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Ultra-High Performance Liquid Chromatography Coupled with Time-of-flight Mass Spectrometry Screening and Analyzing the Potential Bioactive Compounds from Traditional Chinese Medicine Kaixin San, Using Multivariate Data Processing Approach and Metabolynx Tool

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Abstract

Traditional Chinese medicine (TCM) has been used in clinical practice for several thousand years, and has played an indispensable role in the prevention and treatment of disease, due to it contains multiple ingredients. Kai-Xin-San (KXS) is a TCM formula consisting of four herbs, Ginseng Radix, Polygalae Radix, Poria and Acori Tatarinowii Rhizoma, has been used to treat Alzheimer's disease, depression and Parkinson's disease. However, the constituents absorbed into blood after oral administration of KXS remain unknown. Here, a sensitive and rapid method by ultra performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS/MS) combined with multivariate data processing approach (Mdpa) and Metabolynx$^{(TM)}$ software tools were used for analysis and identification of the bioactive components and their metabolites in rat plasma following oral administration of KXS. A hyphenated MS/MS analyzer was used for the determination of accurate fragment ions and mass spectrometric fragmentation mechanisms, and enhanced data acquisition. Metabolite elucidation was performed using UPLC/Q-TOF-MS/MS coupled with Metabolynx tool. With the established method, a total of 49 peaks were tentatively characterized in vivo based on MS and MS/MS data and comparison with available databases. Of the tentatively characterized 49 compounds in rat plasma, a total of 7 metabolites were detected and identified by comparing their fragmentation patterns using MetaboLynx software tool. On the basis of the chromatographic peak area, the glucuronidated conjugates were identified as major metabolites. Furthermore, this work demonstrated the potential of the UPLC/Q-TOF-MS/MS with Mdpa and MetaboLynx for quite rapid, simple, reliable and automated identification of metabolites of herbal medicine.

Keywords

UPLC/Q-TOF-MS/MS; Kai-Xin-San; identification; multivariate data processing approach; Metabolynx

1. Introduction

Traditional Chinese medicine (TCM) prescription have been widely used to possess antibacterial, antifungal, anticancer, antiviral, anti-inflammatory activity and other pharmacological activities of benefit to humankind [1]. The pharmacological effects of TCM prescription are demonstrated to express their effects through multi-active chemical constituents refers to the total compounds which have active function on some disease [2]. The sources of these effective compounds are complicated, including original compounds from the prescription and the products of processed decoction. For the screening and analysis of effective compounds in TCM prescription, conventional phytochemical approach which isolate and identify individual components one by one could be used to search the lead compounds in herbal medicine, but this strategy is a time-consuming, and labor intensive [3]. In views of this, it is essential to develop novel and available methods which could overcome all these limitations, to reveal the complex compounds in TCM prescription.

Recently, high-resolution ultra high-performance liquid chromatography equipped with electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/Q-TOF-MS/MS) has been widely used to identify and analyze the chemical compounds in TCM. In particular, it provides accurate precursor and/or product ions information with a mass error of less than 5 ppm, which substantially enhances the constituent characterization reliability [5]. A new method based on UPLC/Q-TOF-MS/MS combined with multivariate data processing approach (Mdpa) has been widely introduced for the screening and analysis of herbal medicine [6-8]. With the advantages of short analysis time as well as high accuracy of mass values, UPLC/Q-TOF-MS/MS with Mdpa provide an efficient strategy for rapid screening, and identification of the active components in herbal medicine [9]. Substantial progress has been made in using the MetaboLynx^{TM} software to further clarify the metabolic profile. With key parameters carefully set, MetaboLynx is able to show the presence of a wide range of metabolites with only a limited requirement for manual intervention and data interpretation time.

Kai-Xin-San (KXS), firstly recorded in the Chinese ancient medical prescription book *Qian-Jin-Yao-Fang* around 1300 years ago, is a TCM prescription consisting of four herbs, Ginseng Radix, Polygalae Radix, Poria, and Acori Tatarinowii Rhizoma [10]. It has been used for Asian countries to treat Alzheimer's disease, Parkinson's disease, etc [11-13]. Recently, chemical analysis study of KXS has rarely been reported that the polygalaxanthone III, ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, and ginsenoside Rg1 were determined in the plasma of rat after oral administration of KXS by ultra-fast liquid chromatography with tandem mass spectrometry [14]. However, until now, the study on systematically chemical profile of the effective ingredients in the KXS prescription is still not fully known. In this paper, a reliable UPLC/Q-TOF-MS/MS combined with Mdpa method was developed for carrying out a comprehensive characterization of the major potential bioactive constituents and metabolites of KXS. To our best knowledge, this is the first systematical study on screening the bioactive components in KXS.

2. Material and methods

2.1 Chemicals and materials

Methanol and acetonitrile (HPLC grade) was purchased from Merck (Darmstadt, Germany); leucine enkephalin was purchased from Sigma-Aldrich (MO, USA); deionized water (18.2 MΩ) was further purified using a Milli-Q system (Millipore, Billerica, USA); HPLC grade formic acid was purchased from Kermel Chemical Reagent Co., Ltd (Tianjin, China); formic acid was purchased from (DIKMA, USA). Ginseng Radix, Poria, Polygalae Radix, Acori Tatarinowii Rhizoma was purchased from Harbin Tongrentang Drug Store (Harbin, China), and authenticated by Prof. Xijun Wang, Department of Pharmacognosy of Heilongjiang University of Chinese Medicine. Voucher specimens were deposited at the authors' laboratory.

2.2 Preparation of KXS samples

The KXS was prepared in the following procedure: Ginseng Radix, Poria, Polygalae Radix, Acori Tatarinowii Rhizoma in proportion 3:3:2:2, were ground into crude powders, mixed, and then reflux extraction in a rotary evaporator with 6 times volume of 70% ethanol for 2 h twice, then the filtrate was dried under freeze-drying. The freeze-dried powder was dissolved in water, ultrasonic for 10min, made a concentration of 0.08g/ml solution.

2.3 Animals handling

Male Wistar rats (300±10 g) were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were housed in an animal room (24 ± 2◦C, 40% relative humidity). A 12-h dark/light cycle was set, and given water and fed normal food for 1week before the experiment. All rats were randomly divided into 2 groups of 3 rats each: control group and dosed group. Rats were orally administered KXS extract at a dose of 1ml /100g body weight. The control group was orally administrated with an equivalent volume of distilled water. After 60 min, the rats were anaesthetized by intraperitoneal injection of 3% pentobarbital sodium (0.3 mL/100g body weight).

