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Characterization of Protostane Triterpenoids in Dried Tuber of *Alisma orientalis* by Q-TOF Mass Spectrometry in Both Positive and Negative Modes

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Dried tuber of *Alisma orientalis* (Ze Xie) is a traditional Chinese medicine. It has been characterized by high concentrations of protostane triterpenoids, which are believed to play a crucial role for its clinical curative effect. This study describes a reliable and sensitive method for the analysis of protostane triterpenoids in Dried tuber of *Alisma orientalis*. The protostane triterpenoids were studied by HPLC/Q-TOF-mass spectrometry. A total of 28 protostane triterpenoids were simultaneously detected in Q-TOF-MS and 15 of them were newly found in Ze Xie. Especially, we focused on the CID MS/MS fragmentation patterns of 3 major components with different MS/MS collision energy at 15 or 35 eV and obtained extensive fragmentation information in structural elucidation. This is the first report regarding to the CID MS/MS fragmentation patterns of protostane triterpenoids in Ze Xie with both positive and negative modes. The results suggest that the method could be applied to analysis of protostane triterpenoids in Ze Xie and other herbs.

Key Words: *Alisma orientale*, Ze Xie, Protostane triterpenoids, Q-TOF-MS.

INTRODUCTION

Alisma orientalis belongs to *Alismataceae* and its dried tuber is known in traditional Chinese medicine (TCM) as Ze Xie. Ze Xie has been recorded in various editions of Pharmacopoeias in China. As a valuable therapeutic TCM, Ze Xie is prescribed in many classic TCM formulas for treatment of oliguresis and edema. The hypolipidemic effect of Ze Xie has been reported in our previous study[1]. The major components of Ze Xie are terpenes including protostane triterpenoids, kaurane diterpenes and guaiane sesquiterpenes[2-7]. Twenty protostane triterpenoids in Ze Xie have been characterized by Q-TOF-MS with positive mode[8]. In the present study, a chemical profile of Ze Xie was established by HPLC/Q-TOF-MS in both positive and negative modes. The structures of known components in Ze Xie were identified by comparing their retention times, high-resolution molecular weight and CID fragmentation patterns with their corresponding compounds reported in the literature. The unknown components were characterized by comparing the CID fragmentation behaviours in both positive and negative modes between the known and unknown components in Ze Xie.

EXPERIMENTAL

HPLC/MS grade acetonitrile was purchased from Fisher Scientific Company. Water was prepared using an Easy pure II UF ultrapure water system (Barnstead, USA). MS grade formic acid was purchased from Sigma-Aldrich (USA).

The plant of *A. orientalis* was grown in Sichuan Province, China. Dried tubers of *A. orientalis* (Ze Xie) were purchased from Xianning kang Jin Chinese Herbal Pieces Co., Ltd. (Hubei, China). The herb was identified and authenticated by the taxonomist of Key Laboratory of Chinese Medicine Resource and Compound Prescription (Hubei University of Chinese Medicine), Ministry of Education. A voucher specimen (No. 040) was deposited in herbarium of the Key Laboratory.

Sample preparation: Ze Xie was ground to a fine powder. Two g of Ze Xie powder was added with 10 mL of acetonitrile and vortexed vigorously for 2 min followed by sonication for 5 min. The mixture was centrifuged and the supernate as an

extract was removed carefully. The extraction process was repeated one time with the same solvent (10 mL). Two extracts were combined and directly analyzed by LC-MS/MS.

LC/MS analysis: The phytochemicals in an extract of Ze Xie were directly analyzed by LC-MS/MS. LC-MS/MS was performed using a MicrOTOF-Q II Focus mass spectrometer (Bruker Daltonics) equipped with an Agilent 1100 series liquid chromatograph. A 250 mm × 4.6 mm i.d. Acclaim C_{18} column (Dionex, USA) was used at a flow rate of 0.5 mL min^{-1}. The injection volume was 10 µL. The HPLC gradient was acetonitrile-formic acid (99.9 : 0.1, v/v) (B) in water-formic acid (99.9 : 0.1, v/v) (A). The following binary gradient with linear interpolation was used: 0 min, 35 % B; 20 min, 55 % B; 60 min, 65 % B; 75 min, 100 % B; 80 min, 35 % B. HPLC chemical profiles of Ze Xie extract were determined by a diode-array detector (DAD) set at four wavelengths of 200, 245, 280 and 350 nm. TIC chemical profiles of the extract were established in both positive and negative modes. HR-MS/MS analysis were carried out using a MicrOTOF-Q II Focus mass spectrometer fitted with an ESI source operating in Auto-MS^n mode to obtain fragment ion. Conditions for ESI-MS analysis of HPLC peaks in both negative- and positive-ion mode included a capillary voltage of 3500 V, an end plate offset of -500 V, a drying gas flow of 6 mL/min and a dry heater temperature of 180 °C and a nebulizer pressure of 3.0 bar. The MS collision energy was set at 10 eV and the MS/MS collision energy was set at 15 or 35 eV. The outflows were divided into three shares and only one share entered the MS detector.

RESULTS AND DISCUSSION

Chemical profile of Ze Xie extract in positive mode was essentially similar to that previously reported [8]. However, more phytochemicals were detected in the extract by Q-TOF-MS in the present study. Five major peaks **6**, **20**, **21**, **24** and **28** (Fig. 1) have been identified as alisol C, alisol A, alisol A 23-acetate, alisol A 24-acetate and alisol B according to their molecular formula resulted from high-resolution MS and the same CID mass spectra with those reported in the literature[8]. Furthermore, compounds **3**-**5**, **10**-**12**, **14**-**18**, **22**, **23**, **26** and **27** were newly found in Ze Xie extract. The structures of the compounds identified or characterized were shown in Fig. 2. Since chemical profiles of Ze Xie extract obtained in analyses of HPLC with DAD were not characteristic, the HPLC DAD-chromatograms were not shown here.

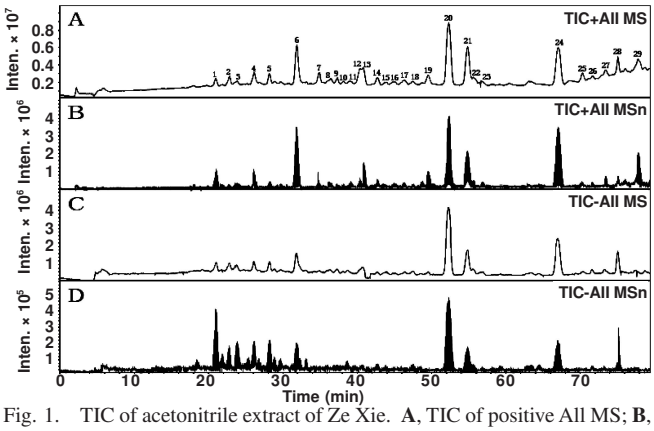
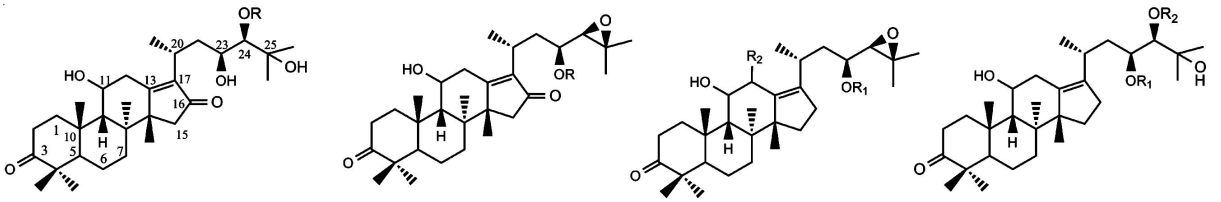


