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•Original article•

Rapid identification of stigmastane-type steroid saponins from *Vernonia amygdalina* leaf based on *α*-glucosidase inhibiting activity and molecular networking

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[ABSTRACT] Steroid saponins are secondary metabolites with multiple medicinal values that are found in large quantities in natural medicines, especially *Vernonia amygdalina*, a famous nature medicine for the treatment of tonsillitis, diabetes, pneumonia. The current study was designed to combine molecular networking (MN) with diagnostic ions for rapid identification of $\Delta^{7,9(11)}$ stigmastane-type saponins which were the *α*-glucosidase inhibitory active substances in *V. amygdalina*. First, the *α*-glucosidase inhibitory activities of five $\Delta^{7,9(11)}$ stigmastane-type steroid saponins that were previously isolated were screened, which indicated that the $\Delta^{7,9(11)}$ stigmastane-type steroid saponin was one of the active constituents responsible for ameliorating diabetes. Furthermore, a strategy was proposed to identify stigmastane-type steroid saponins and verify the plausibility of derived fragmentation pathways by applying MN, MolNetEnhancer and unsupervised substructure annotation (MS2LDA). Based on this strategy, other seven $\Delta^{7,9(11)}$ stigmastane-type steroid saponins were identified from this plant. Our research provide scientific evidence for the antidiabetic potential of the steroid saponin-rich extract of *V. amygdalina* leaf.

[KEY WORDS] $\Delta^{7,9(11)}$ Stigmastane-type steroid saponins; *α*-Glucosidase inhibitory activity; Fragmentation pathways; Diagnostic ions; Molecular networking

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Introduction

Vernonia amygdalina, a member of the genus Vernonia with bottle green leaves, is used not only as a conventional medicine but also food in tropical Africa [1]. Its pharmaceutical activity has been confirmed by numerous medical researches, such as anti-malaria, anti-cancer, anti-nociceptive, antioxidant and anti-inflammatory effects [2]. People in Nigeria and South Africa often use it to ameliorate diabetes [3]. *V. amygdalina* contains various secondary metabolites including steroidal saponins, flavonoids, phenol and oxalate [4]. The common constituents in *V. amygdalina* leaves are $\Delta^{7,9(11)}$ stigmastane-type steroid saponins which are characterized by broad structural diversity and biological activity [5], and have attracted extensive research interest. The main feature of $\Delta^{7,9(11)}$ stigmastane-type steroid saponin lies in the core part including a four-ring structure perhydrocyclopentanophenanthrene, where C-10 and C-13 are substituted by methyl, C-3 is substituted by hydroxyl or glycone, and the common type of monosaccharide is *β*-D-glucose [6]. According to our previous studies, a total of 14 $\Delta^{7,9(11)}$ stigmastane-type steroid saponins were isolated from *V. amygdalina*, and their anti-inflammatory and multidrug resistance reversal activities were evaluated [7, 8]. Therefore, the antidiabetic ability of $\Delta^{7,9(11)}$ stigmastane-type steroid saponins requires further exploration.

In recent years, tandem mass spectrometry (MS/MS) fragmentation has played an irreplaceable role in structural characterization of natural products. However, it is still challenging to quickly and accurately identify stigmastane-type steroid saponins by MS/MS alone. Accordingly, molecular networking (MN) and several tools (such as MolNetEnhancer [9] and MS2LDA [10]) are broadly used. MolNetEnhancer is a workflow annotating the substructure of natural products

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based on diagnostic ions and neutral losses generated in MS/MS spectra [9]. All nodes are hierarchically chemically annotated by ClassyFire [11]. MS2LDA identifies molecular substructures by extracting frequently co-occurring fragments and neutral losses (called Mass2Motifs or motifs) from MS/MS data [10]. In order to perform an in-depth study in $\Delta^{7,9(11)}$ stigmastane-type saponins, we used a combination of molecular networking and MS/MS analysis to identify the trace and difficult-to-isolate stigmastane-type saponins in *V. amygdalina*.

In this study, the *α*-glucosidase inhibitory activities of five $\Delta^{7,9(11)}$ stigmastane-type saponins that were previouly isolated from *V. amygdalina* [7] were evaluated. The results indicated that the component should be one of the active constituents responsible for antidiabetic treatment in this plant. In order to investigate the active constituents, we proposed a strategy for rapid identification of stigmastane-type steroid saponins based on fragmentation pathways with a combination of classical MN, MolNetEnhancer and MS2LDA. Based on this strategy, seven $\Delta^{7,9(11)}$ stigmastane-type steroid saponins were identified from *V. amygdalina*. These findings provide theoretical basis and experimental foundation for the research and development of the hypoglycemic activity of this type of compounds.

Materials and Methods

Plant materials

The leaves of *Vernonia amygdalina* were collected from Guangdong Province in China in December 2017 and authenticated by Professor ZHANG Mian, Department of Pharmacognosy, China Pharmaceutical University. The voucher specimen (NFY201712) was deposited in Department of Natural Medicinal Chemistry, China Pharmaceutical University.

