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## Characterization, quantitation, similarity evaluation and combination with $\text{Na}^+, \text{K}^+$ -ATPase of cardiac glycosides from *Streblus asper*

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### ARTICLE INFO

#### Keywords:

*Streblus asper*  
HPLC-Q-TOF-MS  
 $\text{Na}^+, \text{K}^+$ -ATPase  
Enzyme kinetics  
Molecular docking  
Quantitation  
Chemometrics

### ABSTRACT

*Streblus asper* Lour. (Moraceae) is a medicinal plant in Asian countries including India and Thailand, possessing activities of anti-tumor, anti-allergy, anti-parasitic and anti-bacterial. In this paper, characterization, quantitation and similarity evaluation of cardiac glycosides in different parts of *S. asper* were investigated by HPLC-Q-TOF-MS and chemometric methods. Then, the inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase activity by the compounds isolated from *S. asper* was measured. Meanwhile, enzyme kinetics and molecular docking were determined to exhibit the combination modes between cardiac glycosides and  $\text{Na}^+, \text{K}^+$ -ATPase. As a result, twenty peaks of cardiac glycosides were assigned. Strophanthidin-3-O- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  4)-6-deoxy- $\beta$ -D-allopyranoside (1), glucostrebluside (2), strebluside (4) and mansonin (8) with a significant activity of inhibiting  $\text{Na}^+, \text{K}^+$ -ATPase ( $\text{IC}_{50}$  7.55–13.60  $\mu\text{M}$ ) were chosen for the determination of enzyme kinetics, exhibiting anticompetitive inhibitory characteristics towards  $\text{Na}^+, \text{K}^+$ -ATPase. Compound 4 could reasonably bind to the active sites of  $\text{Na}^+, \text{K}^+$ -ATPase, proved by molecular docking. Furthermore, the contents of the major compounds in four different parts of *S. asper* were extremely different, analyzed by chemometric methods, similarity analysis and principle compounds analysis. All these findings indicated that the contents of major compounds in different parts of *S. asper* were extremely different with a significant activity of inhibiting  $\text{Na}^+, \text{K}^+$ -ATPase, providing a reference for determination of effective part and administered dosage. The combination modes between cardiac glycosides and  $\text{Na}^+, \text{K}^+$ -ATPase were also revealed by enzyme kinetics and molecular docking, which provided a basis for further study of pharmacological activity.

### 1. Introduction

Traditional medicinal plants have been recognized for their therapeutic benefits for centuries. *Streblus asper* Lour. (Moraceae), is a traditionally used medicinal plant in Asian countries, such as India and Thailand [1]. This plant is known to produce steroids, lignans [2], flavonoids and triterpenoids [3]. Several remarkable pharmacological applications of this plant have been reported. The bark extract has been used in relief of fever, diarrhea [4], toothache, gingivitis [5] and inflammation [6]. Moreover, the polyphenolic rich fraction from its stem also has insecticidal action [7]. The methanol extracts of the *S. asper* heartwood and root exhibited an anti-HBV activity [8–10]. *S. asper* leaf extract possesses an inhibitory effect on subgingival biofilm formation

[11,12]. Bactericidal activity was found in the 50% ethanol (v/v) extract of *S. asper* leaf [13]. *S. asper* leaf extract also possesses both longevity effect and neuroprotective activity against glutamate-induced cell death, supporting its therapeutic potential for the treatment of age-associated neurodegenerative diseases [14].

Cardiac glycosides are a kind of chemical compounds which were sufficient in *S. asper*, used clinically for arrhythmia and heart failure, inhibiting  $\text{Na}^+, \text{K}^+$ -ATPase specifically [15]. Apart from its function on the ion pump,  $\text{Na}^+, \text{K}^+$ -ATPase interacts with different signaling proteins, and many of these have been studied for their roles in reducing cancer cell viability [16]. Inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase induced  $\text{Ca}^{2+}$  accumulation and increased reactive oxygen species (ROS), followed by growth arrest and cell death [17].  $\text{Na}^+, \text{K}^+$ -ATPase inhibition also

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<https://doi.org/10.1016/j.bioorg.2019.03.049>

Received 17 January 2019; Received in revised form 9 March 2019; Accepted 16 March 2019

Available online 19 March 2019

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activated signal transduction pathways, including Src, EGFR and MAPK, and also reduced p53 synthesis [18]. Owing to the involvement of  $\text{Na}^+, \text{K}^+$ -ATPase in numerous cellular functions, using cardiac glycosides to alter downstream signaling might be one strategy to repurpose this kind of molecules for cancer treatment.

However, the cardiac glycosides in *S. asper* were lack of study and the investigation of heartwood of *S. asper* was not sufficient. In addition, literature of the determination of cardiac glycosides in different parts of *S. asper* is still limited as well as the combination modes between cardiac glycosides and  $\text{Na}^+, \text{K}^+$ -ATPase were not clear.

In this paper, four parts of *S. asper* were investigated by HPLC and the HPLC-Q-TOF-MS method was developed for qualitative analysis of cardiac glycosides in heartwood of *S. asper*. Comparing leaf, root, stem and heartwood of *S. asper*, heartwood part was the most complicated part with numerous peaks, which was chosen for HPLC-Q-TOF-MS analysis. Then, the inhibition of  $\text{Na}^+, \text{K}^+$ -ATPase activity by the compounds isolated from *S. asper* was measured. Also, the active compounds were chosen for the determination of enzyme kinetics as well as quantitation with an established HPLC-UV method, one of which was chosen to exhibit the binding mode between  $\text{Na}^+, \text{K}^+$ -ATPase and cardiac glycosides by molecular docking. Besides, chemometrics including similarity analysis (SA) and principal compounds analysis (PCA) were utilized for the study of chemical fingerprint and classification of different parts of *S. asper*.