Fig. 1. TIC of acetonitrile extract of Ze Xie. **A**, TIC of positive All MS; **B**, TIC of positive All MS^n; **C**, TIC of negative All MS and **D**, TIC of negative All MS^n

CID mass fragmentation behaviours of major protostane triterpenoids: HR-MS in positive mode of compound **6** exhibited an ion at *m/z* 487.3110 corresponding to $C_{30}H_{47}O_5$



No.	R
1	H
2	Ac
3	Olefination derivative of 1
4*	Isomer of 2
5*	Dihydro derivative of 1
10*	Oxygen derivative of 2
17*	Isomer of 1
18*	Isomer of 10

No.	R
6	H
7	Ac
11*	Isomer of 6
12*, 14*, 15*	Isomer of 7
27*	Olefination derivative of 7

No.	R_1	R_2
8	Ac	OH
23*	Isomer of 8	
28	H	H

No.	R_1	R_2
16*	Isomer of 20	
20	H	H
21	Ac	H
22*	Isomer of 21	
24	H	Ac
26*	Isomer of 21	

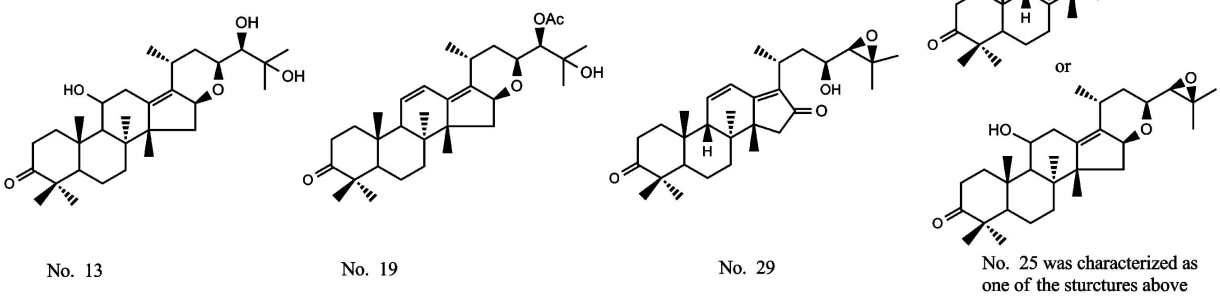


Fig. 2. Structures of the compounds identified in Ze Xie. *: Since NMR data and the corresponding standards of these compounds were not available, identifications of these compounds could not be completed by the LC-MS/MS in this study

$[M+H]^+$ of alisol C (calculated for $C_{30}H_{47}O_5$, 487.3423). The structure of compound **6** was identified as alisol C based on CID product ions. The product ions $[M+H-H_2O]^+$ at *m/z* 469 and $[M+H-H_2O-H_2O]^+$ at *m/z* 451 were generated by losing a series of H_2O from a protonated molecular ion $[M+H]^+$ at *m/z* 487. The product ion $[M+H-H_2O-C_4H_8O]^+$ at *m/z* 397 was formed by cleavage of C_{23}-C_{24} bond *via* hydrogen rearrangement at C_{23}-OH from ion at *m/z* 469, as reported previously[8]. In addition, three product ions at *m/z* values 381, 367 and 353 were detected when MS/MS collision energy was set at 35 eV (Fig. 3), which supported the structural elucidation. In negative mode, this compound exhibited an adduct of molecular ion $[M+HCOOH-H]^-$ at *m/z* 531.3280 corresponding to $C_{31}H_{47}O_7$ (m.w. calculated for $C_{31}H_{47}O_7$, 531.3321). The product ion $[M-H-30]^-$ at *m/z* 455 was formed by the neutral loss of 30 Da (CH_2O) from parent ion at *m/z* 485 (Fig. 4) and this typical fragment was a common pathway for CID of triterpenoids[9]. The product ion at *m/z* 367 was formed by elimination of a C_2H_2O group and a H_2O from the ion at *m/z* 427. The product ion at *m/z* 399 was generated due to the cleavage of a carbon bond at C_{24} position and a neutral loss of 16 Da (CH_4) at C_{21} position to form a stable conjugated system. Interestingly, the product ion at *m/z* 427 from parent ion at *m/z* 485 was detected when MS/MS collision energy was set at a lower value of 15 eV, but not detected in the condition of high MS/MS collision energy 35 eV, which indicated the structure of this product ion was very unstable. Compound **6** has been identified as alisol C by Q-TOF-MS with positive mode[8].

In comparison with TIC in positive mode previously reported[8], compound **20** (Fig. 1) is a major component in Ze Xie and has been identified as alisol A. This structural assignment was confirmed by the ion at *m/z* 535.4588 in negative MS (Fig. 5) in accord with the $C_{31}H_{51}O_7$, a $[M+HCOOH-H]^-$ of alisol A. HR-MS of compound **20** in positive mode exhibited a $[2M+H]^+$ of alisol A at *m/z* 981.6715 corresponding to $C_{60}H_{101}O_{10}$ (calculated for $C_{60}H_{101}O_{10}$, 981.7395). A protonated molecular ion $[M+H]^+$ at *m/z* 491 was detected, although the peak intensity was very weak. Product ions at *m/z* 473, 455, 437 and 419 were generated by eliminating a series of H_2O after depolymerization of $[2M+H]^+$ at *m/z* 981. As shown in Fig. 6, the MS/MS collision energy of 35 eV was used to obtain extensive fragmentation information for this compound. The negative product ions from parent ion $[M+HCOOH-H]^-$ at *m/z* 535 shown in Fig. 5 confirm this result. In negative mode with MS/MS collision energy of 15 eV, parent ion $[M+HCOOH-H]^-$ at *m/z* 535 generated two product ions 489 and 471. When MS/MS collision energy was 35 eV, compound **20** gave a ion at *m/z* 1025.8271 corresponding to $C_{61}H_{101}O_{12}$ $[2M + HCOOH-H]^-$ (calculated for $C_{61}H_{101}O_{12}$, 1025.7293). The $[2M + HCOOH-H]^-$ as a parent ion yielded product ions at *m/z* 535, 489, 471, 435, 413, 395, 377, 353 and 339 with a