Chemicals and reagents

All the solvents used for extraction and dimethyl sulfoxide (DMSO) were of analytical grade and purchased from Hanbon Sci. & Tech. (Huaian, China). LC-MS grade methanol (MeOH) and acetonitrile (ACN) used for LC-MS/MS experiments were obtained from Merck Co., Ltd. (Darmstadt, Germany). Pure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA). p-Nitrophenyl pyranoside (PNPG) was acquired from Aladdin (Shanghai, China). *α*-Glucosidase was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acarbose hydrate (purity ≥ 98%) was from Macklin Inc. (Shanghai, China). Silica gel (200−300 mesh) was obtained from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China).

Extraction and fractionation

The air-dried and powdered leaves of *V. amygdalina* (120 g) were extracted with 95% EtOH under reflux for three times. The dark green solid extract obtained by filtration and concentration was dissolved in water. The water-soluble object was partitioned with petroleum ether (PE), dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) successively. The *n*-BuOH fraction, which showed potent *α*-glucosidase inhibitory effect, was further fractionated. The *n*-BuOH fraction (5 g) was successively separated by silica gel column chromatography with CH_2Cl_2-MeOH (20 : 1, 5 : 1 and 0 : 1, *V/V*) to obtain three subfractions Frs. A−C. Fr. B was further divided into three subfractions Frs. B1−B3 using an ODS column gradient elution of MeOH-H_2O (55 : 45, 65 : 35 and 75 : 25, *V/V*). Under the guidance of UV spectrogram, Fr. B2 was enriched and then dissolved in MeOH for LC-MS/MS analysis. The scheme shown in Fig. 1 illustrates the experimental procedures usded in the curent study.

α-Glucosidase inhibitory assay

The *α*-glucosidase inhibitory assay was perfromed according to previous reports [12] with minor modification. Acarbose was used as the positive control, while PNPG was used as the substrate. Various concentrations of each extract (2.5 μL) were added to PBS (47.5 μL, pH 6.8), followed by the addition of *α*-glucosidase (10 μL; 0.7 $U \cdot mL^{-1}$). After incubation at 37 °C for 10 min, PNPG (40 μL; 1.25 $mmol \cdot L^{-1}$) was added. Then, Na_2CO_3 solution (50 μL; 0.1 $mol \cdot L^{-1}$) was added into each well after reaction at 37 °C for 5 min. The absorbance was measured at 405 nm with a microplate reader (SpectraMax Plus384, Molecular Devices LLC., Sunnyvale, CA, USA). The results are expressed as a percentage of *α*-glucosidase inhibition and calculated according to the following equation:

$$\text{inhibition rate}(\%) = \frac{(A_{control} - A_{blank}) - (A_{sample} - A_{blank})}{A_{control} - A_{blank}} \times 100\%$$

where $A_{control}$ is the absorbance without inhibitors; A_{sample} is the absorbance of the sample; and A_{blank} is the background absorbance.

Docking studies

The detailed experimental procedure of molecular docking was shown in Supporting Information.

HPLC-Q-TOF-MS analysis

An Agilent 1290 Infinity HPLC instrument (Agilent, Waldbronn, Germany) was used. The sample was used a Shimadzu Shim-pack VP-ODS column (4.6 mm × 150 mm, 5 μm) with a pre-column. The mobile phase consisting of A (ACN) and B (H_2O) was used after sample injection (2 μL) with a gradient elution as follows: 0−30 min, 35%−45% A; 30−35 min, 45%−70% A; 35−40 min, 70% A; and 40−45 min, 70%−100% A. The flow rate was 0.6 $mL \cdot min^{-1}$ at a column temperature of 30 °C. An Agilent 6520 B Q-TOF-MS (Agilent Technologies, Santa Clara, CA, USA), equipped with an electrospray ionization (ESI) source, was used with the following operating parameters: drying gas (N_2) flow rate, 10 $L \cdot min^{-1}$; drying gas temperature, 320 °C; nebulizer, 45 psi; capillary, 4000 V; fragmentor voltage, 160 V; skimmer, 65 V; and OCT 1 RF Vpp, 750 V. The acquisition rate for each analytical operation was three spectra per second in the positive ion mode. MS full scan analysis was performed to obtain an accurate mass-to-charge ratio (*m/z*) and retention time of the target steroid saponins. The collision energy (CE) was adjusted from 10, 20 and 30 eV, at a mass range of 50−1500 *m/z* and MS/MS range of 50−1500 *m/z*. All the operations, acquisition, and data analysis were performed on