## 2. Results and discussion

### 2.1. Optimization of chromatographic conditions

To achieve a better chromatographic behavior of *S. asper*, various HPLC parameters were also investigated. Detector (UV and ELSD), UV detection wavelength (220 and 254 nm), column temperature (30, 35 and 40 °C) and flow rate (0.9, 1.0 and 1.1 mL·min<sup>-1</sup>) were optimized. The detector of UV, detection wavelength of 220 nm, column temperature of 40 °C and flow rate of 1.0 mL·min<sup>-1</sup> provided a better absorption, a sufficiently large number of detectable peaks and better peak resolution on the HPLC chromatogram. As a result, the best chromatographic behavior was achieved as follows: Inertsil ODS-SP column (4.6 mm × 250 mm, 5 μm), methanol (A) and water (B) as mobile phase, detector of UV, wavelength of 220 nm, column temperature at 40 °C and flow rate of 1.0 mL·min<sup>-1</sup>. Four parts of *S. asper* under the optimal condition were shown in Fig. 1.

### 2.2. Identification of cardiac glycosides

Comparing leaf, root, stem and heartwood of *S. asper*, heartwood part was the most complicated part with numerous peaks, which was

chosen for further analysis. Chemical constituents of heartwood part of *S. asper* were identified by the developed HPLC-Q-TOF-MS method in positive ion modes. The total ion chromatograms (TIC) with numbered peaks are illustrated in Fig. 2. Twenty compounds of cardiac glycosides were identified or tentatively identified from *S. asper* (Table 1). Among them, several compounds were unambiguously identified by comparing with their reference standards on retention times and MS/MS data. Besides them, other compounds were tentatively inferred based on their fragmentation pathways and previous reports. All information about these compounds was shown in Fig. 3, Table 1 and Supplementary Data.

Cardiac glycosides are the major group of bioactive compounds from *S. asper*. The molecular weight of the compounds were determined by the predominant ion  $[\text{M} + \text{H}]^+$  and/or  $[\text{M} + \text{Na}]^+$  in full-scan mass spectra. Further structural information was obtained by referring to previous reports on fragmentation behaviors of cardiac glycosides.

Peak 1 exhibited sodiated molecule  $[\text{M} + \text{Na}]^+$  at  $m/z$  719. Comparing with a reference standard and literature, it was tentatively assigned as strophanthidin-3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 → 4)-6-deoxy- $\beta$ -D-allopyranoside [19]. In the positive ion mode of peak 1, the  $m/z$  405 ion  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_{12}\text{H}_{22}\text{O}_9]^+$  was produced by loss of 310 Da, corresponding to two rhamnose units. A product ion at  $m/z$  341  $[\text{M} + \text{H} - \text{C}_{12}\text{H}_{22}\text{O}_9 - \text{H}_2\text{O} - \text{CO}]^+$  was produced from  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_{12}\text{H}_{22}\text{O}_9]^+$ , losing two  $\text{H}_2\text{O}$  units and a CO unit. And also, a product ion at  $m/z$  261  $[\text{M} + \text{H} - \text{C}_{12}\text{H}_{21}\text{O}_9 - 2\text{H}_2\text{O} - \text{CO} - \text{HCHO} - \text{CH}_3\text{OH}]^+$  was produced from  $[\text{M} + \text{H} - \text{C}_{12}\text{H}_{21}\text{O}_9 - \text{H}_2\text{O} - \text{CO}]^+$ , losing a  $\text{H}_2\text{O}$  unit, a HCHO unit and a  $\text{CH}_3\text{OH}$  unit.

Based on retention times, mass spectrum of the reference standards and literature, peak 2, peak 4 and peak 7 were confirmed as glucostrebloside [20,21], strebloside [3,21], and glucokamaloside [20,21], respectively. Peak 2 exhibited  $[\text{M} + \text{Na}]^+$  ion at  $m/z$  763 and  $[\text{M} + \text{H} + \text{H}_2\text{O}]^+$  at  $m/z$  759. Fragment ion  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_{14}\text{H}_{26}\text{O}_{10}]^+$  at  $m/z$  405 due to a loss of a methylated rhamnose unit and a glucose unit. Product ion  $[\text{M} + \text{H} - \text{C}_{14}\text{H}_{26}\text{O}_{10}]^+$  at  $m/z$  387, product ion  $[\text{M} + \text{H} - \text{C}_{14}\text{H}_{26}\text{O}_{10} - \text{H}_2\text{O}]^+$  at  $m/z$  369, product ion  $[\text{M} + \text{H} - \text{C}_{14}\text{H}_{26}\text{O}_{10} - \text{H}_2\text{O} - \text{CO}]^+$  at  $m/z$  341 and product ion  $[\text{M} + \text{H} - \text{C}_{14}\text{H}_{26}\text{O}_{10} - 3\text{H}_2\text{O} - \text{CO}]^+$  at  $m/z$  305 were all produced from  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_{14}\text{H}_{26}\text{O}_{10}]^+$ , losing different units of  $\text{H}_2\text{O}$  and CO. Peak 4 exhibited  $[\text{M} + \text{Na}]^+$  ion at  $m/z$  601. Fragment ion  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_8\text{H}_{16}\text{O}_5]^+$  at  $m/z$  405 due to a loss of a methylated rhamnose unit. Product ion  $[\text{M} + \text{H} - \text{C}_8\text{H}_{16}\text{O}_5 - \text{CO}]^+$  at  $m/z$  359 was produced from  $[\text{M} + \text{H} + \text{H}_2\text{O} - \text{C}_8\text{H}_{16}\text{O}_5]^+$ , losing different a  $\text{H}_2\text{O}$  unit and a CO unit. Production  $[\text{M} + \text{H} - \text{C}_8\text{H}_{16}\text{O}_5 - \text{CO} - 2\text{H}_2\text{O}]^+$  at  $m/z$  323 was produced from  $[\text{M} + \text{H} - \text{C}_8\text{H}_{16}\text{O}_5 - \text{CO}]^+$ , losing different two  $\text{H}_2\text{O}$  units. Also, production  $[\text{M} + \text{H} - \text{C}_8\text{H}_{16}\text{O}_5 - 2\text{CO} - 2\text{H}_2\text{O} - \text{CH}_3\text{OH}]^+$  at  $m/z$  263 was produced from  $[\text{M} + \text{H} - \text{C}_8\text{H}_{16}\text{O}_5 - \text{CO} - 2\text{H}_2\text{O}]^+$ , losing different a CO unit and a  $\text{CH}_3\text{OH}$  unit. Peak 7 exhibited  $[\text{M} + \text{Na}]^+$  ion at  $m/z$