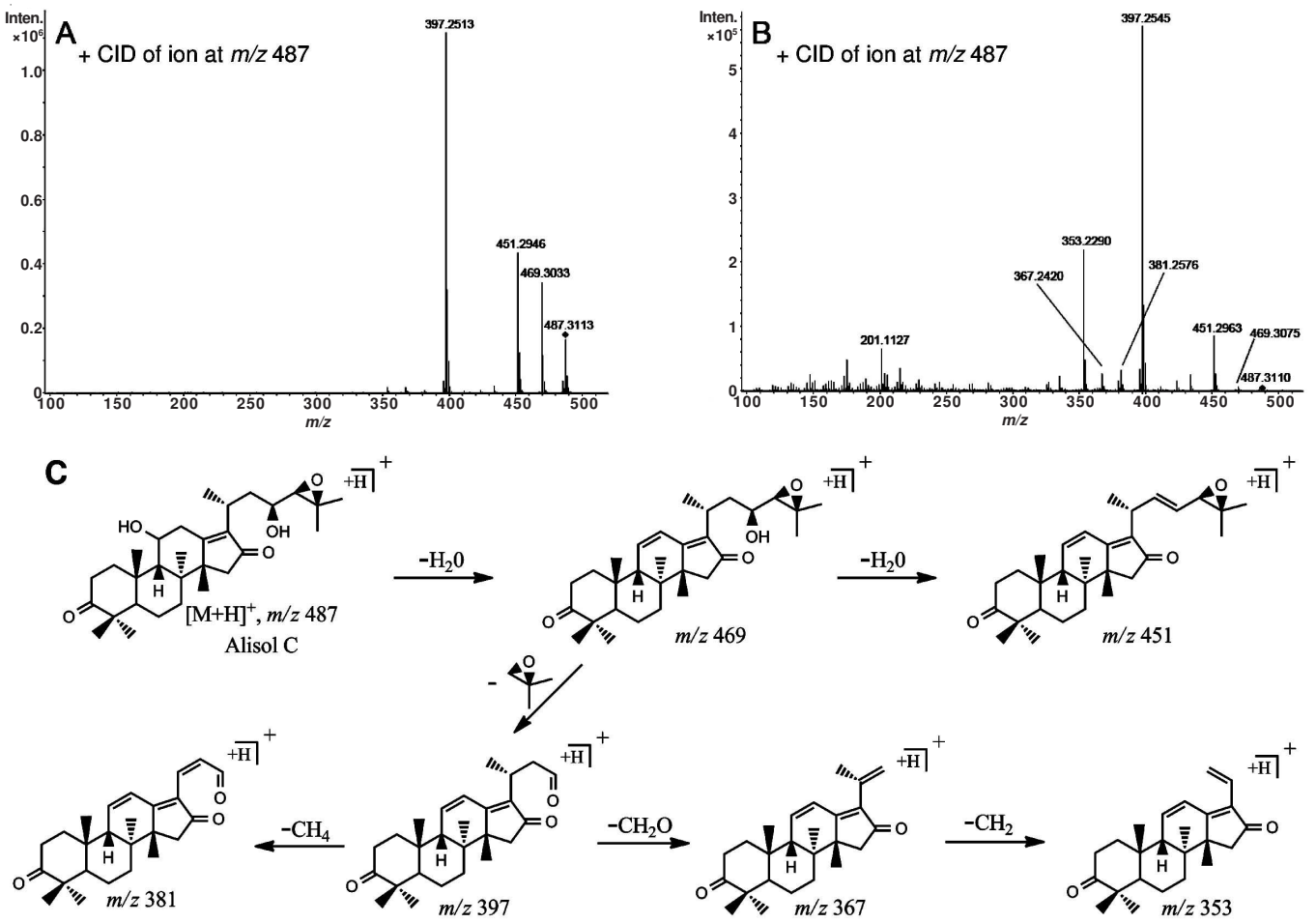


Fig. 3. CID MS/MS positive mass spectrum obtained from $[M+H]^+$ of compound **6** at *m/z* 487.3110: **A**, with MS/MS collision energy at 15 eV; **B**, with MS/MS collision energy at 35 eV. **C**, proposed fragmentation pathway of compound **6**

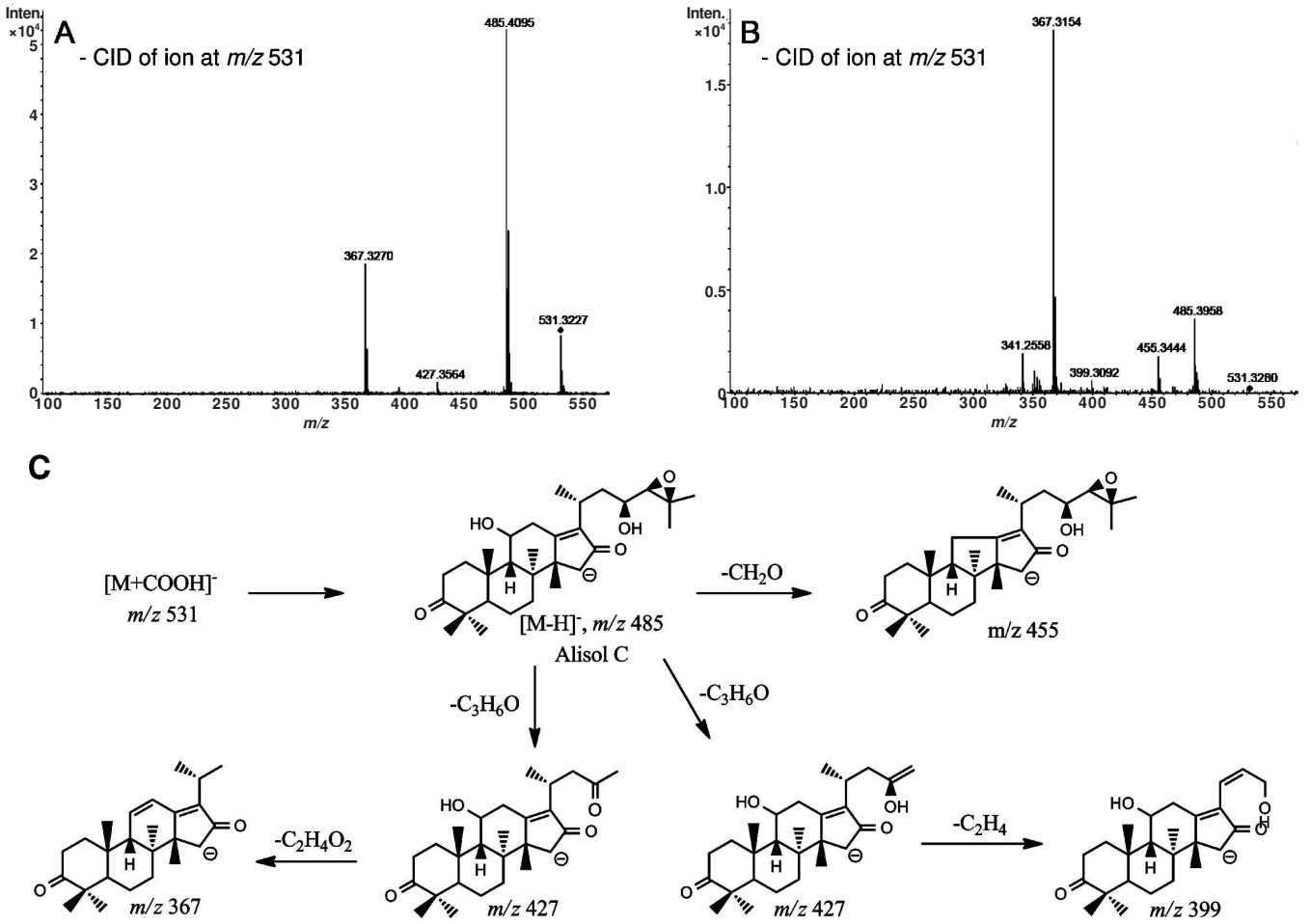


Fig. 4. CID MS/MS negative mass spectrum obtained from $[M+HCOOH-H]^-$ of compound **6** at *m/z* 531.3280: **A**, with MS/MS collision energy at 15 eV; **B**, with MS/MS collision energy at 35 eV. **C**, proposed fragmentation pathway of compound **6**

MS/MS collision energy of 35 eV. The ion at *m/z* 535 was alisol A-formic acid adduct ($[M+HCOOH-H]^-$). The ions at *m/z* 471 and 435 were generated by losing a series of H_2O from the molecular ion $[M-H]^-$ at *m/z* 489. The ion at *m/z* 413 was generated by loss of C_3H_4 in the side chain from the ion at *m/z* 453 and followed by a series elimination of H_2O to yield ions at *m/z* 395 and 377. All HR-MS and MS/MS data supported the assignment of alisol A to compound **20**.