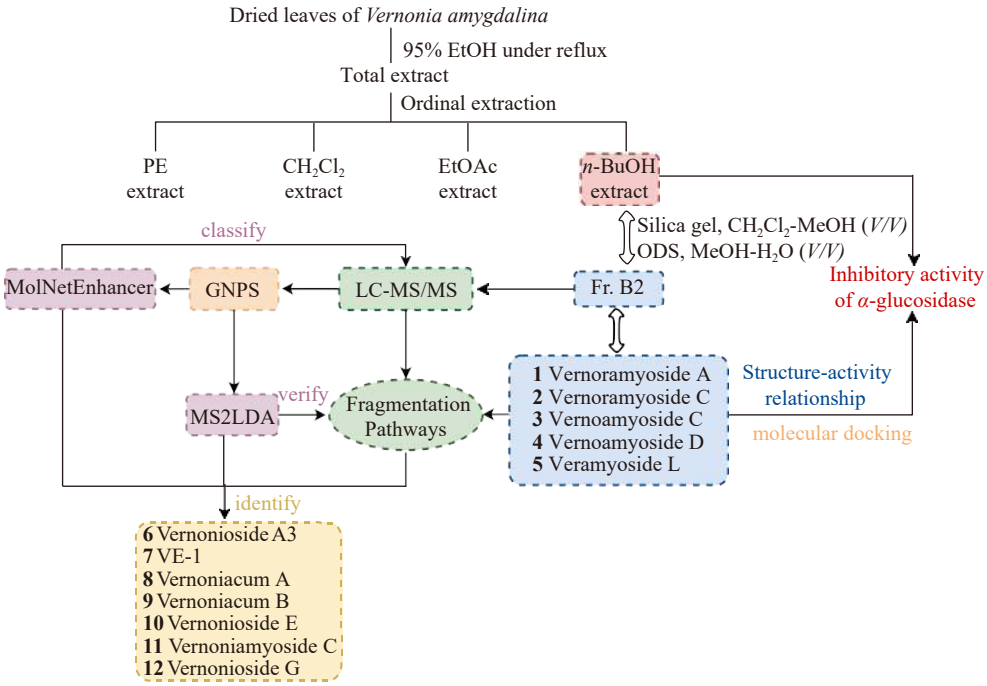

Fig. 1 Schematic diagram of the experiment conducted in *V. amygdalina*

MassHunter Workstation v.B.08.00. Data retrieval was conducted using Micronmr and Sci-finder databases (https://origin-scifinder.cas.org/scifinder/).

Molecular networking

The MS/MS spectrum of Fr. B2 was converted to .mzML file format by ProteoWizard (http://proteowizard.sourceforge.io/). Molecular networking was created using .mzML file with the online web-workflow at GNPS (http://gnps.ucsd.edu) [13]. The MN was generated using the minimum cosine score of 0.6. The other parameters used the default values. The MN was also submitted to MolNetEnhancer [9] and MS2LDA [14] (http://ms2lda.org/) workflows. The bin width was set to 0.01 and other parameters were default for MS2LDA. The data were visualized in Cytoscape (version 3.9.0) [15]. All the networks can be checked online (links are provided in the Supporting Information).

Data analysis

Extrapolation by linear regression analysis yielded half maximal inhibitory concentration (IC_{50}) values. Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) and expressed as mean ± SD from triplicate determinations.

Results and Discussion

Compounds **1**–**5** (Fig. 2) were isolated from the *n*-BuOH extract in our previous research [7], namely vernoramyoside A (**1**, 0.0125%), vernoramyoside C (**2**, 0.0375%), vernoamyoside C (**3**, 0.0125%), vernoamyoside D (**4**, 0.01%) and veramyoside L (**5**, 0.005%).

α-Glucosidase inhibitory activities of $\Delta^{7,9(11)}$ stigmastane-type saponins and extracts

The *α*-glucosidase inhibitory activities of five crude extracts and compounds **1**–**5** were evaluated (Table 1). The *n*-BuOH extract (IC_{50} = 43.49 ± 2.09 μg·mL^{-1}) was more active than other extract fractions, followed by the 95% EtOH extract (IC_{50} = 85.07 ± 2.16 μg·mL^{-1}). Moreover, the inhibitory activities of compounds **1**–**5** against *α*-glucosidase were different. Compounds **1** and **2** exhibited obvious activity. According to the results metioned above, $\Delta^{7,9(11)}$ stigmastane-type steroid saponins were supposed to be the active components of *V. amygdalina* leaves exerting *α*-glucosidase inhibitory effects. Consequently, the structure-activity relationship between *α*-glucosidase inhibitory activity and the compounds was investigated.

Among compounds **1**, **4** and **5**, compound **1** (IC_{50} = 134.35 ± 1.56 μg·mL^{-1}) exhibited the strongest activity, while **4** (IC_{50} = 285.22 ± 7.26 μg·mL^{-1}) and **5** (IC_{50} = 636.39 ± 4.24 μg·mL^{-1}) had moderate activity. The similarity in their structure is the same side chain group at the C-17 position. Therefore, it is possible that this group is effective for *α*-glucosidase inhibition. Furthermore, the differences in structural formula are the substituents at the C-16 position, namely carbonyl (**1**), hydroxyl (**4**) and acetoxy (**5**). Consequently, we speculated that carbonyl group greatly enhances the *α*-glucosidase inhibitory effect. Compound **2** (IC_{50} = 181.29 ± 1.84 μg·mL^{-1}) exhibited stronger activity compared with compound **3** (IC_{50} = 845.64 ± 1.63 μg·mL^{-1}). The side chain groups at the C-17 position in their structure differ by methoxy. Therefore, it is possible that the presence of methoxy decreases the ability to inhibit *α*-glucosidase. The speculation about structure-activity relationship needs to be further confirmed. If the speculation is confirmed, the efficiency of screenign *α*-glucosidase inhibitory compounds will be largely improved.