2.4. Preparation of plasma samples in vivo

The blood samples were collected from hepatic portal vein at 1h after administration and the rats were sacrificed. Then, the plasma was separated immediately by centrifuging at 13 000rpm for 10min at 4 ◦C. All samples were stored at −80 ◦C until analysis. 8 mL methanol was added to 2.0 mL blood samples and then vortexed for 30 s, ultrasound and centrifuged at 13000 rpm for 10min at 4 ◦C, the supernatant was dried under a stream of nitrogen gas at 45 ◦C. Each dried sample was reconstituted in 200 μl methanol prior to analyses, centrifuged at 13000 rpm for 10min at 4 ◦C. The sample was filtered through a 0.22 um membrane, and 5ul aliquot was injected for UPLC/MS analysis.

2.5 Ultra-high performance liquid chromatography condition

Chromatographic separation for samples was performed using a Waters Acquity™ ultra performance LC system controlled with Masslynx (V4.1) and controlled by MassLynx software (Version 4.1). Separation was performed with an acquity UPLC HSS C_{18} column (100 mm × 2.1 mm i.d., 1.8 μm particle size, Waters Corporation, Milford, MA, USA) maintained at 40 ◦C. A gradient with eluent A (HCOOH: H_2O=0.1: 100, v/v) and B (HCOOH: CH_3CN= 0.1:100, v/v) was used at a flow rate of 0.3 ml/min. The linear elution gradient program was used as follows: 0-1.0 min, 2-20 % A; 1.0-4.0 min, 20-21% A; 4.0-6.5 min, 21-30% A; 6.5-9.5 min, 30-35% A; 9.5-14.0 min, 35-50% A; 14.0-16.0 min, 50-52 % A; 16.0-16.5 min, 52-82 % A; 16.5-19.0 min; 82-83 % A; 19.0-19.5 min, 83-100 % A; 19.5-20.0 min, 100-2 % A; 20.0-21.0 min, 2 % A. The sample-tray temperature was kept at 4°C.

2.6 Mass spectrometry condition

MS instrument consisted of a Waters Synapt™ QTOF/MS (Waters Corp., Milford, MA, USA). Ionization was performed in both positive and negative ion modes. The MS source temperature was set at 110 °C, and the desolvation temperature was set at 300 °C with desolvation gas flow at 600 L/h. The capillary voltage was 3 kV. The mass spectra were recorded across the range of m/z 100 to 2700 Da, with accurate mass measurement of all mass peaks. Ar gas was used as collision gas at a pressure of 0.2Mpa. The collision energy was set as 10-30 eV for low-energy scans, and 30–50 eV for high-energy scans. MS/MS experiment was operated for major metabolites to obtain additional information from product ions. Leucine-enkephalin was used as the lock mass generating a reference ion for accurate mass acquisition. The instrument was controlled by Masslynx 4.1 (Waters Corp.).

2.7 Multivariate data processing approach

The raw data of all tested samples were analyzed by the MarkerLynx and EZINFO software (Waters Corp., USA) for preliminary phytochemical screening. The three-dimensional data comprising peak number, sample name and ion intensity were analyzed by principal component analysis (PCA) and orthogonal partial least-squared discriminant analysis (OPLS-DA) in the EZINFO 2.0 software. For further confirmed the structure and the source of the metabolites, all data were introduced to MassFragment™ software tool. The ions which were present in the dosed group and absent in the control group were extracted with the help of the VIP plot of OPLS-DA, and further these ions were identified with a combination of elemental composition tool and MS/MS fragment mass spectra.

2.8 Metabolynx processing approach for metabolites analysis

Expected and unexpected metabolites were found out using Metabolynx software (Waters Corporation, Milford, USA) by automatically comparing MS(E) data from the sample and control. The metabolites were reliably characterized by

accurate MS/MS spectra and their different fragmentation pathways. It employs an extensive list of potential biotransformation reaction consists of deglycosylation, ringcleavage, dehydroxylation, decarbonylation, methylation, sulfation, hydrogenation andhydroxylation. Metabolynx^{TM} in combination with the elemental composition of the substrate molecules generated a series of extracted ion chromatograms. The extracted ion chromatograms which present in the analyte were evaluated relative to the controls ample by comparing their retention time and peak area. The method parameters for data processing were set as follows: retention time range 0.1-20.0 min, mass range 50-1500 Da, retention time tolerance 0.1 min, mass tolerance 0.05 Da, noise elimination level 5, peak intensity threshold 50. If different peaks were found in the analyte by comparing with control sample, only those peaks with the area ratio of analyte to control sample to be above 3 were considered to be the compound- related metabolites.

3. Results and discussion

3.1 Optimization of chromatographic and mass spectrometry conditions

UPLC and MS conditions were optimized to obtain better detection. UPLC chromatograms with good separation, different mobile-phase compositions were screened (data not shown) and found that acetonitrile and formic acid was the most suitable eluting solvent system. The mobile phase played an important role in achieving good chromatographic behavior and appropriate ionization. A mixture of 0.1% v/v aqueous formic acid and acetonitrile was finally chosen as the preferred mobile phase because it produced the desired separation and acceptable tailing factors within the 20 min run time. In the course of optimizing separation conditions, mobile phase, gradient program, column temperature and detection wavelength were investigated. The final results showed that best resolution, shortest analysis time and lowest pressure variations were achieved when a gradient elution mode composed of acetonitrile (containing 0.1% formic acid, eluent A) and water (containing 0.1% formic acid, eluent B) was programmed as follows: 0-1.0 min, 2-20 % A; 1.0-4.0 min, 20-21% A; 4.0-6.5 min, 21-30% A; 6.5-9.5 min, 30-35% A; 9.5-14.0 min, 35-50% A; 14.0-16.0 min, 50-52 % A; 16.0-16.5 min, 52-82 % A; 16.5-19.0 min; 82-83 % A; 19.0-19.5 min, 83-100 % A; 19.5-20.0 min, 100-2 % A; 20.0-21.0 min, 2 % A. The most efficient way to obtain rapid analysis time, while generating high column efficiency and resolution, is by utilizing a small particle size column. Therefore, chromatographic analysis was executed through a 1.8 μm particle size HSS C_{18} column. Considering sensitivity and resolution, the ultimate flow rate was optimized at 0.3 mL/min.

Furthermore, in order to reduce the column pressure resulting from a higher flow rate, the liquid chromatography column temperature was set at 40◦C. TOF/MS enables peak deconvolution of analytes of differing m/z after ionization was utilized in order to achieve fast scanning characteristics for rapid chromatographic separations. For the MS conditions, a series of parameters including the ion spray voltage, turbo spray temperature, declustering potential, collision energy, nebulizer gas, heater gas and curtain gas were all optimized. Both positive and negative ion modes

were employed to screen as many potential compound signals as possible. The negative ion mode provided higher signal intensity and had ability to detect more peak signal perhaps because of the existence of some compounds easy to ionize in the negative mode. The negative ion mode was employed because of its increased sensitivity to the signals of the common constituents compared with the positive ion mode. Furthermore, some ions were only observed in the negative ion mode, which is helpful for the structural determination. As a result, the negative ion mode was used. The base peak ion (BPI) chromatogram in rat plasma after oral administration of KXS analyzed by UPLC/Q-TOF-MS/MS in positive ion mode and negative ion mode was shown in Figure 1.