Alisol A 23-acetate and alisol A 24-acetate as major components have been identified in Ze Xie[8]. In positive mode, compounds **21** (HR-MS: *m/z* 515.3374) and **24** (HR-MS: *m/z* 515.3406) had the same ions in accordance with a $C_{32}H_{51}O_5$, a formula of alisol A acetate $[M+H-H_2O]^+$ (m.w. calculated for $C_{32}H_{51}O_5$, 515.3736). HR-MS of these two compounds with negative mode exhibited almost identical $[M+HCOOH-H]^-$ ion at *m/z* 577.3496 and 577.3190 corresponding to $C_{33}H_{53}O_8$ $[M+HCOOH-H]^-$ (m.w. calculated for $C_{33}H_{53}O_8$, 577.3740). Acetylation is a common biosynthetic pathway of protostane triterpenoid. Many protostane triterpenoids in Ze Xie have acetyl derivatives. For MS/MS of $[M+H-H_2O]^+$ from compounds **21** and **24**, the product ions at *m/z* 455, 437, 419, 383, 365, 339 were identical to that of compound **20** (alisol A). The product ions at *m/z* 497 and 479 were generated by elimination of one or two H_2O from $[M+H-H_2O]^+$ at *m/z* 515 followed by loss of a acetyl group to yield ions at *m/z* 455 and 437. Based on the retention time on HPLC and different peak abundance in TIC mass spectrum, compound **21** and **24** were identified as alisol A 23-acetate and alisol A 24-acetate, as reported previously[8]. In negative mode with a high MS/MS collision energy of 35 eV, these two compounds exhibited ions at *m/z* 1109.8260 and 1109.8035, respectively and both were in accord with the $C_{65}H_{105}O_{13}$ formula, a $[2M+HCOOH-H]^-$ of alisol A acetate (m.w. calculated for $C_{65}H_{105}O_{13}$, 1109.7504). Unlike alisol A, only three product ions $[M+HCOOH-H]^-$ at *m/z* 577, $[M-H]^-$ at *m/z* 531 and $[M-H\text{-acetyl-}H_2O]^-$ at *m/z* 471 were observed from CID of $[2M+HCOOH-H]^-$.

Alisol B has been reported to be a major component in Ze Xie[8]. HR-MS of compound **28** showed a ion at *m/z* 945.9709 corresponding to $C_{60}H_{97}O_8$ formula, a $[2M+H]^+$ of alisol B. The calculated value for $C_{60}H_{97}O_8$ is 945.7183. The ions at *m/z* 455, 437 and 419 were generated by elimination of a series of H_2O from the protonated molecular ion $[M+H]^+$ at *m/z* 473. Like alisol C, a ion at *m/z* 383 was formed by cleavage of C_{23}-C_{24} bond *via* hydrogen rearrangement at C_{23}-OH, then followed by elimination of a H_2O to yield a ion at *m/z* 365 (Fig. 7). Meanwhile, the ions at *m/z* 353 and 341 were generated by loss of C_5H_6 or C_6H_8O from the ion at *m/z* 419 and 437, respectively. In negative mode, this compound yielded base peak ion $[M + HCOOH-H]^-$ at *m/z* 517 and a product ion $[M-H_2O]^-$ at *m/z* 453. All MS data supported the assignment of alisol B to compound **28**.

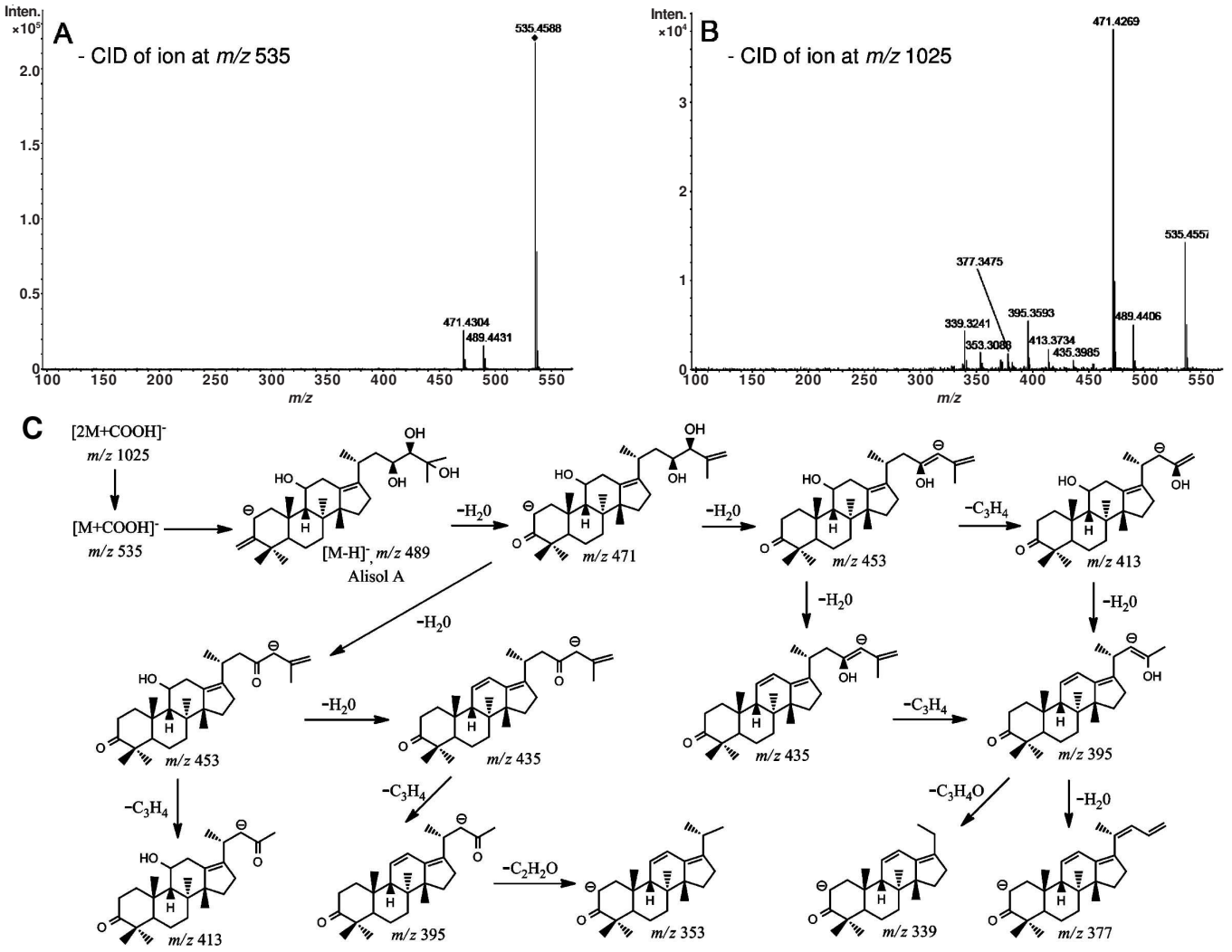


Fig. 5. CID MS/MS negative mass spectrum obtained from: **A**, $[M+HCOOH-H]^-$ of compound **20** at *m/z* 535.4588 with MS/MS collision energy at 15 eV; **B**, $[2M+HCOOH-H]^-$ of compound **20** at *m/z* 1025.8271 with MS/MS collision energy at 35 eV. **C**, proposed fragmentation pathway of compound **20**