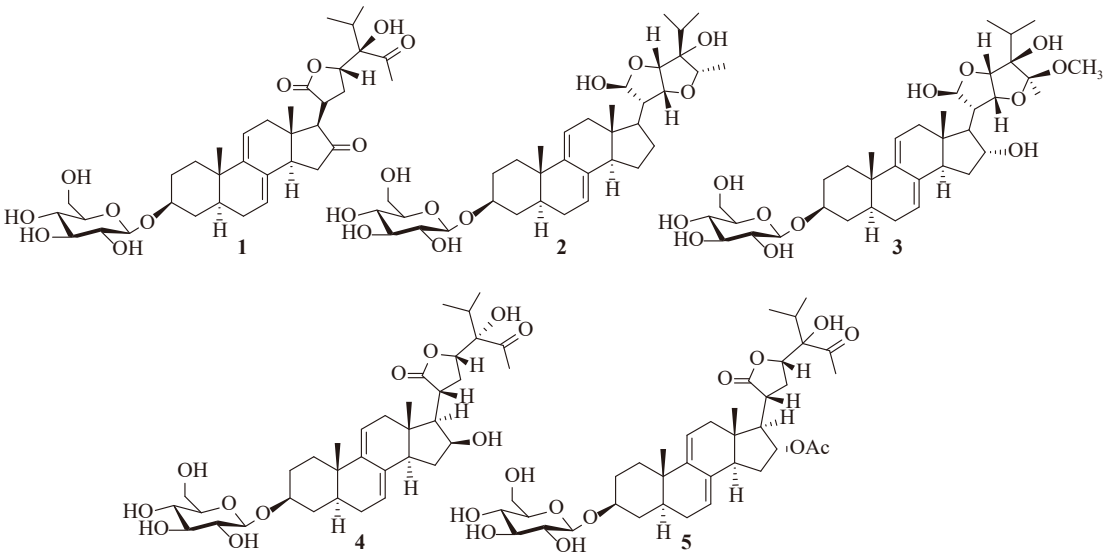

Fig. 2 Chemical structures of 1−5

Table 1 *α*-Glucosidase inhibitory activity of crude extracts and compounds[a]

Crude extracts	IC_{50} (μg·mL^{-1})	Compd.	IC_{50} (μg·mL^{-1})
95% EtOH	85.07 ± 2.16	**1**	134.35 ± 1.56
PE	>1000	**2**	181.29 ± 1.84
CH_2Cl_2	489.32 ± 10.96	**3**	845.64 ± 1.63
EtOAc	505.30 ± 13.15	**4**	285.22 ± 7.26
n-BuOH	43.49 ± 2.09	**5**	636.39 ± 4.24
Positive control	Acarbose		
	279.35 ± 7.50		

[a]Values are expressed as means ± standard deviations (n = 3).

Molecular docking between ligands and enzyme proteins is pivotal for their interaction [16]. To determine the binding mode of the compounds to brewer's yeast *α*-glucosidase (*α*GHY), docking analysis was performed using the crystalline structure of *α*GHY (PDB ID 3A4A). The binding modes between **1**, **2** and *α*GHY active site are shown in Fig. S1. Furthermore, compounds **1** and **2** had lower free binding energy (−6.41 kcal·mol^{-1} and −8.47 kcal·mol^{-1}, respectively) than acarbose hydrate (−2.00 kcal·mol^{-1}). From the perspective of energy, it is indicated that compounds **1** and **2** can combine with the active cavity and bind to *α*GHY in a stable manner.

Our study suggested that steroid saponin-rich *V. amygdalina* extracts have promising potential in ameliorating diabetes. With regard to the considerable activity of $\Delta^{7,9(11)}$ stigmastane-type steroid saponins, we speculated that they are the active components responsible for antidiabetic treatment in *V. amygdalina*.

Classification of $\Delta^{7,9(11)}$ stigmastane-type saponins in V. amygdalina by MolNetEnhancer

With regard to the complexity of phytochemicals, it is challenging to rapidly and efficiently identify component species in plants. The Global Natural Product Social (GNPS) molecular networking Web-platform supplies in-depth studies of natural products based on unique MS/MS spectral clusters of known and unknown molecular analogs or families [13]. MolNetEnhancer provides a convenient method to classify and annotate the chemical compositions in complex MS/MS data [17, 18]. The MN shown in Fig. 3 was the results by the MolNetEnhancer and Classyfire. The compounds were classified into seven categories according to the classification system of the software workflow, where "steroids and steroid derivatives" account for the majority. According to the isolated compounds **1**−**5**, we determined that nodes in the largest cluster represented $\Delta^{7,9(11)}$ stigmastane-type steroid saponins and their characteristic diagnostic ions through database analysis and literature review. Compound species were quickly identified by MolNetEnhancer from the complicated MS/MS data, which facilitated the analysis of natural products.