3.2 Multivariate statistical analysis

Mdpa converts the multidimensional data space into two matrices known as scores and loadings. In the PCA score plot (Fig. 2A and B), each coordinate represents a sample, and it could be observed that the determined samples are clearly divided into two clusters. For the analysis of the differences in chemical compositions between dosed rat plasma and blank plasma samples, OPLS-DA, a supervised multivariate analysis method was performed. The interest ions which were present only in the dosed group and absent in the control group were extracted easily by VIP plot of OPLS-DA (Fig. 2C and D). In the VIP-plot, each point represents an ion RT–m/z pair; the X-axis represents variable contribution, and the further the ion RT–m/z pair point departs from zero, the more the ion contributes to the difference between the dosed rat plasma and blank plasma samples. Using UPLC/Q-TOF-MS/MS and database-matching techniques, a total of 42 compounds were characterized tentatively. As demonstrated above, 42 interested ions in blood samples were extracted and identified *in vivo*, 21 of them were detected by pattern recognition methods in positive ion mode and others were detected in negative mode. Information regarding the 42 compounds, such as the tR (min), identity, observed m/z values, mass error, molecular formula, and botanical source, MS/MS data is offered in Table S1.

3.3 Metabolites identified using Metabolynx tool

Using Metabolynx™ software, peaks present in the analyte were evaluated relative to the control samples by comparing their retention times with post-acquisition analyses, MS spectra and peak area. When a peak was included as a metabolite, the peak area in the analyte had to be at least 5 times greater than that of the control. After post-acquisition data processing of plasma samples using a MetaboLynx program, the results demonstrated that the vast majority of the metabolites. The extracted ion chromatogram of plasma samples with Metabolynx tool are presented in Figure 3, and the products were well separated using the developed UPLC method. Through a comprehensive analysis of the peaks, we found 7 peaks compared with blank samples: one parent constituent and three metabolites which indicated that glucuronide conjugation, decarbonylation, and deethylation were the major metabolic pathways of constituents in vivo. Table S2 lists the detailed information of these metabolites, including the retention times,

proposed elemental compositions, and the characteristic fragment ions. Taking an example, in ESI negative ion mode, it gave an $[M-H]^-$ ion at m/z 567.1332, indicating the formula $C_{25}H_{28}O_{15}$ (calc. 568.1428). According to its retention time and MS data, the compound was identified as polygalaxanthone Ⅲ (Figure 4). Similarly, other compounds could also be characterized according to the above-mentioned methods.

Previous studies examined the uptake of ginsenosides Rg1, Re and Rb1 to the brain after oral administration of KXS preparation [15]. The results suggested that the presence of delivering servant in the KXS formula promoted the initial absorption of ginsenosides Rg1 and Re in the gastrointestinal tract, but unlikely affected the brain-to-plasma AUC ratios. A study showed that KXS aqueous extract significantly ameliorated the depressive symptoms, including the reduced preference index and prolonged latency to novelty-suppressed feeding [16]. Simultaneously, KXS exerts its antidepressant-like and nootropic effects by modulating the HPA axis, monoamine neurotransmitter and cholinergic systems. Moreover, the mechanism of its antidepression action was preliminary explored [17]. The expressions of the molecular bio-markers relating to depression in rat brains were altered by the treatment of KXS. It suggested that the anti-depressant-like action of KXS might be mediated by an increase of neurotransmitters and expression of neurotrophic factors and its corresponding receptors in the brain.

In this paper, integrated UPLC/Q-TOF-MS/MS with Mdpa and Metabolynx software tools were performed to screen the bioactive compounds from KXS, one of the most well-known TCM prescription, which is clinically effective for Alzheimer's disease and Parkinson's disease, was chosen as a case. Although KXS has been used in clinic widely, the bioactive ingredients and metabolites of KXS are not fully known. The bioactive compounds in KXS could be discovered by comparative analysis of the chemical profiles of control plasma and dosed plasma, and then identified based on their MS and MS/MS spectra. The data sets of retention time (RT)-m/z pairs, ion intensities and sample codes were further processed with Mdpa to generate a VIP-plot. Furthermore, the established method allowed the detection of low-abundance metabolites along with their structural elucidation. Mdpa method indicated that 42 components in the KXS were absorbed into the rat's body. In the present study, metabolites in vivo system of Wistar rats were identified and elucidated by UPLC/Q-TOF-MS/MS. The automated data analysis (MetaboLynx) was developed and validated for simultaneous identification and determination of metabolites produced by body in vitro. Expected and unexpected metabolites were detected by Metabolynx software, which could automatically compare MS data from the sample and control [18-20]. In addition, 7 components might be metabolites of some components in the KXS. From these results, it could be concluded that the proposed method could be used to simultaneously analyze and screen the multiple absorbed bioactive constituents and metabolites in TCM. The present method provides a quick and reliable analytical tool to investigate the prototype components absorbed and metabolite metabolism of TCM. In order to illustrate the dynamic metabolism profiles of absorbed constituents and metabolites in vivo, further

research would be required on pharmacokinetics to understand the absorption, distribution and excretion of these components.

4. Conclusion

In this work, we demonstrated the use of UPLC/Q-TOF-MS/MS coupled with automated with Mdpa and Metabolynx data analysis for the first time for structural characterization of global compounds in rat plasma following oral administration of KXS. After the automated analysis, a total of 49 compounds were characterized tentatively. The proposed method was appropriate for rapid screening and identification of absorbed and metabolic components of KXS. Additionally, we could conclude that the 49 compounds including 42 components from KXS and 7 metabolites were simultaneously determined, and provided essential data for further pharmacological studies of KXS. This approach yielded a series of potential bioactive compounds in a high-throughput manner, which is useful for the drug discovery of compounds from KXS. This method could be developed as an integrated approach for screening and identifying the active ingredients in TCM prescription and provided helpful chemical information for further pharmacology and active mechanism research.

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Competing financial interests

The authors declare no competing financial interests.

