyl-lup-20(29)-en-28-oic acid) is a pentacyclic triterpene which can be found in many plant sources. It was well documented that betulinic acid has multiple pharmacological activities including anti-HIV, anti-inflammatory, anti-malarial, anthelmintic, and anticancer activities [29]. There are multiple mechanisms for its anticancer effects, such as inducing apoptosis, anti-angiogenesis, anti-metastasis, anti-multidrug resistance [30]. In our study, betulinic acid exhibited potent anticancer on melanoma cells. As shown in Fig. 5, betulinic acid inhibited cancer cell proliferation and induced cell cycle arrest at G0/G1 phase. Moreover, betulinic acid induced cell apoptosis, increased the levels of cellular ROS and caused dysfunction of mitochondria.

4. Conclusions

We developed an integrated UPLC–MS-based metabolomics analysis and bioactivity-guided multivariate data analysis for the fast identification of anticancer compound(s) in herb extract. The data showed that betulinic acid was a potent anticancer com-

pound in Forsythiae Fructus. The results of *in vitro* anticancer assays confirmed the prediction of metabolomics analysis. This study demonstrated that the integrated approach could serve as a useful tool for the fast identification of active compounds in herbs.

Author contributions

Conceived and designed the experiments: JB CH. Performed the experiments: JB RD YL FL CZ XJ. Analyzed the data: JB FL CH JBW. Contributed reagents/materials/analysis tools: PL KW YW. Wrote the paper: JB RD CH.

Conflict of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jpba.2018.03.020.

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