Identification of minor protostane triterpenoids in Ze Xie: Compounds **1**, **2**, **7**, **8**, **13**, **19** and **29** have been reported in Ze Xie[8], which were also identified in the present study using HR-molecular ion and its product ions. Compound **25** exhibited a ion at *m/z* 471.3109 corresponding to $C_{32}H_{50}O_6$, a $[M + H]^+$ of 11-deoxy-alisol C or 16, 23-oxido-alisol B, which have been reported in Ze Xie[8]. In negative mode, an expected formic acid adduct $[M + HCOOH-H]^-$ was observed at *m/z* 515.3483. The $[M + H]^+$ and $[M + HCOOH-H]^-$ suggested the molecular formula $C_{32}H_{50}O_6$ for this compound. Together with the product ions at *m/z* 453, 435, 413, 381 and 363 from parent ion $[M + H]^+$ at *m/z* 471, compound **25** was characterized as 11-deoxy-alisol C or 16, 23-oxido-alisol B and final identification of compound **25** could not be completed by the LC-MS/MS in this study.

Based on the MS and fragmentation patterns of components discussed above**,** almost identical MS and fragmentation patterns (Table-1) suggested that compound **4** was a isomer of compound **2**; compound **11** was a isomer of compound **6**; compounds **12**, **14** and **15** were the isomers of compound **7**; Compound **16** was a isomer of compound **20**; Compound **17** was a isomer of compound **1**; compounds **22** and **26** were the isomers of compound **21** and compound **23** was a isomer of **8**. As neither NMR data of these minor compounds nor the corresponding standards were available, identification of **4**, **11**, **12**, **14**, **15**, **16**, **17**, **22, 23** and **26** could not be completed by the LC-MS/MS in this study.

HR-MS in positive mode of compound **3** showed a ion at *m/z* 503.3004 corresponding to $C_{30}H_{47}O_6$ (m.w. calculated for $C_{30}H_{47}O_6$, 503.3372). $C_{30}H_{47}O_6$ formula accorded a $[M-H_2O+H]^+$ of dehydro-16-oxo-alisol A. In negative mode, compound **3** gave a ion at *m/z* 547.3876 corresponding to $C_{31}H_{47}O_8$ (m.w. calculated for $C_{31}H_{47}O_8$, 547.3271), which was in accord with $[M+HCOOH-H]^-$ of dehydro-16-oxo-alisol A. The fragmentation pattern of compound **3** was similar to that of 16-oxo-alisol A. In positive mode, the product ions at *m/z* 485, 467 and 413 were two hydrogens less than the ions at *m/z* 487, 469 and 415 generated from 16-oxo-alisol A (compound **1**) suggesting that compound **3** was an olefination derivative of compound **1**. Although the exact position of this double bond could not be determined by MS data, the little difference of retention time and the same UV max. at 222 nm between compounds **1** and **3** indicated that the conjugated system of protostane triterpenoid skeleton of compound **3** was not changed