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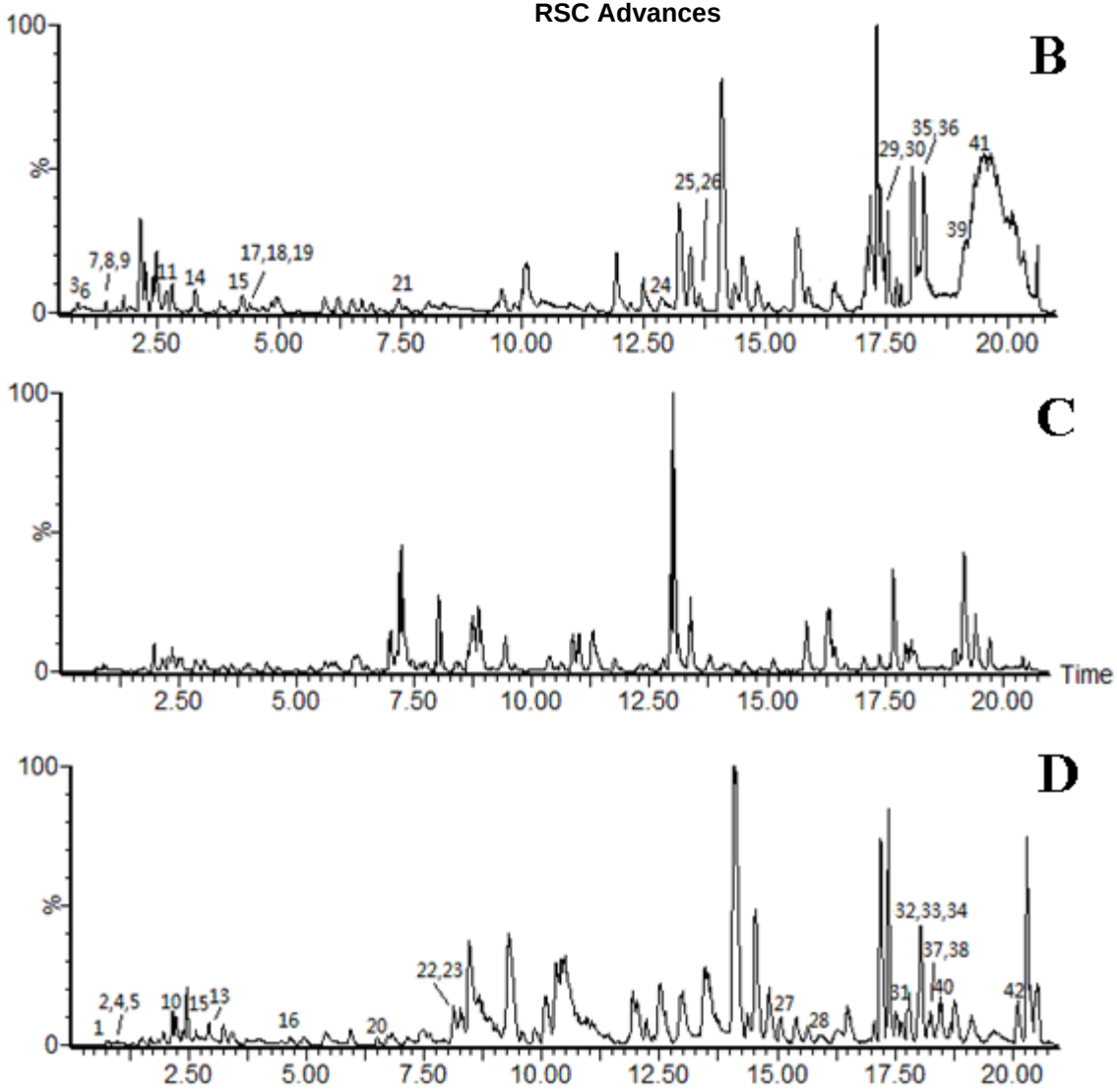

Figure 1. ESI base peak ion (BPI) chromatogram of the KXS analyzed by UPLC/QTOF MS.

Positive ion mode (A, KXS serum; B, control serum); negative ion mode (C, KXS serum; D, control serum). (The peak numbers were displayed in Table 1).