Fragmentation patterns of isolated $\Delta^{7,9(11)}$ stigmastane-type saponins

Compounds **1**−**5** revealed that their substituents at the C-16 position were commonly carbonyl, hydroxyl, acetoxy or unsubstituted. The major difference in the structure of these compounds lay in the side chain attached to C-17. The fragment ion, *m/z* 631, was frequently present in cleavage, albeit in different structures. The diagnostic ion at *m/z* 431 $[C_{25}H_{35}O_6]^+$ often appeared due to the loss of the C-16 substituent and the C-17-linked side chain. During the fragmentation, all the compounds mentioned lost one molecule of glucose unit (162 Da) at the C-3 position. The parent nucleus structure was formed after the loss of the C-16 substituent (if had), the C-17-linked side chain, and one molecule of glucose unit. There were three forms of parent nucleus fragment structures. First, if there was no substituent at the C-16 position, the final parent nucleus fragment ion was *m/z* 271 $[C_{19}H_{27}O]^+$. Second, the final parent nucleus fragment ion

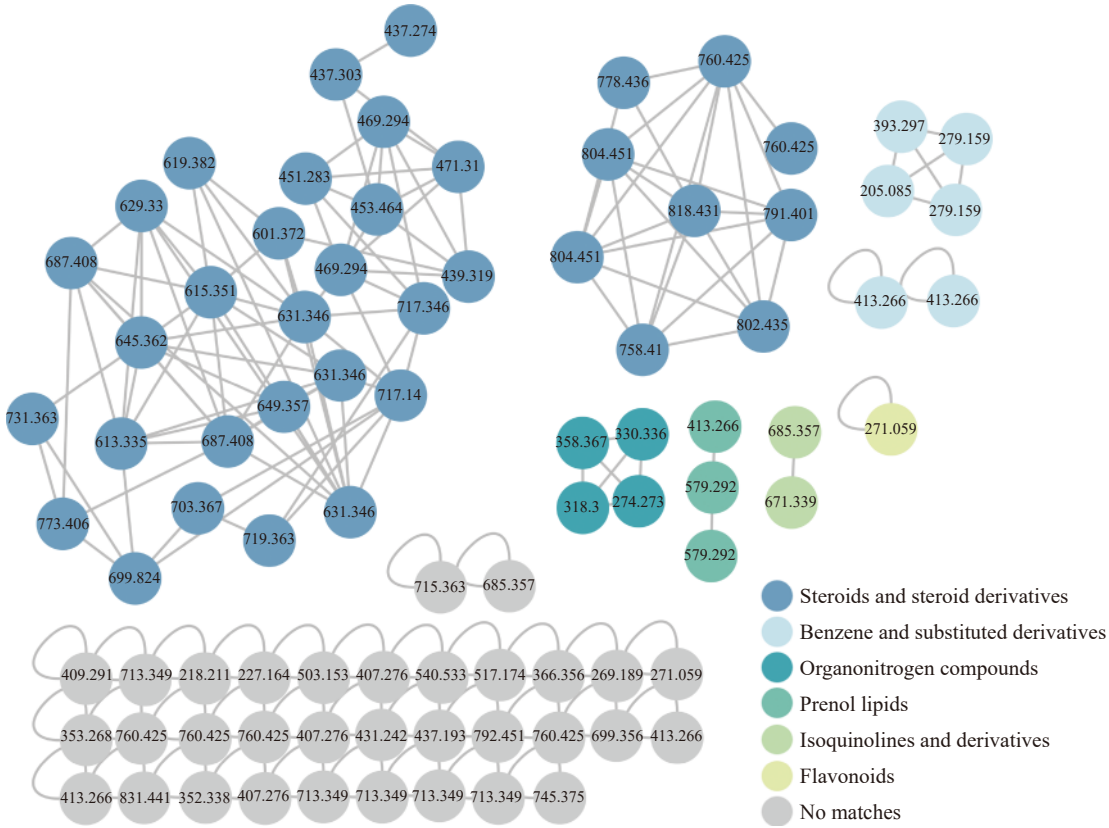

Fig. 3 Molecular network for annotation of Fr. B2 at the class level by MolNetEnhancer and ClassyFire

formed was *m/z* 269 $[C_{19}H_{25}O]^+$ when the substituent at the C-16 position was hydroxyl or acetoxy. Finally, if the substituent at the C-16 position was carbonyl, *m/z* 267 $[C_{19}H_{23}O]^+$ would undergo H rearrangement and keto-enol tautomerism. Then, the three forms of parent nucleus were often stripped of one molecule of H_2O (18 Da) to *m/z* 253 $[C_{19}H_{25}]^+$, *m/z* 251 $[C_{19}H_{23}]^+$ and *m/z* 249 $[C_{19}H_{21}]^+$, respectively. The HPLC chromatograms and the fragmentation patterns are shown in Fig. S2 and Fig. S3.

The fragment ions at *m/z* 271, 253, *m/z* 269, 251 and *m/z* 267, 249 can be used as three sets of key ions for the identification of $\Delta^{7,9(11)}$ stigmastane-type steroid saponins. Moreover, fragment ions at *m/z* 431 and neutral loss 162 Da are characteristic in most $\Delta^{7,9(11)}$ stigmastane-type saponins in fragmentation patterns. These fragmentation behaviors and diagnostic ions of $\Delta^{7,9(11)}$ stigmastane-type steroid saponins provid a solid foundation for rapid and high-efficiency structural elucidation of similar metabolites in plant-derived medicines.