TABLE-1
Q-TOF-MS DATA OF COMPOUNDS DETECTED IN ACETONITRILE EXTRACT OF ZE XIE

No.	Rt (min)	Identification	m.f.	Ionization mode	Product ions from CID, m/z (relative intensity %)	
					Parent ion	Product ions
1	21.0	16-oxo-alisol A	$C_{30}H_{48}O_6$	Positive	$[M+H]^+$ 505.3213 (9.2)	487 (21.2), 469 (23.6), 451 (9.4), 415 (100), 397 (2.1)
				Negative	$[M+HCOOH-H]^-$ 549.3395 (10.2)	485 (100), 503 (16.3), 427 (11.9), 369 (26.9)
2	22.7	16-oxo-alisol A 24-actetate	$C_{32}H_{50}O_7$	Positive	$[M-H_2O+H]^+$ 529.3184 (53.8)	511 (8.3), 497 (2.4), 487 (1.7), 469 (60.8), 451 (100), 415 (22.3), 397 (11.1), 379 (2.6), 353 (2.7),
				Negative	$[M+HCOOH-H]^-$ 591.3963 (30.4)	545 (17.9), 527 (100), 485 (13.1), 467 (62.1), 367 (3.4)
3*	23.9	dehydro-16-oxo-alisol A	$C_{30}H_{46}O_6$	Positive	$[M+H]^+$ 503.3004 (9.3)	485 (51.2), 467 (19.1), 445 (32.4), 427 (8.3), 413 (100), 395 (5.7), 353 (1.8)
				Negative	$[M+HCOOH-H]^-$ 547.3876 (13.7)	503 (53.2), 501 (21.3), 483 (12.6), 443 (15.5), 425 (100), 407 (11.8), 353 (18.4), 327 (21.0)
4*	26.1	Isomer of 2	$C_{32}H_{50}O_7$	Positive	$[M+H]^+$ 547.3876 (1.6)	529 (100), 511 (5.8), 469 (28.4), 451 (63.2), 433 (4.0), 415 (22.6), 397 (1.1)
				Negative	$[M+HCOOH-H]^-$ 591.3677 (8.2)	545 (28.0), 527 (100), 485 (12.0), 467 (39.0), 367 (2.5)
5*	28.2	dihydro-16-oxo-alisol A	$C_{30}H_{50}O_6$	Positive	$[M+Na]^+$ 529.3163 (100)	511 (2.0), 451 (1.1)
				Negative	$[M+HCOOH-H]^-$ 551.3886 (100)	505 (42.4), 487 (1.7)
6	31.8	Alisol C	$C_{30}H_{46}O_5$	Positive	$[M+H]^+$ 487.3110 (0.03)	469 (1.0), 451 (14.8), 433 (4.3), 423 (2.6), 397 (100), 381 (5.6), 367 (4.5), 353 (38.4)
				Negative	$[M+HCOOH-H]^-$ 531.3280 (0.48)	485 (20.2), 455 (9.9), 399 (3.2), 367 (100), 351 (6.1), 341 (10.8)
7	34.7	Alisol C 23-acetate	$C_{32}H_{48}O_6$	Positive	$[M+H]^+$ 529.3134 (2.3)	511 (71.6), 493 (6.9), 469 (20.8), 451 (100), 433 (28.2), 397 (15.7)
				Negative	$[M+HCOOH-H]^-$ 573.3621 (19.7)	527 (100), 509 (25.4), 485 (14.1), 468 (17.1),
8	36.2	Alisol N 23-acetate	$C_{32}H_{50}O_6$	Positive	$[M+H]^+$ 531.3277 (7.5)	513 (73.9), 495 (11.2), 471 (12.7), 453 (100), 435 (47.2), 417 (5.6), 399 (24.3), 381 (42.7), 363 (14.7), 355 (10.2)
				Negative	$[M+HCOOH-H]^-$ 575.3586 (12.0)	529 (100), 487 (13.5), 469 (19.9), 451 (6.5)
9	37.2	Unknown		Positive	$[M+H]^+$ 543.3247 (100)	525 (1.2), 495 (2.2), 431 (1.3), 413 (2.8)
				Negative	$[M+HCOOH-H]^-$ 587.4219 (100)	541 (16.2), 519 (34.2)
10*	38.1	Hydroxy-16-oxo-alisol A 24-actetate	$C_{32}H_{50}O_8$	Positive	$[M-H_2O+H]^+$ 545.3097 (100)	527 (27.5), 509 (11.3), 485 (59.9), 467 (97.7), 449 (37.4), 431 (48.8), 413 (58.1), 387 (69.0), 369 (16.6)
				Negative	$[M-H]^-$ 561.3960 (100)	517 (19.1), 501 (30.9)
11*	39.1	Isomer of 6	$C_{30}H_{46}O_5$	Positive	$[M+H]^+$ 487.3419 (33.6)	469 (97.4), 451 (13.5), 427 (5.1), 397 (100), 381 (10.5), 355 (14.5)
				Negative	$[M+HCOOH-H]^-$ 531.3570 (41.1)	485 (100), 427 (21.9), 353 (12.3), 327 (14.5)
12*	40.3	Isomer of 7	$C_{32}H_{48}O_6$	Positive	$[M-H_2O+H]^+$ 511.3088 (100)	493 (2.7), 451 (45.6), 433 (78.4), 397 (10.5), 353 (2.8)
				Negative	$[M+HCOOH-H]^-$ 573.3566 (14.3)	527 (100), 467 (14.6)
13	40.8	Alisol F	$C_{30}H_{48}O_5$	Positive	$[M-H_2O+H]^+$ 471.3115 (1.8)	453 (3.6), 395 (2.9), 381 (14.2), 363 (3.7), 339 (100), 321 (5.2), 297 (11.1)
				Negative	$[M+HCOOH-H]^-$ 533.3478 (100)	487 (34.2), 449 (15.6), 409 (16.8)
14*	42.7	Isomer of 7	$C_{32}H_{48}O_6$	Positive	$[M+H]^+$ 529.3166 (100)	511 (10.1), 469 (31.2), 451 (57.8), 433 (5.7), 415 (15.9)
				Negative	$[M+HCOOH-H]^-$ 573.3899 (3.1)	527 (2.7), 509 (100), 467 (4.2), 449 (3.3)
15*	44.6	Isomer of 7	$C_{32}H_{48}O_6$	Positive	$[M+H]^+$ 529.3134 (17.3)	511 (100), 493 (7.2), 469 (10.2), 451 (17.4), 433 (8.1), 397 (11.9), 353 (2.6)
				Negative	$[M+HCOOH-H]^-$ 573.3139 (29.5)	527 (100), 503 (12.7), 485 (23.2), 467 (78.8)
16*	45.1	Isomer of 20	$C_{30}H_{50}O_5$	Positive	$[M+Na]^+$ 513.3455 (100)	495 (3.2), 455 (1.8), 437 (6.3), 383 (2.2)
				Negative	$[M+HCOOH-H]^-$ 535.2821 (100)	489 (83.4)

17*	46.4	Isomer of 1	$C_{30}H_{48}O_6$	Positive	$[M-H_2O+H]^+$ 487.3075 (100)	469 (35.9), 451 (56.4), 413 (14.2), 397 (49.7), 379 (17.5)
				Negative	$[M+HCOOH-H]^-$ 549.4380 (100)	503 (45.8), 485 (27.9), 443 (10.3), 383 (28.1)
18*	47.5	Isomer of 10	$C_{32}H_{50}O_8$	Positive	$[M-H_2O+H]^+$ 545.3069 (84.1)	485 (9.2), 467 (22.3), 449 (7.2), 431 (39.5), 413 (100), 387 (65.2), 369 (13.8), 353 (8.8), 341 (7.4)
				Negative	$[M-H]^-$ 561.3654 (100)	501 (27.6), 475 (1.4)
19	49.5	Alisol O	$C_{32}H_{48}O_5$	Positive	$[M+H]^+$ 513.3183 (6.5)	495 (11.6), 455 (3.0), 435 (10.2), 417 (3.4), 381 (30.9), 363 (7.0), 339 (100)
20	52.3	Alisol A	$C_{30}H_{50}O_5$	Positive	$[2M+H]^+$ 981.6715 (0.9)	491 (0.8), 473 (1.6), 455 (8.3), 437 (11.4), 419 (4.9), 383 (69.2), 365 (100), 339 (46.8)
				Negative	$[2M+HCOOH-H]^-$ 1025.8271 (0.2)	535 (37.4), 489 (13.2), 471 (100), 435 (2.4), 413 (5.8), 395 (14.4), 377 (4.6), 353 (5.0), 339 (11.3)
21	54.8	Alisol A 23-acetate	$C_{32}H_{52}O_6$	Positive	$[M-H_2O+H]^+$ 515.3374 (67.8)	497 (52.7), 479 (11.7), 455 (19.8), 437 (100), 419 (65.3), 383 (28.2), 365 (44.0), 339 (40.8)
				Negative	$[2M+HCOOH-H]^-$ 1109.8260 (5.3)	577 (100), 531 (23.4)
22*	55.6	Isomer of 21	$C_{32}H_{52}O_6$	Positive	$[M-H_2O+H]^+$ 515.3561 (20.6)	497 (76.6), 479 (19.8), 455 (14.2), 437 (100), 419 (21.3), 383 (66.0), 365 (57.1), 339 (48.2)
				Negative	$[M+HCOOH-H]^-$ 577.3954 (100)	531 (9.2), 469 (5.1)
23*	56.9	Isomer of 8	$C_{32}H_{50}O_6$	Positive	$[M-H_2O+H]^+$ 513.3424 (90.8)	495 (14.9), 451 (53.4), 435 (20.1), 417 (11.7), 399 (5.3), 381 (75.3), 363 (33.1), 339 (100)
				Negative	$[M+HCOOH-H]^-$ 575.3679 (100)	529 (9.4)
24	67.0	Alisol A 24-acetate	$C_{32}H_{52}O_6$	Positive	$[M-H_2O+H]^+$ 515.3406 (100)	497 (7.5), 455 (2.4), 437 (20.8), 419 (13.1), 383 (44.5), 365 (45.0), 339 (38.3)
				Negative	$[2M+HCOOH-H]^-$ 1109.8035 (0.8)	577 (100), 531 (27.9), 471 (7.4)
25	70.2	11-deoxy-alisol C or 16, 23-oxido-alisol B	$C_{30}H_{46}O_4$	Positive	$[M+H]^+$ 471.3109 (18.8)	453 (26.8), 435 (23.1), 413 (12.9), 381 (78.6), 363 (22.8), 339 (100)
				Negative	$[M+HCOOH-H]^-$ 515.3483 (50.7)	469 (100)
26*	71.6	Isomer of 21	$C_{32}H_{52}O_6$	Positive	$[M-H_2O+H]^+$ 515.3311 (13.8)	497 (16.4), 457 (5.1), 437 (16.3), 419 (12.0), 383 (100), 365 (53.4), 339 (34.6)
				Negative	$[M+HCOOH-H]^-$ 577.3953 (100)	531 (13.6)
27*	73.4	Olefination adduct of 7	$C_{32}H_{46}O_6$	Positive	$[M+H]^+$ 527.3314 (11.2)	509 (3.8), 469 (84.5), 451 (96.7), 433 (14.2), 397 (100)
28	75.0	Alisol B	$C_{30}H_{48}O_4$	Positive	$[2M+H]^+$ 945.5709 (2.6)	473 (17.6), 455 (100), 437 (81.9), 419 (21.8), 383 (20.3), 365 (4.5), 341 (21.5)
				Negative	$[M+HCOOH-H]^-$ 517.4447 (100)	453 (2.8)
29	77.7	Alisol L or its isomer	$C_{30}H_{44}O_4$	Positive	$[M+H]^+$ 469.3019 (100)	451 (23.8), 433 (2.4), 411 (4.0), 397 (4.3), 381 (4.7), 339 (14.4)