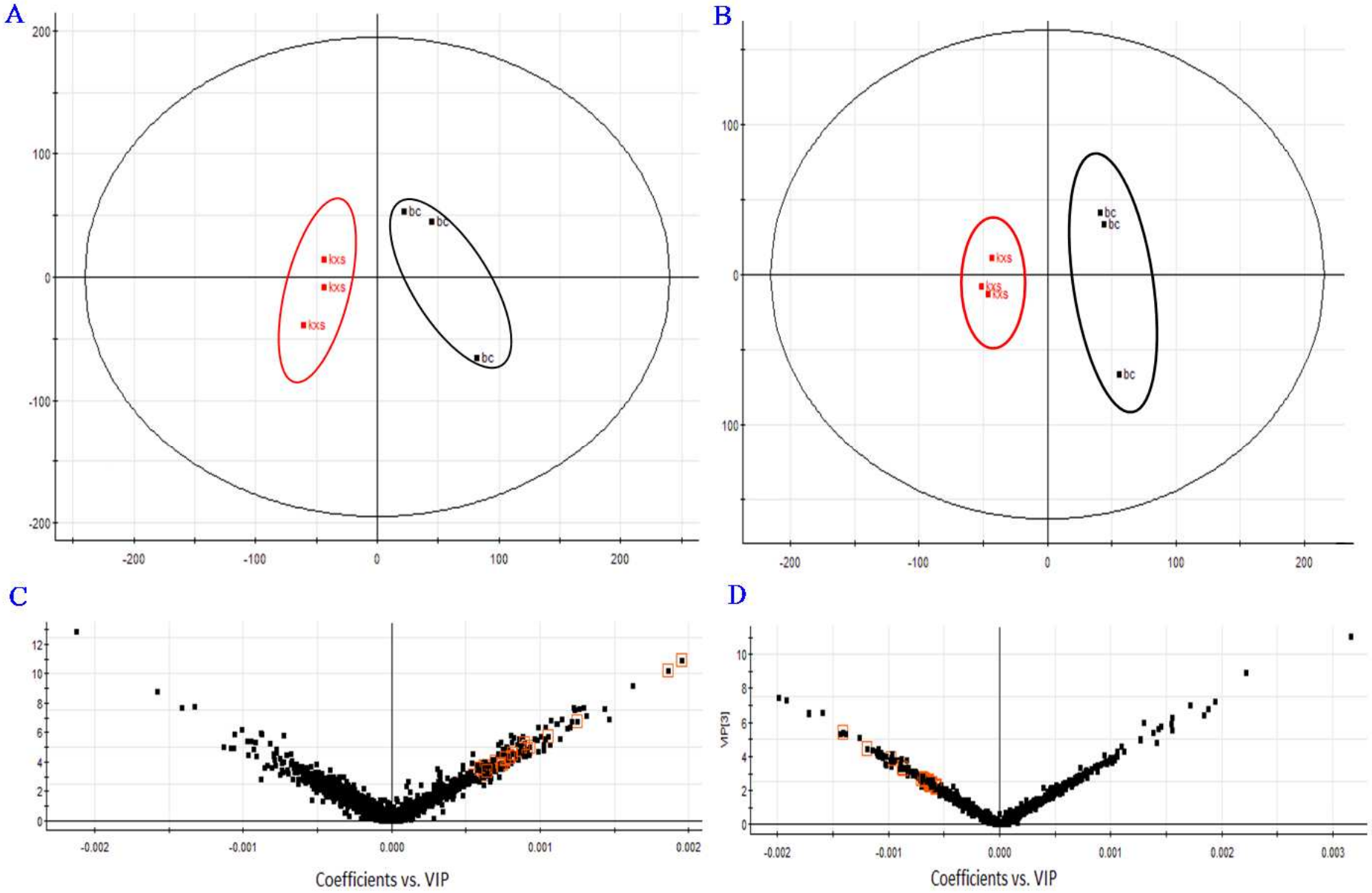

Figure 2. Multivariate statistical analysis of constituents in serum dosed with KXS.

PCA scores plot (A, positive ion mode; B, negative ion mode) of dosed rat serum and control rat serum; the VIP plot of OPLS-DA of dosed rat serum and control rat serum (C, positive ion mode; D, negative ion mode). bc: Controls

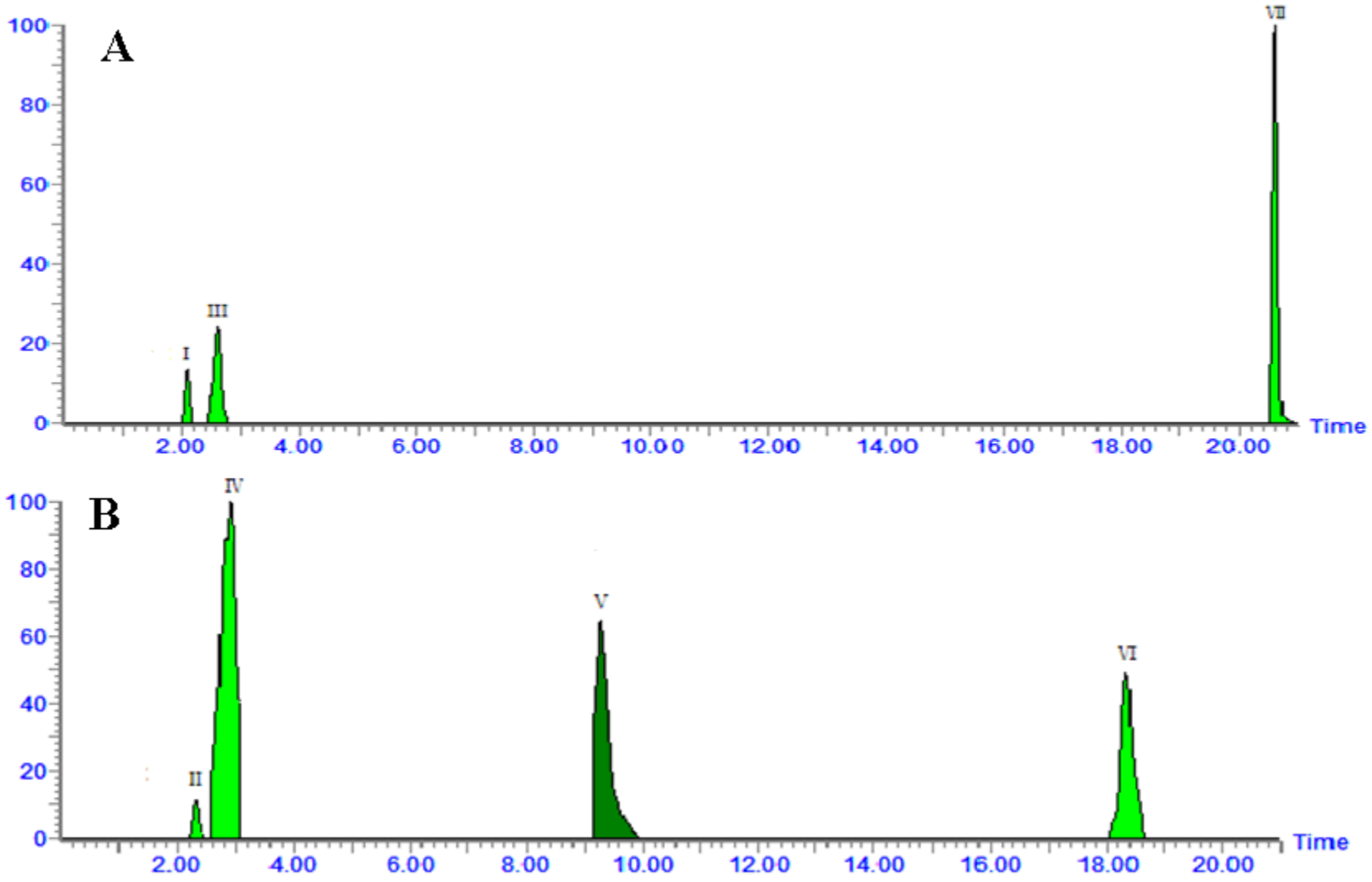

Figure 3: The extracted ion chromatogram of serum treated accordingly to experimental schemes with Metabolynx tool. Extracted ion chromatograms of metabolites with Metabolynx in positive ion mode (A), and extracted ion chromatograms of metabolites with Metabolynx in negative ion mode (B).