Substructure annotation of $\Delta^{7,9(11)}$ stigmastane-type saponins by MS2LDA

MS2LDA utilizes GNPS and repeated interactions (shared fragmentation patterns and/or neutral loss) to extract fragment ions from the substructural diversity of each natural product [10]. In the study, nodes were connected by Mass2motifs and cosine (Fig. 4). The nodes of *m/z* 687.408, 615.351, 601.372 and 619.382 connected by motif_169 collectively included the diagnostic ion *m/z* 431, which implied that these ions showed the same substructure during cleavage. The neutral loss 180 Da, included in motif_86, resulted from removal of one molecule of glucose and water. Motif_20 and motif_86 had the characteristic fragment ion *m/z* 251. With regard to the fragmentation patterns, the original structures of the ions (including those before cleavage), connected by motif_169, motif_20 and motif_86, were $\Delta^{7,9(11)}$ stigmastane-type saponins substituted with hydroxyl or acetoxy groups at the C-16 position. MS2LDA can reveal some spectral features involved in fragmentation clustering. During MS2LDA application, it is clearly showed the presence or absence of a substituent at the C-16 position of a stigmastane-type steroid saponin and the type of substituent, which can be used to identify the compounds based on MS/MS spectra, with improved efficiency and accuracy. Meanwhile, MS2LDA and Mass2Motifs results verified the plausibility of the putative fragmentation pathways.

Identification of $\Delta^{7,9(11)}$ stigmastane-type saponins in V. amygdalina

The enrichment was conducted by HPLC-DAD method using the characteristic UV spectrum (Fig. 5A). However, it is still challenging to use MS/MS spectrum to identify trace and difficult-to-isolated $\Delta^{7,9(11)}$ stigmastane-type saponins. Multiple methods are suggested, for instance molecular networking as an efficient choice. On the basis of compounds **1**−**5**, combined with UV spectra and fragment ions, we identi-

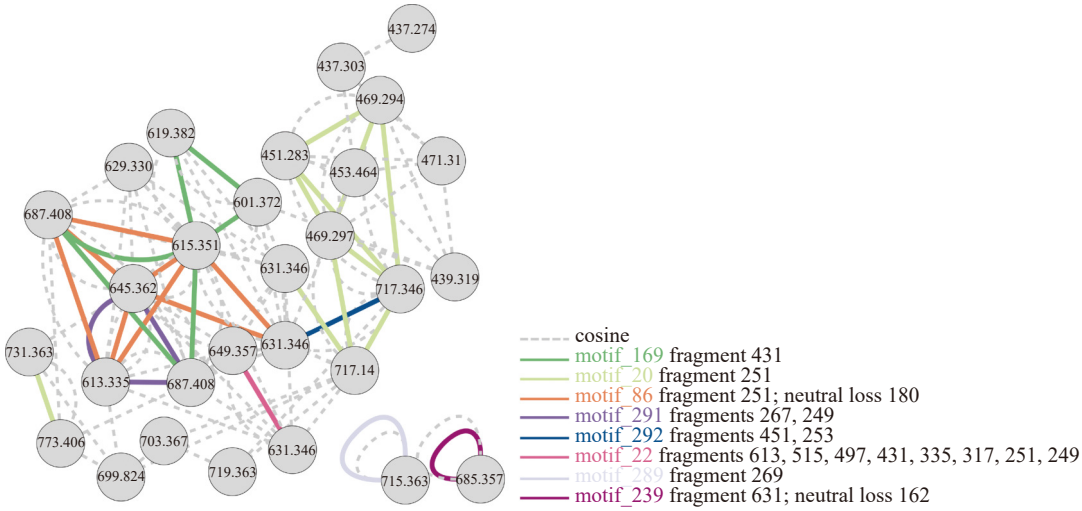

Fig. 4 MN layout representing the Mass2motifs interactions that cluster the nodes