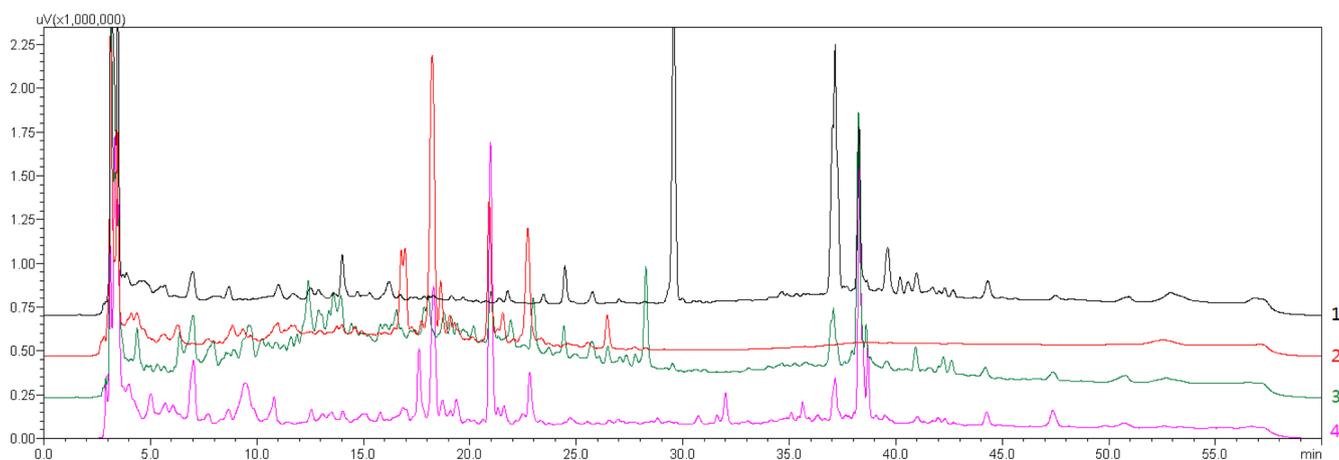


Fig. 1. HPLC chromatogram of four parts of *S. asper* (1-leaf, 2-root, 3-stem, 4-heartwood).

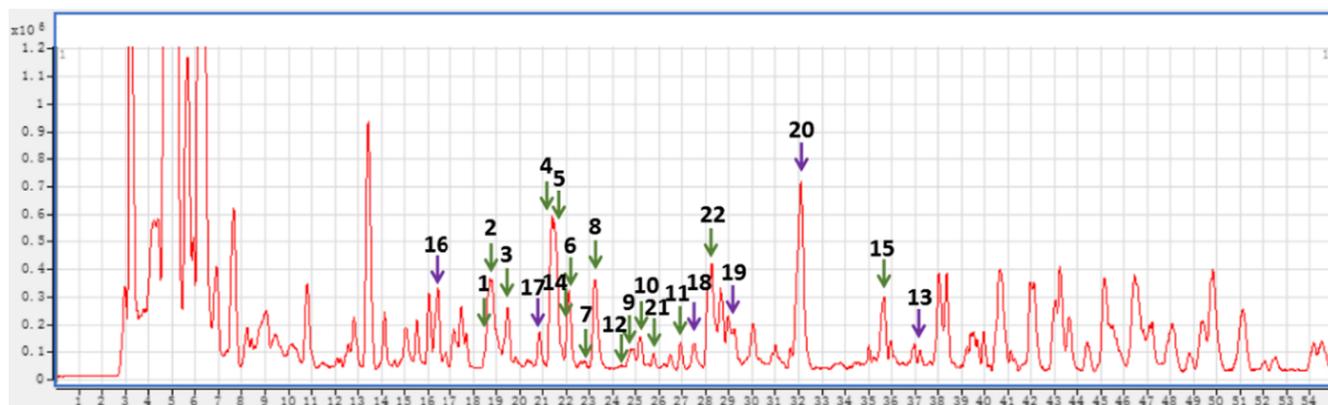


Fig. 2. The typical total ion chromatograms of *S. asper* acquired by HPLC-Q-TOF-MS in positive ion mode.