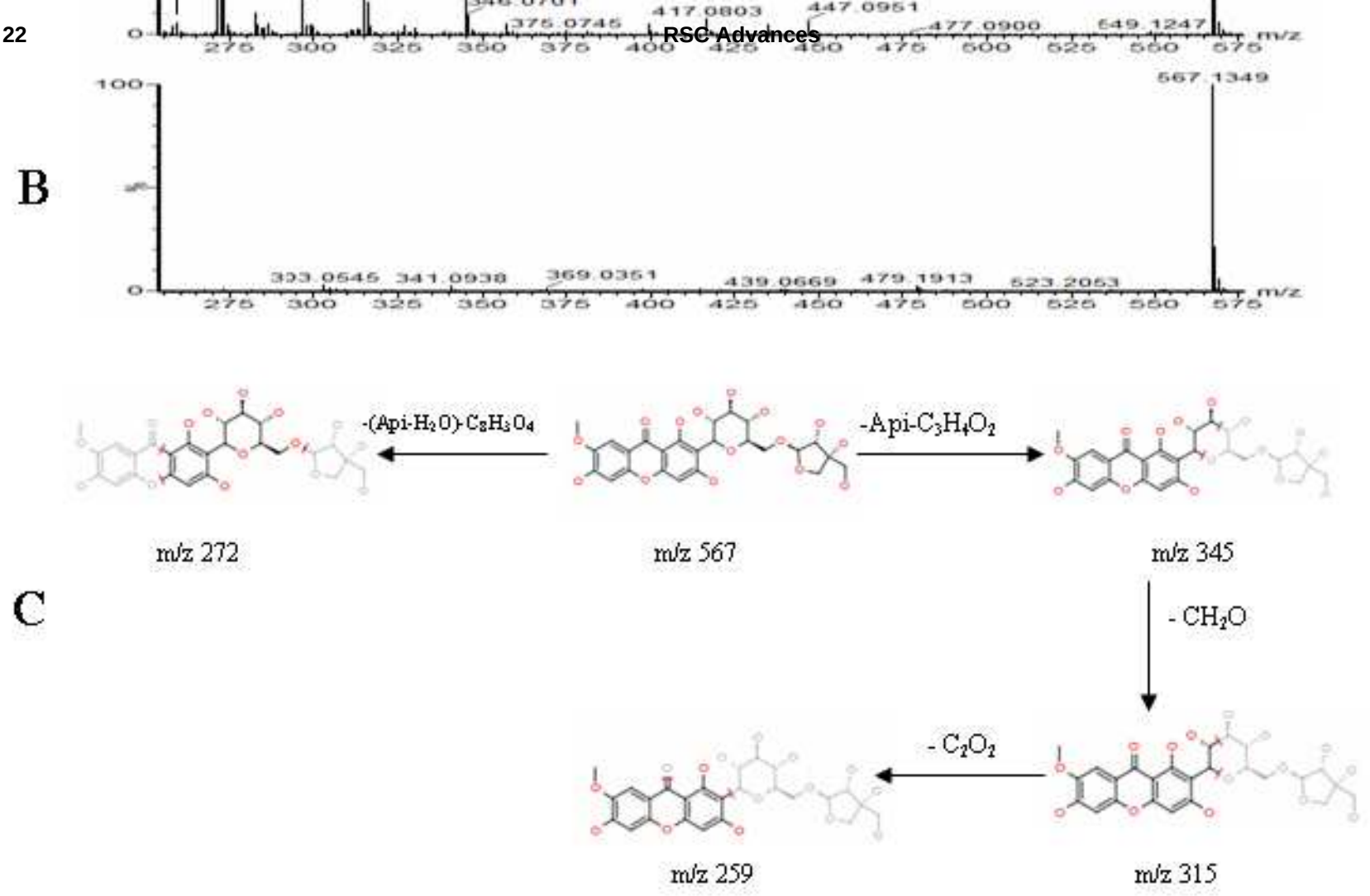

Figure 4. The mass spectra of polygalaxanthone Ⅲ in negative mode.

MS^E spectra and fragment assignment at high collision energy (A); the mass spectrum at low collision (B); proposed fragmentation pathways of Polygalaxanthone Ⅲ (C).

Table S1. The identified results of the constituents in rat plasma after oral administration of KXS by UPLC/Q-TOF-MS/MS and multivariate data processing approach.

No	Rt/ min	Compound	Positive (m/z)		Negative (m/z)		Formula	MW(Da)	Fragment ions (m/z)	Origin
1	0.83	Bergapten	—	—	215.0344	0	$C_{12}H_8O_4$	216.0423	215[M-H]$^-$,179[M-H-3C]$^-$, 161[M-H-C_3H_2O]$^-$,89[M-H-$C_7H_{10}O_2$]$^-$	d
2	0.90	Mannose	—	—	179.0556	1.1	$C_6H_{12}O_6$	180.0634	179[M-H]$^-$,161[M-H-H_4O]$^-$,96[M-H-CH_8O_4]$^-$	a
3	0.93	Valine	118.0863	2.9	—	—	$C_5H_{11}NO_2$	117.0790	103[M+H-NH]$^+$,100[M+H-H_2O]$^+$	d
4	0.93	Dimethyl(R)-(+)-malate	—	—	161.0398	5	$C_6H_{10}O_5$	162.0528	148[M+H-CH_3]$^+$,119[M+H-C_2H_4O]$^+$, 161[M-H]$^-$,142[M-H-H_2O]$^-$, 99[M-H-2CH_2OH]$^-$,57[M-H-2CH_2OH-CO-CH_2]$^-$	b
5	1.12	Sucrose	—	—	341.1084	-9.1	$C_{12}H_{22}O_{11}$	342.1162	341[M-H]$^-$,179[M-H-(Glc-H_2O)]$^-$, 161[M-H-Glc]$^-$,119[M-H-(Glc-H_2O)-CO_3]$^-$, 113[M-H-Glc-CH_4O_2]$^-$	a, c
6	1.54	Nicotinic acid	124.0399	-9.7	—	—	$C_6H_5NO_2$	123.0320	124[M+H]$^+$,108[M+H-O]$^+$, 106[M+H-H_2O]$^+$,80[M+H-C_2H_6N]$^+$	a
7	1.62	Benzoic acid	123.0446	1.8	—	—	$C_7H_6O_2$	122.0368	123[M+H]$^+$,108[M+H-O]$^+$	d
8	1.62	Adenine	136.0623	1.2	—	—	$C_5H_5N_5$	135.0545	136[M+H]$^+$,119[M+H-H_3N]$^+$,109[M+H-CHN]$^+$	b

9	1.62	Adenosine	268.1046	1.8	—	—	$C_{10}H_{13}N_5O_4$	267.0968	268$[M+H]^+$,136$[M+H-C_4H_{10}N_3O_2]^+$,119$[M+H-C_5H_{13}N_2O_3]^+$	a
10	2.41	Sibiricose A1	—	—	547.1663	-1.5	$C_{23}H_{32}O_{16}$	548.1741	547$[M-H]^-$,385$[M-H-(Fru-H_2O)]^-$,367$[M-H-Fru]^-$, 325$[M-H-C_{11}H_{10}O_5]^-$,223$[M-H-Fru-(Glc-2H_2O)]^-$, 205$[M-H-Fru-(Glc-H_2O)]^-$	c
11	2.61	Ethyl benzoylacetate*	193.0821	-2.6	—	—	$C_{11}H_{12}O_3$	192.0786	193$[M+H]^+$,165$[M+H-CO]^+$,150$[M+H-CO-CH_3]^+$, 135$[M+HCO-2CH_3]^+$	c
12	2.63	Salicylic acid	—	—	137.0239	-1.9	$C_7H_6O_3$	138.0317	137$[M-H]^-$,108$[M-H-2O]^-$	a
13	2.95	PolygalaxanthoneⅢ	—	—	567.1332	-5.3	$C_{25}H_{28}O_{15}$	568.1428	569$[M+H]^+$,551$[M+H-H_2O]^+$,317$[M+H-Api-C_4H_6O_3]^+$, 287$[M+H-(Api-H2O)-C_6H_{14}O_4]^+$, 567$[M-H]^-$,447$[M-H-C_7H_4O_2]^-$,417$[M-H-Api]^-$, 345$[M-H-Api-C_3H_4O_2]^-$,315$[M-H-Api-C_4H_6O_3]^-$, 272$[M-H-(Api-H_2O)-C_8H_3O_4]^-$,259$[M-H-Api-C_6H_6O_5]^-$	c
14	3.62	1-(3,4-Dimethoxyphenyl)ethan-1-one	181.0865	-2.2	—	—	$C_{10}H_{12}O_3$	180.0786	181$[M+H]^+$,166$[M+H-CH_3]^+$, 151$[M+H-2CH_3]^+$,121$[M+H-CH_3-C_2H_5O]^+$	c