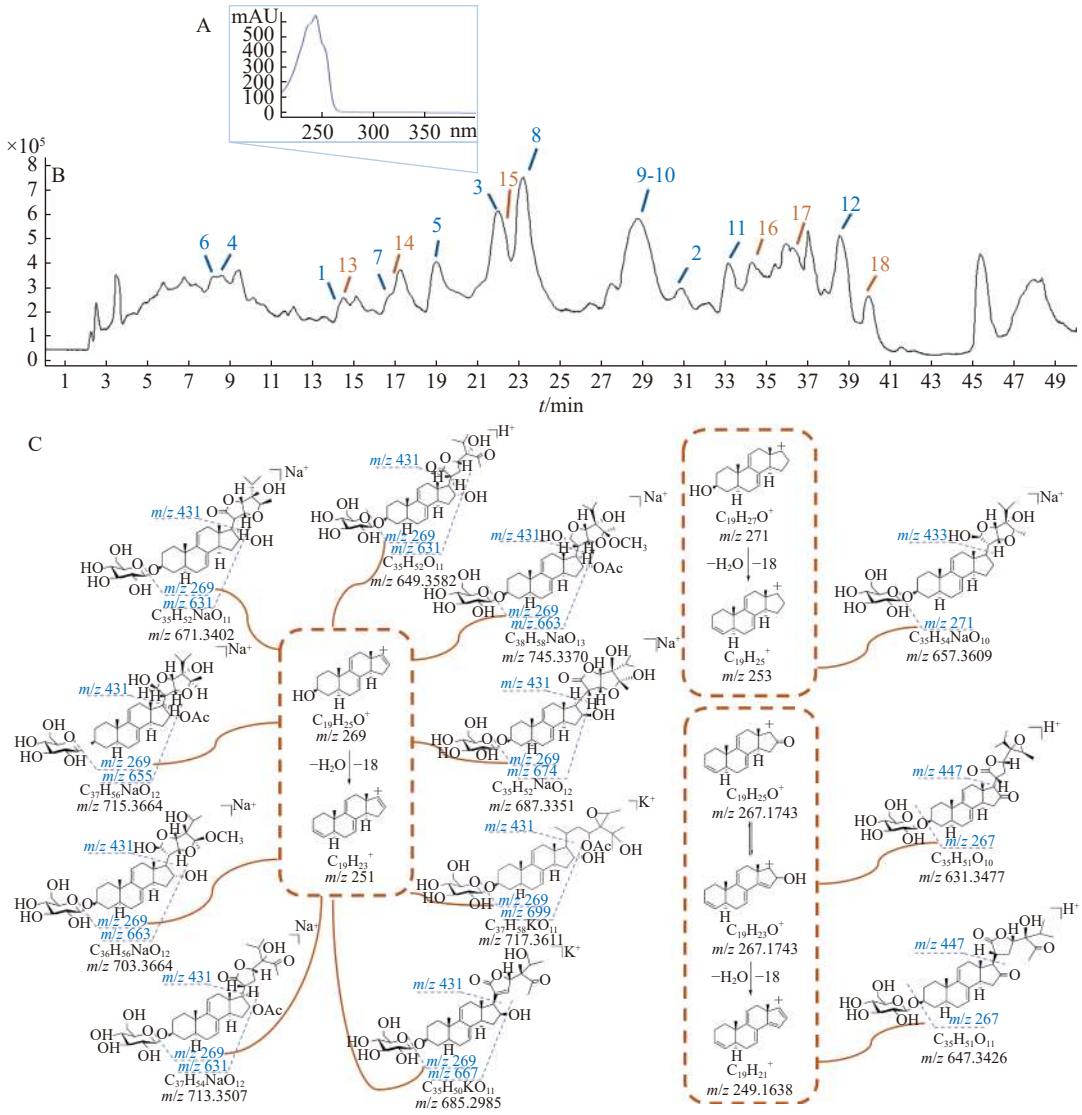

Fig. 5 (A) UV Spectrogram of compound 3. (B) ESI (+)-TIC scan mass spectrum of the extract enriched from *V. amygdalina* leaves. Compounds 1–12 and compounds 13–18 were known and unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponins identified from Fr. B2. (C) The main fragmentation behaviors of 1–12 and the structure of parent nucleus formed after fragmentation

fied seven known and six unknown $\Delta^{7,9(11)}$ stigmastane-type saponins in Fr. B2 with the assistance of MolNetEnhancer and MS2LDA. For example, according to the databases and MolNetEnhancer, node *m/z* 715.363 was VE-1 [19] or vernonioside S [20]. The C-16 position of VE-1 was substituted by an acetoxyl group, while vernonioside S was unsubstituted at the C-16 position. The node was tagged by motif_289, which contained fragment *m/z* 269 (Fig. 4). As *m/z* 269 was one of the diagnostic ions to identify $\Delta^{7,9(11)}$ stigmastane-type saponins, node 715.363 was determined to be VE-1 with the C-16 position substituted by acetoxy. MS2LDA not only assisted manual identification of the compounds by MS/MS, but also improved the accuracy of identification. Mass2Motifs results also validated the reliability of the fragmentation patterns. However, node *m/z* 715.363 was annotated as "no matches" (Fig. 3), indicating that GNPS need to be improved. Compounds **1**−**12** were tentatively assigned by matching the empirical molecular formula with those recorded in databases. The ESI (+)-TIC scan mass spectrum is shown in Fig. 5B. As the compounds contained glucose group, unsharp peak shapes were seen. The major fragmentation pathways of compounds **1**−**12** were deduced (Fig. 5C). The detailed information of these components [6-8, 19, 21-25] which have been identified from Fr. B2 are summarized in Table 2 and Fig. S3.