*Compounds newly found in Ze Xie.

and suggested that the position of this double bond was situated in side chain.

HR-MS in negative mode of compound **5** showed a base peak at *m/z* 551.3886 corresponding to $C_{31}H_{51}O_8$, (m.w. calculated for $C_{31}H_{51}O_8$, 551.3584), which was two more hydrogens than compound **1** (16-oxo-alisol A). In positive mode MS, a prominent ion at *m/z* 529.3163 corresponding to $C_{30}H_{50}O_6Na$ (calculated for $C_{30}H_{50}O_6Na$, 529.3505) was detected. $C_{31}H_{51}O_8$ and $C_{30}H_{50}O_6Na$ should be assigned to $[M + HCOOH-H]^-$ and $[M + Na]^+$ of dihydro-16-oxo-alisol A. The product ions at *m/z* 505 and 487 of $[M + HCOOH-H]^-$ at *m/z* 551 were $[M-H]^-$ and $[M-H_2O-H]^-$, respectively. Thus, compound **5** was characterized as a di-hydrogenated derivative of compound **1**. CID of $[M + HCOOH-H]^-$ and $[M+Na]^+$ of this compound did not reveal any other characteristic fragments in neither high (35 eV) nor low (15 eV) MS/MS collision energy and position of di-hydrogenation could not be determined.

HR-MS in positive mode of compounds **10** and **18** yielded base peaks at *m/z* 545.3097 and *m/z* 545.3069, respectively, were in accordance with a $C_{32}H_{49}O_7$ formula (m.w. calculated for $C_{32}H_{49}O_7$, 545.3478). In negative mode HR-MS, these two compounds gave base peaks at *m/z* 561.3960 and 561.3654 corresponding to $C_{32}H_{49}O_8$ formula (m.w. calculated for $C_{32}H_{49}O_8$, 561.3427). Based on the MS behaviours in positive and negative modes of protostane triterpenoids in Ze Xie, $C_{32}H_{49}O_7$ formula in positive mode and $C_{32}H_{49}O_8$ formula in negative mode were assigned as $[M + H-H_2O]^+$ and $[M-H]^-$ of compounds **10** and **18**. The same MS data suggested that compounds **10** and **18** were isomers. The $[M + H-H_2O]^+$ at *m/z* 545 and its product ions at *m/z* 527, 485, 467, 431, 413 and 369 were exactly one oxygen more than that of compound **2** (16-oxo-alisol A 24-actetate), $[M + H-H_2O]^+$ at *m/z* 529 as well as its product ions at *m/z* 511, 469, 451, 415, 397 and 353. The MS data comparison suggested that one hydrogen of compound

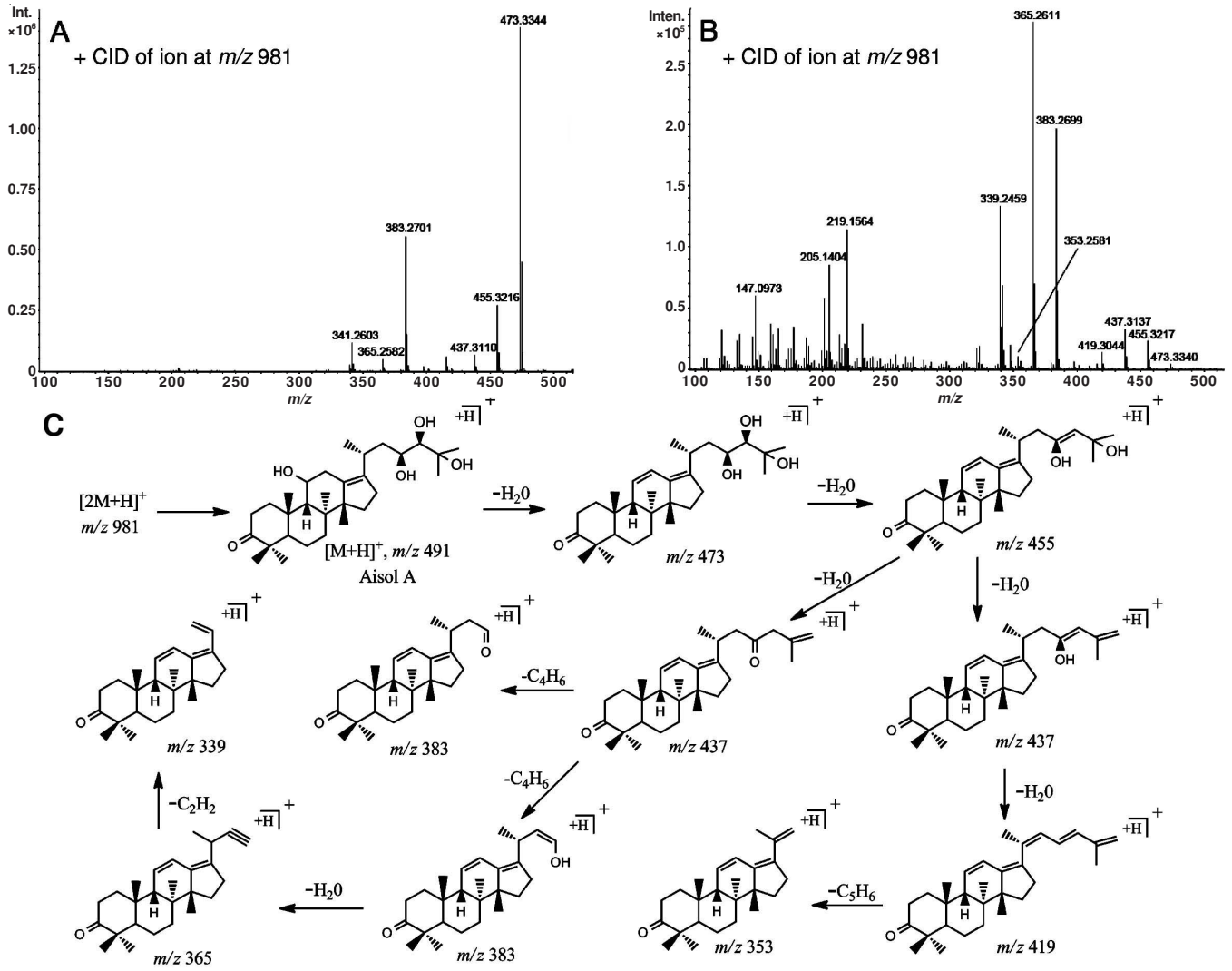


Fig. 6. CID MS/MS positive mass spectrum obtained from $[2M+H]^+$ of compound **20** at *m/z* 981.6715: **A**, with MS/MS collision energy at 15 eV; **B**, with MS/MS collision energy at 35 eV. **C**, proposed fragmentation pathway of compound **20**