749, as well as  $[M + H + H_2O - C_{14}H_{26}O_{10}]^+$ , losing a glucose unit and a methylated rhamnose unit.

Peak 5, peak 8 and peak 11 were identified as mansonin-19-carboxylic acid [19], mansonin [3] and kamaloside [21,24] by comparing with the reference standards and literature. Peak 5 exhibited  $[M + Na]^+$  ion at  $m/z$  617. Fragment ion  $[M + H + H_2O - C_8H_{16}O_5]^+$  at  $m/z$  421 due to a loss of a methylated rhamnose unit. Product ion  $[M + H - C_8H_{16}O_5 - H_2O]^+$  at  $m/z$  385 and product ion  $[M + H - C_8H_{16}O_5 - 2H_2O]^+$  at  $m/z$  367 were all produced from  $[M + H + H_2O - C_8H_{16}O_5]^+$ , losing different units of  $H_2O$ . Product ion  $[M + H - C_8H_{16}O_5 - H_2O - CO_2]^+$  at  $m/z$  341, product ion  $[M + H - C_8H_{16}O_5 - 2H_2O - CO_2]^+$  at  $m/z$  323, product ion  $[M + H - C_8H_{16}O_5 - 3H_2O - CO_2]^+$  at  $m/z$  305, product ion  $[M + H - C_8H_{16}O_5 - 3H_2O - 2CO_2]^+$  at  $m/z$  261 and product ion  $[M + H - C_8H_{16}O_5 - 3H_2O - 3CO_2]^+$  at  $m/z$  217 were all produced from the previous fragment ion, losing different units of  $H_2O$  and  $CO_2$ . Peak 8 showed  $[M + Na]^+$  ion at  $m/z$  601 in positive ion mode and exhibited product ion  $[M + H + H_2O - C_8H_{16}O_5]^+$  at  $m/z$  405. Also, product ion  $[M + H - C_8H_{16}O_5 - CO]^+$  at  $m/z$  359 was produced from  $[M + H + H_2O - C_8H_{16}O_5]^+$ , losing a  $H_2O$  unit and a  $CO$  unit. Peak 11 exhibited  $[M + Na]^+$  ion at  $m/z$  587, producing  $[M + Na - 2CH_3OH]^+$  ion at  $m/z$  523, which lost two  $CH_3OH$  units. It produced a  $[M + H + H_2O - C_8H_{16}O_5]^+$  ion at  $m/z$  391. Product ion  $[M + H + 3H_2O - C_8H_{16}O_5]^+$  at  $m/z$  427 and product ion  $[M + H - H_2O - C_8H_{16}O_5]^+$  at  $m/z$  355 were all produced from the previous fragment ion, adding or losing different units of  $H_2O$ . Product ion  $[M + H - C_8H_{16}O_5 - H_2O - C_3H_7 - 3OH]^+$  at  $m/z$  261 was produced from the previous fragment ion, losing different units of  $C_3H_7$  and  $OH$ .

Comparing with the literature, peak 3 and peak 6 were tentatively identified as glucogitodimethoside [20,21] and gitodimethoside [21,22]. Both of them exhibited  $[M + Na]^+$  at  $m/z$  765 and 603, respectively. Peak 3 exhibited fragment ion  $[M + H + H_2O - C_6H_{12}O_6]^+$  at  $m/z$  581 due to a loss of a glucose unit. And both of them exhibited fragment ion at  $m/z$  389, referring to  $[M + H + H_2O - C_{14}H_{26}O_{11} - H_2]^+$  and  $[M + H + H_2O - C_8H_{16}O_6 - H_2]^+$ , respectively, with the former losing a glucose unit and a methylated glucose unit. Product ion  $[M + H - C_{14}H_{26}O_{11} - H_2]^+$  at  $m/z$  371 was exhibited from  $[M + H + H_2O - C_{14}H_{26}O_{11} - H_2]^+$ , losing a  $H_2O$  unit. It also exhibited fragment ion  $[M + H + H_2O - C_{14}H_{26}O_{11} - CH_3OH]^+$  at  $m/z$  359, which lost a glucose unit, a methylated glucose unit and a  $CH_3OH$  unit. Losing a  $H_2O$  unit subsequently, it produced fragment ion  $[M + H - C_{14}H_{26}O_{11} - CH_3OH]^+$  at  $m/z$  341. Peak 6 exhibited fragment ion  $[M + H - C_8H_{16}O_6 - H_2]^+$  at  $m/z$  371, produced from  $[M + H + H_2O - C_8H_{16}O_6 - H_2]^+$ . It also exhibited fragment ion  $[M + H + H_2O - C_8H_{16}O_6 - CH_3OH]^+$  at  $m/z$  359, which lost a methylated glucose unit and a  $CH_3OH$  unit. Losing a  $H_2O$  unit subsequently, it produced fragment ion  $[M + H - C_8H_{16}O_6 - CH_3OH]^+$  at  $m/z$  341.