Table 2 Identification of the chemical constituents of *V. amygdalina* leaves by HPLC-Q-TOF-MS

No.	t_R (min)	Ion mode	Formula	Putative compounds	Mass (*m/z*)	Major fragment ions (*m/z*)	ref.
1	14.170	$[M + H]^+$	$C_{35}H_{51}O_{11}$	Vernoramyoside A	647.3378	629.3301, 503.1513, 449.2666, 267.1717, 249.1645	[7]
2	30.871	$[M + Na]^+$	$C_{35}H_{54}NaO_{10}$	Vernoramyoside C	657.3579	617.3665, 601.3704, 469.2933, 455.3143, 437.3044, 253.1944	[7]
3	22.131	$[M + Na]^+$	$C_{36}H_{56}NaO_{12}$	Vernoamyoside C	703.3650	645.3629, 631.3470, 469.2933, 431.2416, 269.1895, 251.1808	[8]
4	8.464	$[M + H]^+$	$C_{35}H_{53}O_{11}$	Vernoamyoside D	649.3545	631.3471, 613.3361, 515.2598, 497.2512, 335.2029, 317.1903, 251.1742	[8]
5	18.794	$[M + Na]^+$	$C_{37}H_{54}NaO_{12}$	Veramyoside L	713.3486	631.3456, 469.2937, 451.2829, 431.2373, 269.1884, 251.1779	[6]
6	8.188	$[M + H]^+$	$C_{35}H_{51}O_{10}$	Vernonioside A3	631.3464	613.3339, 515.2655, 497.2537, 335.1985, 317.1909, 267.1717, 249.1628	[21]
7	16.648	$[M + Na]^+$	$C_{37}H_{56}NaO_{12}$	VE-1	715.3641	631.3467, 619.3827, 615.3519, 469.2932, 431.2420, 269.1891, 251.1783	[19]
8	23.200	$[M + Na]^+$	$C_{35}H_{52}NaO_{11}$	Vernoniacum A	671.3401	631.3475, 613.3367, 515.2624, 469.2938, 431.2425, 317.1852, 269.1895, 251.1760	[22]
9	28.916	$[M + Na]^+$	$C_{38}H_{58}NaO_{13}$	Vernoniacum B	745.3759	717.3468, 613.3361, 469.2933, 451.2826, 431.2398, 269.1889, 251.1766	[22]
10	29.666	$[M + K]^+$	$C_{37}H_{58}KO_{11}$	Vernonioside E	717.3483	613.3349, 601.3720, 469.2923, 437.3042, 431.2439, 269.1913, 251. 1775	[23]
11	33.133	$[M + K]^+$	$C_{35}H_{50}KO_{11}$	Vernoniamyoside C	685.3580	631.3476, 469.2942, 451.2833, 431.2425, 269.1883, 251.1785	[24]
12	38.516	$[M + Na]^+$	$C_{35}H_{52}NaO_{12}$	Vernonioside G	687.4081	631.3469, 613.3379, 515.2612, 431.2415, 407.2763, 269.2081	[25]
13	14.353			unknown	629.3298	503.1540, 467.2780, 271.0592	
14	16.762			unknown	619.3828	615.3509, 601.3730, 431.2415, 269.1888	
15	22.472			unknown	645.3628	631.3465, 469.2925, 451.2836, 431.2385, 317.1865, 269.1886, 251.1768	
16	34.095			unknown	719.3643	631.3459, 517.2472, 469.2929, 453.2990, 335.2187, 271.1999, 253.1926	
17	36.246			unknown	699.3561	629.3303, 537.3064, 469.2932, 267.1564, 249.1612	
18	39.866			unknown	773.4084	701.3513, 517.2423, 409.2914, 269.2105, 251.1811	

In addition, compounds **13**−**18**, six unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponins in *V. amygdalina* were found in Fr. B2. Compounds **13** and **16** were presumed to be unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponins without substituents at the C-16 position. Compounds **14**, **15** and **18** were speculated to be unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponins with hydroxyl or acetoxy substituents at the C-16 position. The results were consistent with the Mass2Motifs analysis in MS2LDA. Compound **17** was presumed to be an unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponin with the C-16 position substituted by a carbonyl group.

In summary, MolNetEnhancer quickly classified the compounds in complex MS/MS spectra. Then the target compound species were preliminarily identified. For $\Delta^{7,9(11)}$ stigmastane-type steroid saponins, the presence or absence of a substituent at the C-16 position and the type of substituent was evident by MS2LDA analysis. In the case of multiple candidate compounds, the annotations of Mass2Motifs in MS2LDA were used to screen the compounds, with improved identification accuracy and efficiency. However, it is difficult to accurately identify isomers using this strategy alone. To overcome this difficulty, more studies should be

conducted subsequently.

Conclusion

In this work, five $\Delta^{7,9(11)}$ stigmastane-type steroid saponins isolated from *V. amygdalina* are determined with considerable α-glucosidase inhibitory activities, which indicates that the $\Delta^{7,9(11)}$ stigmastane-type steroid saponin is one of the active constituents responsible for ameliorating diabetes in this plant. In order to investigate the active constituents that are trace elements and difficult-to-isolated, a strategy for rapid identification of stigmastane-type steroid saponins is proposed based on fragmentation pathways with a combination of classical MN, MolNetEnhancer and MS2LDA. As a result, seven known and six unknown $\Delta^{7,9(11)}$ stigmastane-type steroid saponins are identified. The method is effective in the constitutive study to investigate the antidiabetic activity of stigmastane-type steroid saponins and reveals that *V. amygdalina* extracts exhibit promising potential in ameliorating diabetes. Our workflow provides a rapid and efficient method to analyze natural medicines.

Supplementary Material

Supporting information can be acquired by e-mail to the corresponding author.

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