15	4.42	2-O-Methyl-α-D-glucopyranose*	195.0601	-1.2	—	—	$C_7H_{14}O_6$	194.0790	195$[M+H]^+$,165$[M+H-C_2H_3NO-CHN]^+$, 137$[M+H-C_2H_3NO-CH_2O-CHN]^+$	c
16	4.44	M1*	—	—	257.0838	-3.5	$C_8H_{18}O_9$	258.0784	257$[M-H]^-$,221$[M-H-2H_2O]^-$,179$[M-H-2H_2O-C_2H_6O_3]^-$	a
17	4.45	M2*	410.1240	3.4	—	—	$C_{18}H_{19}NO_{10}$	409.1009	410$[M+H]^+$,193$[M+H-C_2H_3NO-C_5H_4O_6]^+$, 165$[M+H-C_2H_3NO-C_5H_4O_6-2CH_2]^+$	a
18	4.47	M3*	181.0830	-1.1	—	—	$C_{10}H_{12}O_3$	180.0786	181$[M+H]^+$,151$[M+H-2CH_3]^+$,136$[M+H-3CH_3]^+$	d
19	4.48	M4*	298.1275	-5.2	—	—	$C_{14}H_{19}NO_6$	297.1212	298$[M+H]^+$,136$[M+H-CHN-C_{10}H_9N_2O_2]^+$, 91$[M+H-CHN-C_{12}H_{14}N_2O_3]^+$	d
20	6.31	2-Hydroxybenzonic acid	—	—	137.0239	-4.4	$C_7H_6O_3$	138.0317	137$[M-H]^-$,93$[M-H-CO_2]^-$	c
21	7.59	[(2S)-5-Oxotetrahydro-2-furanyl] methyl benzoate*	221.0785	-1.3	—	—	$C_{12}H_{12}O_4$	220.0736	221$[M+H]^+$,206$[M+H-CH_3]^+$,191$[M+H-2CH_3]^+$, 135$[M+H-2CH_3-2CO]^+$	c
22	7.78	Dibutyl oxalate	—	—	201.1129	-1	$C_{10}H_{18}O_4$	202.1205	201$[M-H]^-$,183$[M-H-H_2O]^-$,139$[M-H-C_2H_5-CH_4-OH]^-$	a
23	7.84	3,4,5-Trimethoxy cinnamic acid	—	—	237.076	7.2	$C_{12}H_{14}O_5$	238.0841	237$[M-H]^-$,197$[M-H-C_3H_4]^-$,193$[M-H-CO_2]^-$	c

					3				
24	12.80	M5*	221.0795	0.9	—	—	$C_8H_{12}O_7$	220.0583	221[M+H]$^+$,206[M+H-CH_3]$^+$,191[M+H-2CH_3]$^+$, 135[M+H-2CH_3-C_3H_5O]$^+$	c
25	13.76	Acoronene	235.1698	1.1	—	—	$C_{15}H_{22}O_2$	234.1620	235[M+H]$^+$,217[M+H-CH_6]$^+$, 189[M+H-C_3H_{10}]$^+$,119[M+H-$C_7H_{16}O$]$^+$	d
26	13.81	M6*	373.1637	3.3	—	—	$C_{17}H_{24}O_9$	372.1420	373[M+H]$^+$,355[M+H-H_2O]$^+$,151[M+H-$C_5H_8O_6$-2CH_3-CH]$^+$	d
27	14.97	Poricoic acid H	—	—	499.3424	-0.8	$C_{31}H_{48}O_5$	500.3502	499[M-H]$^-$,481[M-H-H_2O]$^-$, 419[M-H-C_6H_8]$^-$,389[M-H-C_7H_{10}-O]$^-$	b
28	15.76	Poricoic acid G	—	—	485.3267	-3.5	$C_{30}H_{46}O_5$	486.3345	487[M+H]$^+$,407[M+H-H_2O-CH_2O_2-CH_4]$^+$, 201[M+H-$C_{19}H_{26}O_2$]$^+$,159[M+H-$C_8H_{12}O_2$-$C_{11}H_{24}O_2$]$^+$, 485[M-H]$^-$,425[M-H-CH_4-CO_2]$^-$, 387[M-H-C_6H_9-OH]$^-$,369[M-H-C_6H_{12}-CO_2]$^-$	b
29	17.59	Gomisin A	417.1875	1.2	—	—	$C_{23}H_{28}O_7$	416.1835	417[M+H]$^+$,224[M+H-$C_{11}H_{13}O_3$]$^+$, 193[M+H-$C_{12}H_{16}O_4$]$^+$,165[M+H-$C_{14}H_{20}O_4$]$^+$	a
30	17.62	3β,16α-Dihydroxylanosta-7,9(11), 24-trien- 21-oic acid	471.3474	-5.9	—	—	$C_{30}H_{46}O_4$	470.3396	471[M+H]$^+$,453[M+H-H_2O]$^+$, 407[M+H-CH_2O_2-H_2O]$^+$,313[M+H-$C_8H_{12}O_2$-H_2O]$^+$, 469[M-H]-,407[M-H-CO_2-H_2O]$^-$,	b