Peak 9 and peak 10 showed precursor ion  $[M + Na]^+$  at  $m/z$  559

and 573, respectively. Based on retention times and literature, peak 9 and peak 10 were confirmed as (+)-3-*O*- $\beta$ -D-fucopyranosylperiplogenin [23] and (+)-3'-*de-O*-methylkamaloside [23], that had the same basic structure. Both of them showed fragment ion at  $m/z$  391, losing the sugar moiety respectively. Peak 9 also showed  $[M + H]^+$  at  $m/z$  537. Fragment ion  $[M + H - C_6H_{12}O_5 - H_2O]^+$  at  $m/z$  355, fragment ion  $[M + H - C_6H_{12}O_5 - H_2O - C_3H_7]^+$  at  $m/z$  312, fragment ion  $[M + H - C_6H_{12}O_5 - H_2O - C_3H_7 - CO]^+$  at  $m/z$  284 and fragment ion  $[M + H - C_6H_{12}O_5 - 3H_2O - C_3H_7]^+$  at  $m/z$  276 were produced from  $[M + H]^+$ , losing different units of  $H_2O$ ,  $C_3H_7$  and  $CO$ . Also, peak 10 produced fragment ion  $[M + H + H_2O - C_7H_{14}O_5 - CH_3OH]^+$  at  $m/z$  359 and  $[M + H - C_7H_{14}O_5 - CH_3OH - C_2H_5]^+$  at  $m/z$  312, losing a methylated rhamnose unit, a  $CH_3OH$  unit and a  $C_2H_5$  unit.

Peak 12 was precisely characterized as 11,19-dihydroxydigitoxigenin-3-*O*- $\beta$ -D-(3'-*O*-methyl)-glucopyranoside [25,26] by comparing with reported reference. It showed precursor ion  $[M + Na]^+$  at  $m/z$  605 with product ion  $[M + H - 2H_2O - C_7H_{14}O_6]^+$  at  $m/z$  353,  $[M + H - 2H_2O - C_7H_{14}O_6 - 2CH_3OH - CO]^+$  at  $m/z$  261 and  $[M + H - 3H_2O - C_7H_{14}O_6 - 2CH_3OH - CO]^+$  at  $m/z$  243, losing a methylated glucose unit,  $CH_3OH$  and  $CO$  units.

Peak 13 showed  $[M + Na]^+$  ion at  $m/z$  589 in positive ion mode. It also presented fragment ion  $[M + H + H_2O - C_7H_{14}O_6 - CH_3OH]^+$  at  $m/z$  359 which was tentatively assigned as sarmethoside [20,21] based on MS/MS data from the literature, but still needs to be further confirmed by reference standard.

Peak 14 presented  $[M + H]^+$  ion at  $m/z$  553, producing the fragment ion  $[M + H - H_2O - OCH_3]^+$  at  $m/z$  504 and  $[M + H - H_2O - OCH_3 - 2OH]^+$  at  $m/z$  470. It also exhibited a fragment ion  $[M + Na + H_2O - C_6H_{12}O_5]^+$  at  $m/z$  428, losing a rhamnose unit, being tentatively characterized as strophanolloside [21].

Peak 15 showed precursor ion  $[M + Na]^+$  at  $m/z$  737 and  $[M + H]^+$  at  $m/z$  715 with product ion  $[M + Na - CO_2]^+$  at  $m/z$  693,  $[M + Na + H_2O - C_6H_{12}O_5 - 2H]^+$  at  $m/z$  589 and  $[M + H + H_2O - C_6H_{12}O_5 - 2H]^+$  at  $m/z$  567, losing a rhamnose unit. It also produced a ion  $[M + Na + 2H_2O - C_{12}H_{22}O_9]^+$  at  $m/z$  463, losing two rhamnose units.

Peak 16, 17 and 18 all exhibited precursor ion  $[M + Na]^+$  at  $m/z$  619, 573 and 593, respectively. Losing methylated rhamnose,  $CO$ ,  $OH$  and  $CH_2OH$  units, peak 16 showed product ions  $[M + H + H_2O - C_8H_{16}O_5 - 2CO]^+$  at  $m/z$  367,  $[M + H - C_8H_{16}O_5 - 2CO - 2OH]^+$  at  $m/z$  315 and  $[M + H - C_8H_{16}O_5 - 3CO - 2OH - CH_2OH]^+$  at  $m/z$  256, produced sequentially. Losing rhamnose,  $CO_2$  and  $CH_3OH$  units, peak 17 showed product ions  $[M + Na + H_2O - C_6H_{12}O_5]^+$  at  $m/z$  427 and  $[M + Na + H_2O - C_6H_{12}O_5 - CO_2 - CH_3OH]^+$  at  $m/z$  351, produced sequentially. Also, peak 18 exhibited precursor ion  $[M + H]^+$  at  $m/z$  571, after losing glucose,  $CH_3OH$ ,  $H_2O$  and  $CO$  units, it showed product ion  $[M + H - C_6H_{12}O_6 - CH_3OH]^+$  at  $m/z$  359 and