2 was replaced by a hydroxyl in compounds **10** and **18**. However, the precise position of this hydroxyl was difficult to be certain of since NMR data or the corresponding standards of these two compounds were not available. Therefore, compound **10** and **18** were characterized as hydroxylated derivatives of compound **2**.

HR-MS of compound **27** showed a prominent ion at *m/z* 527.3314 corresponding to $C_{32}H_{47}O_6$ (m.w. calculated for $C_{32}H_{47}O_6$, 527.3372), which has two hydrogens less than that of compound **7**. Based on the MS behaviours in positive mode of protostane triterpenoids in Ze Xie, $C_{32}H_{47}O_6$ formula in positive mode was assigned as $[M + H]^+$ of compound **27**. Its product ions at *m/z* 469, 433 and 397 were identical to that of produced by compound **7** (Alisol C 23-acetate), suggesting that these two compound possessed a same protostane triterpenoid skeleton. Therefore, compound **27** was characterized as olefination derivative of compound **7** and had an additional double bond in side chain.

Conclusion

Using HPLC-DAD/HR-ESI-TOF-MS in this study, a reliable and sensitive method has been established for the analysis of protostane triterpenoids in Ze Xie. As a result, a total of 28 protostane triterpenoids were separated and simultaneously detected in Q-TOF-MS and 15 of them were newly found in Ze Xie. Our results showed that most protostane triterpenoids in Ze Xie generated diverse parent ions including $[M + H]^+$, $[M-H_2O + H]^+$ and/or $[2M+H]^+$ in positive mode, which led to a rather difficult identification for their molecular ion. In negative mode, these protostane triterpenoids gave the most prominent ions of formic acid adduct $[M+HCOOH-H]^-$ in their mass spectra. Since authentic standards of many components in Ze Xie are un-available, more reliable analysis of protostane triterpenoids in crude extract of Ze Xie should be performed by comparing both positive and negative to determine the parent ions and their diagnostic CID fragments. This study demonstrated a reliable and sensitive method suitable for the analysis of protostane triterpenoids in Ze Xie and other herbs. Further work is clearly needed to separate pure compounds and identify the new bioactive protostane triterpenoids in Ze Xie by bioassays for determinations of their bioactivities and NMR and other methods for their final structural identification.

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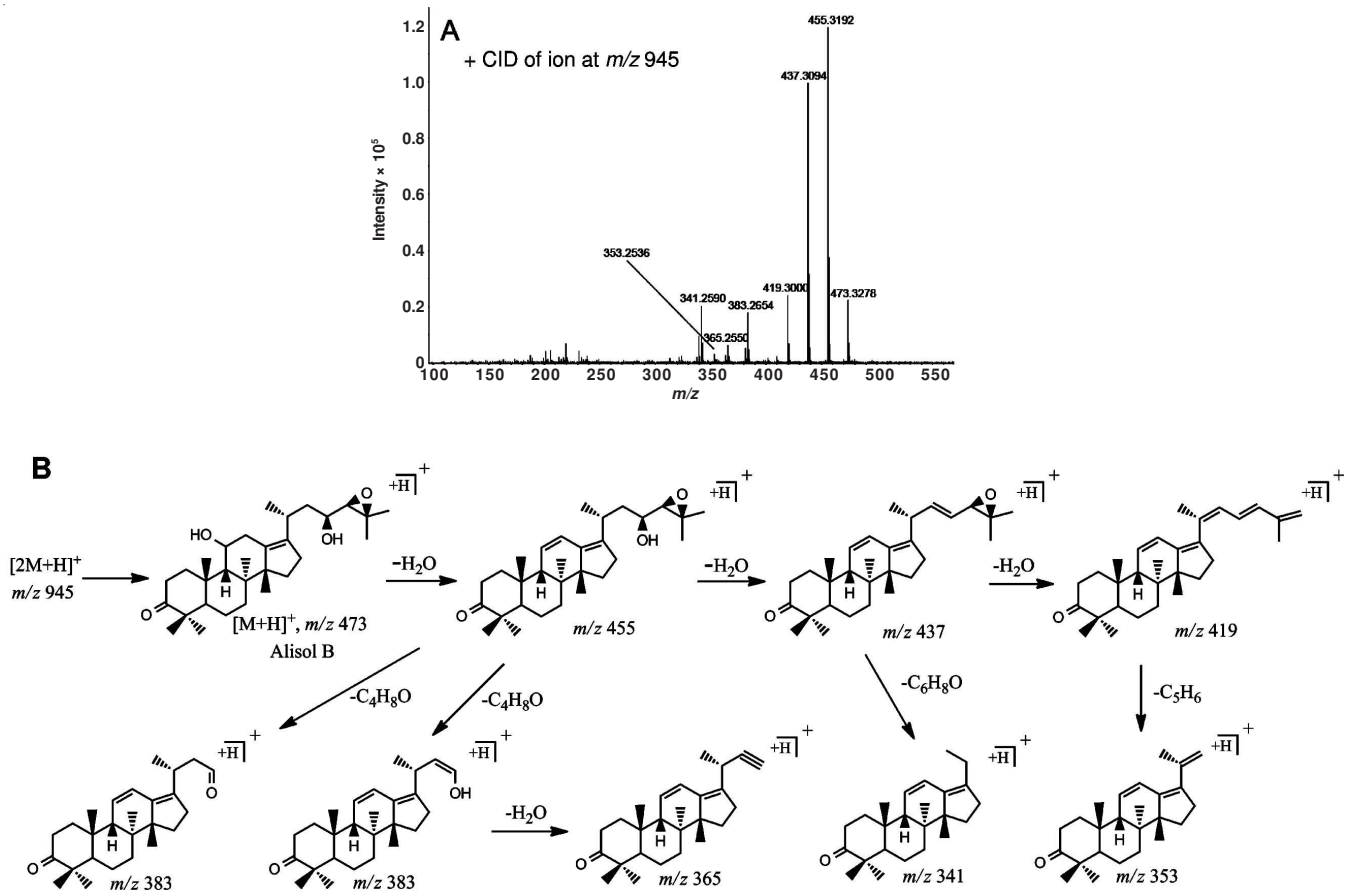

Fig. 7. CID MS/MS negative mass spectrum obtained from $[2M+H]^+$ of compound **28** at *m/z* 945.5709 with MS/MS collision energy at 15 eV (**A**) and its proposed fragmentation pathway (**B**)

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