									367[M-H-C_6H_{12}-H_2O]$^-$	
31	17.66	Poricoic acid B	—	—	483.3111	-1.9	$C_{30}H_{44}O_5$	484.3189	483[M-H]$^-$,439[M-H-CO_2]$^-$, 409[M-H-CO_2-2CH_2]$^-$,367[M-H-2CO_2-2CH_2]$^-$	b
32	17.90	6,7-Dehydroporicoic acid H	—	—	497.3267	-2.6	$C_{31}H_{46}O_5$	498.3345	497[M-H]$^-$,479[M-H-H_2O]$^-$, 453[M-H-2CH_3-CH_2]$^-$,423[M-H-C_2H_6-CO_2]$^-$	b
33	17.97	16-Deoxyporicoic acid B	—	—	467.3161	4.7	$C_{30}H_{44}O_4$	468.3240	469[M+H]$^+$,451[M+H-H_2O]$^+$, 311[M+H-$C_8H_{12}O_2$-H_2O]$^+$,293[M+H-$C_8H_{12}O_2$-CH_8O]$^+$ 467[M-H]$^-$,423[M-H-CO_2]$^-$, 407[M-H-CO_2-CH_4]$^-$,374[M-H-C_7H_9]$^-$	b
34	18.04	Tumulosic acid	—	—	485.3631	2.3	$C_{31}H_{50}O_4$	486.3709	485[M-H]$^-$,441[M-H-CO_2]$^-$,423[M-H-CO_2-H_2O]$^-$	b
35	18.09	cis/trans-Methylisoeugenol	179.0726	6.7	—	—	$C_{11}H_{14}O_2$	178.0994	179[M+H]$^+$,165[M+H-CH_2]$^+$, 151[M+H-2CH_2]$^+$,121[M+H-2CH_3-2CH-2H]$^+$	d
36	18.19	3β-Hydroxy-16α-acetoxy-lanosta-7,9(11),24-trien-21-oic acid	513.3580	-1.9	—	—	$C_{32}H_{48}O_5$	512.3502	513[M+H]$^+$,495[M+H-H_2O]$^+$, 453[M+H-$COCH_3$-OH]$^+$, 355[M+H-$C_8H_{12}O_2$-H_2O]$^+$, 295[M+H-$C_8H_{12}O_2$-CH_4O_2-H_2O]$^+$,	b

37	18.31	Daedaleanic acid A	—	—	481.3318	-3.7	$C_{31}H_{46}O_4$	482.3396	483[M+H]$^+$,465[M+H-H_2O]$^+$, 419[M+H-C_3H_{10}-H_2O]$^+$,309[M+H-$C_9H_{16}O_2$-H_2O]$^+$, 481[M-H]$^-$,437[M-H-CO_2]$^-$, 421[M-H-C_3H_8-O]$^-$,403[M-H-C_3H_8-2HO]$^-$	b
38	18.48	(5ξ,20S)-24-Methylene-3-oxolanosta-7,9(11)-dien-21-oic acid*	—	—	465.3127	-1.9	$C_{31}H_{46}O_3$	466.3447	465[M-H]$^-$,421[M-H-CO_2-2H_2O]$^-$, 403[M-H--2CH_3-CH_2-2H_2O]$^-$	b
39	18.49	Dehydroeburiconic acid	467.3525	0.2	—	—	$C_{31}H_{46}O_3$	466.3447	467[M+H]$^+$,449[M+H-H_2O]$^+$, 311[M+H-$C_9H_{16}O_2$]$^+$,293[M+H-$C_{10}H_{22}O_2$]$^+$	b
40	18.50	Dehydrotu-mulosic acid	—	—	483.3474	-2.3	$C_{31}H_{48}O_4$	484.3553	483[M-H]$^-$,437[M-H-CH_2O_2]$^-$, 421[M-H-CO_2-H_2O]$^-$,337[M-H-C_6H_{12}-CO_2-H_2O]$^-$	b
41	18.71	2,6-Di-sec-butyl-4-methylphenol	221.1905	-7.2	—	—	$C_{15}H_{24}O$	220.1827	221[M+H]$^+$,203[M+H-CH_6]$^+$, 193[M+H-CH_3-CH]$^+$,133[M+H-CH_3-CH-4CH_3]$^+$	a
42	19.99	Pachymic acid	—	—	527.3737	-0.8	$C_{33}H_{52}O_5$	528.3815	527[M-H]$^-$,483[M-H-C_2H_4O]$^-$, 465[M-H-C_2H_4O-H_2O]$^-$,221[M-H-$C_{18}H_{26}O_4$]$^-$	b

Note: a: Ginseng Radix; b: Poria; c: Polygalae Radix; d: Acori Tatarinowii Rhizoma; * means the metabolites;
42 components were identified by pattern recognition methods, 21 of them were detected in positive ion mode and others were detected in negative mode.

Table S2. The identified results of the constituents in rat plasma after oral administration of KXS by Metabolynx software.

No	Rt/ min	Compound	Positive (m/z)		Negative (m/z)		Formula	MW(Da)	Fragment ions (m/z)	Origin
Ⅰ	2.07	3-(2-furyl)-3-oxopropanenitrile	136.0544	0.9	—	—	$C_7H_5NO_2$	135.0320	136$[M+H]^+$,119$[M+H-OH]^+$,109$[M+H-CHN]^+$	d
Ⅱ	2.27	M7	—	—	307.0481	3.7	$C_{14}H_{12}O_8$	308.0532	307$[M-H]^-$,247$[M-H-C_2H_4O_2]^-$,227$[M-H-C_2H_8O_3]^-$	c
Ⅲ	2.61	N-(2,5-Dimethoxyphenyl)-2-methoxyacetamide	226.1137	2.7	—	—	$C_{11}H_{15}NO_4$	225.1001	226$[M+H]^+$,210$[M+H-CH_3]^+$,167$[M+H-CH_3N-2CH_3]^+$, 152$[M+H-CH_3N-3CH_3]^+$	d
Ⅳ	2.91	M8	—	—	289.0375	2.1	$C_{14}H_{10}O_7$	290.0427	289$[M-H]^-$,274$[M-H-CH_3]^-$,263$[M-H-CH_3-2CH]^-$	c
Ⅴ	9.25	M9	—	—	273.0432	4.6	$C_{10}H_{10}O_9$	274.0324	273$[M-H]^-$,193$[M-H-Glc]^-$,163$[M-H-Glc-CH_2O]^-$, 135$[M-H-Glc-CH_2O-C_2H_4]^-$	a
Ⅵ	18.33	M10	—	—	507.3721	4.5	$C_{33}H_{48}O_4$	508.3553	507$[M-H]^-$,461$[M-H-2CH_2-H_2O]^-$	b
Ⅶ	20.60	M11	299.1357	4.6	—	—	$C_{14}H_{18}O_7$	298.1053	299$[M+H]^+$,284$[M+H-CH_3]^+$, 271$[M+H-CO]^+$,134$[M+H-C_5H_{10}O_5-CH_3]^+$	c

Note: a: Ginseng Radix; b: Poria; c: Polygalae Radix; d: Acori Tatarinowii Rhizoma; three metabolites were identified in positive ion mode and four metabolites were identified in negative ion mode. Glc: β-D-glucose; Xyl: β-D-xylose; Fru: β-D-fructose; Api:β-D-Apiose.