**Table 1**  
Identification of twenty compounds from *S. asper* by developed HPLC-Q-TOF-MS.

No.	t <sub>r</sub> (min)	Formula	[M + Na] <sup>+</sup> (m/z) Error (ppm)	[M + H] <sup>+</sup> (m/z) Error (ppm)	Fragment ions in positive (+) mode	Identification
1	18.472	C <sub>35</sub> H <sub>52</sub> O <sub>14</sub>	719.3231 (1.27)		719.3231 [M + Na] <sup>+</sup> 405.2266 [M + H + H <sub>2</sub> O-C <sub>12</sub> H <sub>22</sub> O <sub>9</sub> ] <sup>+</sup> 341.2083 [M + H-C <sub>12</sub> H <sub>22</sub> O <sub>9</sub> -H <sub>2</sub> O-CO] <sup>+</sup>	Strophanthidin-3-O-α-L-rhamnopyranosyl-(1 → 4)-6-deoxy-β-D-allopyranoside [19]
2	18.668	C <sub>37</sub> H <sub>56</sub> O <sub>15</sub>	763.3462 (1.05)		261.1303 [M + H-C <sub>12</sub> H <sub>22</sub> O <sub>9</sub> -2H <sub>2</sub> O-CO-HCHO-CH <sub>3</sub> OH] <sup>+</sup> 763.3462 [M + Na] <sup>+</sup> 759.3982 [M + H <sub>2</sub> O + H] <sup>+</sup> 405.2203 [M + H + H <sub>2</sub> O-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> ] <sup>+</sup> 387.2155 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> ] <sup>+</sup> 369.2023 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> -H <sub>2</sub> O] <sup>+</sup> 341.2081 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> -H <sub>2</sub> O-CO] <sup>+</sup> 305.1862 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> -3H <sub>2</sub> O-CO] <sup>+</sup>	Glucostreblside [20,21]
3	19.428	C <sub>37</sub> H <sub>58</sub> O <sub>15</sub>	765.3655 (1.18)		765.3655 [M + Na] <sup>+</sup> 581.3922 [M + H + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> ] <sup>+</sup> 389.2311 [M + H + H <sub>2</sub> O-C <sub>14</sub> H <sub>26</sub> O <sub>11</sub> -H <sub>2</sub> ] <sup>+</sup> 371.22 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>11</sub> -H <sub>2</sub> ] <sup>+</sup> 359.2194 [M + H + H <sub>2</sub> O-C <sub>14</sub> H <sub>26</sub> O <sub>11</sub> -CH <sub>3</sub> OH] <sup>+</sup> 341.2099 [M + H-C <sub>14</sub> H <sub>26</sub> O <sub>11</sub> -CH <sub>3</sub> OH] <sup>+</sup>	Glucogitodimethoside [20,21]
4	21.53	C <sub>31</sub> H <sub>46</sub> O <sub>10</sub>	601.2967 (1.77)		601.2967 [M + Na] <sup>+</sup> 405.2263 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 359.2201 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -CO] <sup>+</sup> 323.1944 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -CO-2H <sub>2</sub> O] <sup>+</sup> 263.1732 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -2CO-2H <sub>2</sub> O-CH <sub>3</sub> OH] <sup>+</sup>	Streblside [3,21]
5	21.783	C <sub>31</sub> H <sub>46</sub> O <sub>11</sub>	617.2912 (1.18)		617.2912 [M + Na] <sup>+</sup> 421.2211 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 385.1999 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -H <sub>2</sub> O] <sup>+</sup> 367.1953 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -2H <sub>2</sub> O] <sup>+</sup> 341.2088 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -H <sub>2</sub> O-CO <sub>2</sub> ] <sup>+</sup> 323.1965 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -2H <sub>2</sub> O-CO <sub>2</sub> ] <sup>+</sup> 305.1580 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -3H <sub>2</sub> O-CO <sub>2</sub> ] <sup>+</sup> 261.1314 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -3H <sub>2</sub> O-2CO <sub>2</sub> ] <sup>+</sup> 217.1046 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -3H <sub>2</sub> O-3CO <sub>2</sub> ] <sup>+</sup>	Mansonin-19-carboxylic acid [19]
6	22.1	C <sub>31</sub> H <sub>48</sub> O <sub>10</sub>	603.3125 (1.57)		603.3125 [M + Na] <sup>+</sup> 389.2315 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>6</sub> -H <sub>2</sub> ] <sup>+</sup> 371.2211 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>6</sub> -H <sub>2</sub> ] <sup>+</sup> 359.2204 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>6</sub> -CH <sub>3</sub> OH] <sup>+</sup> 341.2097 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>6</sub> -CH <sub>3</sub> OH] <sup>+</sup>	Gitodimethoside [21,22]
7	22.981	C <sub>37</sub> H <sub>58</sub> O <sub>14</sub>	749.3703 (-0.57)		749.3703 [M + Na] <sup>+</sup> 391.0524 [M + H + H <sub>2</sub> O-C <sub>14</sub> H <sub>26</sub> O <sub>10</sub> ] <sup>+</sup>	Glucokamaloside [20,21]
8	23.24	C <sub>31</sub> H <sub>46</sub> O <sub>10</sub>	601.2967 (0.85)		601.2967 [M + Na] <sup>+</sup> 405.2257 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 359.2203 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -CO] <sup>+</sup>	Mansonin [3]
9	25.014	C <sub>29</sub> H <sub>44</sub> O <sub>9</sub>	559.2875 (0.53)	537.3036 (0.27)	559.2875 [M + Na] <sup>+</sup> 537.3036 [M + H] <sup>+</sup> 391.1583 [M + H + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> ] <sup>+</sup> 355.2249 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -H <sub>2</sub> O] <sup>+</sup> 312.2505 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -H <sub>2</sub> O-C <sub>3</sub> H <sub>7</sub> ] <sup>+</sup> 284.1282 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -H <sub>2</sub> O-C <sub>3</sub> H <sub>7</sub> -CO] <sup>+</sup> 276.1674 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -3H <sub>2</sub> O-C <sub>3</sub> H <sub>7</sub> ] <sup>+</sup>	(+)-3-O-β-D-fucopyranosylperiplogenin [23]
10	25.199	C <sub>30</sub> H <sub>46</sub> O <sub>9</sub>	573.3025 (1.63)		573.3025 [M + Na] <sup>+</sup> 359.2203 [M + H + H <sub>2</sub> O-C <sub>7</sub> H <sub>14</sub> O <sub>5</sub> -CH <sub>3</sub> OH] <sup>+</sup> 312.2507 [M + H-C <sub>7</sub> H <sub>14</sub> O <sub>5</sub> -CH <sub>3</sub> OH-C <sub>2</sub> H <sub>5</sub> ] <sup>+</sup>	(+)-3'-de-O-methylkamaloside [23]
11	26.909	C <sub>31</sub> H <sub>48</sub> O <sub>9</sub>	587.3184 (1.15)		587.3184 [M + Na] <sup>+</sup> 523.3090 [M + Na-2CH <sub>3</sub> OH] <sup>+</sup> 427.2658 [M + H + 3H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 391.2439 [M + H + H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 355.2255 [M + H-H <sub>2</sub> O-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> ] <sup>+</sup> 261.1305 [M + H-C <sub>8</sub> H <sub>16</sub> O <sub>5</sub> -H <sub>2</sub> O-C <sub>3</sub> H <sub>7</sub> -3OH] <sup>+</sup>	Kamaloside [21,24]
12	24.751	C <sub>30</sub> H <sub>46</sub> O <sub>11</sub>	605.3283 (1.39)		605.3283 [M + Na] <sup>+</sup> 353.2286 [M + H-2H <sub>2</sub> O-C <sub>7</sub> H <sub>14</sub> O <sub>6</sub> ] <sup>+</sup> 261.1296 [M + H-2H <sub>2</sub> O-C <sub>7</sub> H <sub>14</sub> O <sub>6</sub> -2CH <sub>3</sub> OH-CO] <sup>+</sup> 243.1809 [M + H-3H <sub>2</sub> O-C <sub>7</sub> H <sub>14</sub> O <sub>6</sub> -2CH <sub>3</sub> OH-CO] <sup>+</sup>	11,19-dihydroxydigitoxigenin-3-O-β-D-(3'-O-methyl)-glucopyranoside [25,26]
13	22.008	C <sub>30</sub> H <sub>46</sub> O <sub>10</sub>	589.2970 (1.40)		589.2970 [M + Na] <sup>+</sup> 359.2211 [M + H + H <sub>2</sub> O-C <sub>7</sub> H <sub>14</sub> O <sub>6</sub> -CH <sub>3</sub> OH] <sup>+</sup>	Sarmethoside [20,21]
14	35.979	C <sub>29</sub> H <sub>44</sub> O <sub>10</sub>		553.3003 (0.43)	553.3003 [M + H] <sup>+</sup> 504.3669 [M + H-H <sub>2</sub> O-OCH <sub>3</sub> ] <sup>+</sup> 470.3826 [M + H-H <sub>2</sub> O-OCH <sub>3</sub> -2OH] <sup>+</sup> 428.3355 [M + Na + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> ] <sup>+</sup>	Strophanolloside [21]
15	16.761	C <sub>35</sub> H <sub>54</sub> O <sub>15</sub>	737.3341 (0.42)		737.3341 [M + Na] <sup>+</sup> 715.3342 [M + H] <sup>+</sup>	

(continued on next page)

Table 1 (continued)

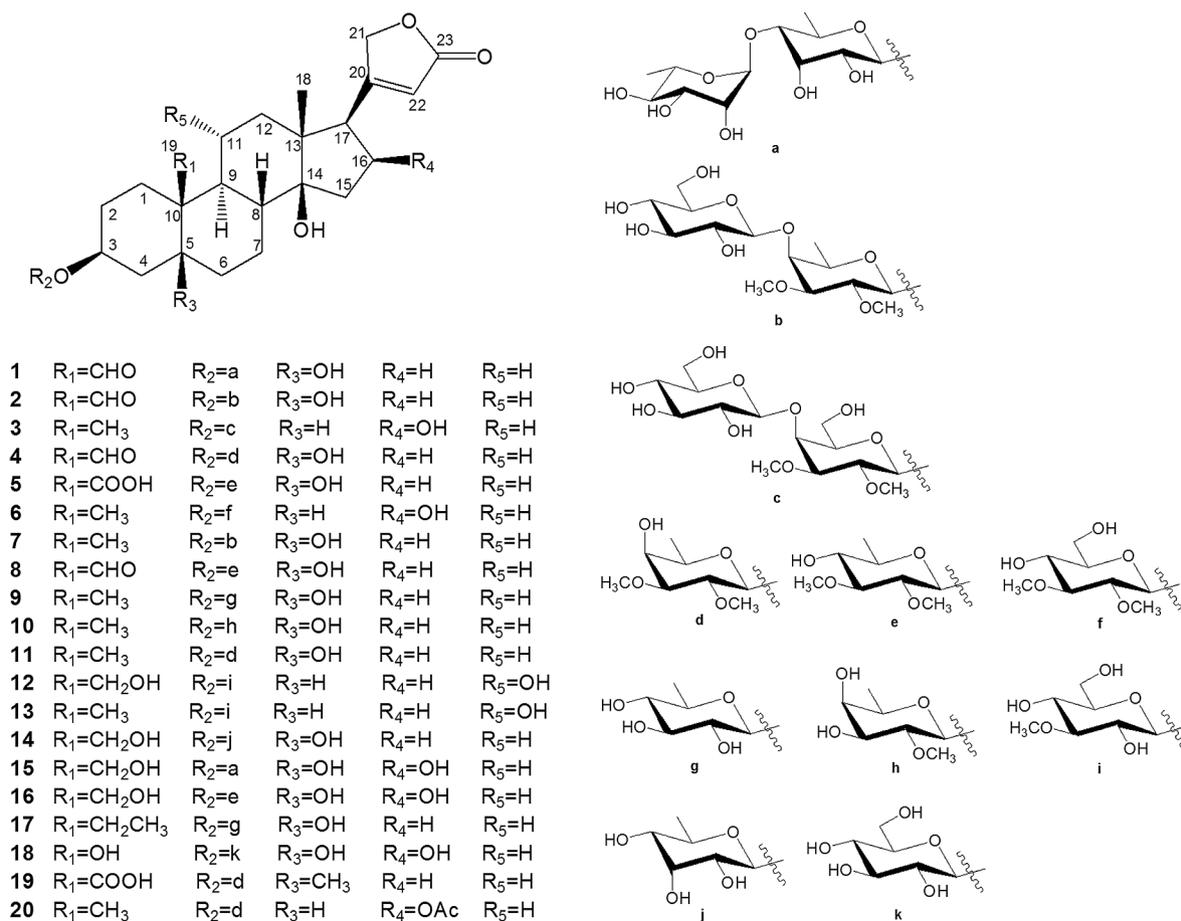
No.	t <sub>R</sub> (min)	Formula	[M + Na] <sup>+</sup> (m/z) Error (ppm)	[M + H] <sup>+</sup> (m/z) Error (ppm)	Fragment ions in positive (+) mode	Identification
16	20.864	C <sub>31</sub> H <sub>48</sub> O <sub>11</sub>	619.3089 (-0.45)		693.3565 [M + Na-CO <sub>2</sub> ] <sup>+</sup>	
					589.2451 [M + Na + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -2H] <sup>+</sup>	
					567.0332 [M + H + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -2H] <sup>+</sup>	
					463.7929 [M + Na + 2H <sub>2</sub> O-C <sub>12</sub> H <sub>22</sub> O <sub>9</sub> ] <sup>+</sup>	
					619.3089 [M + Na] <sup>+</sup>	
					367.2078 [M + H + H <sub>2</sub> O-C <sub>6</sub> H <sub>16</sub> O <sub>5</sub> -2CO] <sup>+</sup>	
17	27.490	C <sub>30</sub> H <sub>46</sub> O <sub>9</sub>	573.3021 (0.59)		315.2058 [M + H-C <sub>6</sub> H <sub>16</sub> O <sub>5</sub> -2CO-2OH] <sup>+</sup>	
					256.1332 [M + H-C <sub>6</sub> H <sub>16</sub> O <sub>5</sub> -3CO-2OH-CH <sub>2</sub> OH] <sup>+</sup>	
					573.3012 [M + Na] <sup>+</sup>	
					427.2655 [M + Na + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> ] <sup>+</sup>	
18	32.002	C <sub>28</sub> H <sub>42</sub> O <sub>12</sub>	593.2609 (-1.16)		351.2133 [M + Na + H <sub>2</sub> O-C <sub>6</sub> H <sub>12</sub> O <sub>5</sub> -CO <sub>2</sub> -CH <sub>3</sub> OH] <sup>+</sup>	
					593.2609 [M + Na] <sup>+</sup>	
					571.2793 [M + H] <sup>+</sup>	
					359.2313 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> -CH <sub>3</sub> OH] <sup>+</sup>	
					345.2163 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> -H <sub>2</sub> O-CO] <sup>+</sup>	
19	25.774	C <sub>32</sub> H <sub>48</sub> O <sub>10</sub>	615.3111 (1.53)		308.1243 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> -CH <sub>3</sub> OH-3OH] <sup>+</sup>	
					286.1412 [M + H-C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> -H <sub>2</sub> O-2CO-CH <sub>2</sub> OH] <sup>+</sup>	
					615.3111 [M + Na] <sup>+</sup>	
					515.2804 [M + H-HCOOH-CH <sub>3</sub> OH] <sup>+</sup>	
					351.2137 [M + H-C <sub>6</sub> H <sub>16</sub> O <sub>5</sub> -H <sub>2</sub> O-CH <sub>3</sub> OH] <sup>+</sup>	
20	28.219	C <sub>33</sub> H <sub>50</sub> O <sub>10</sub>	629.3296 (-1.24)		629.3296 [M + Na] <sup>+</sup>	
					353.2289 [M + H + H <sub>2</sub> O-C <sub>6</sub> H <sub>16</sub> O <sub>5</sub> -CH <sub>3</sub> OH-OCH <sub>3</sub> -OH] <sup>+</sup>	

[M + H-C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>-H<sub>2</sub>O-CO]<sup>+</sup> at m/z 345. After losing three OH units, [M + H-C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>-CH<sub>3</sub>OH]<sup>+</sup> at m/z 359 produced [M + H-C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>-CH<sub>3</sub>OH-3OH]<sup>+</sup> at m/z 308. Meanwhile, after losing CO and CH<sub>2</sub>OH units, [M + H-C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>-H<sub>2</sub>O-CO]<sup>+</sup> at m/z 345 produced [M + H-C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>-H<sub>2</sub>O-2CO-CH<sub>2</sub>OH]<sup>+</sup> at m/z 286.

Furthermore, peak 19 showed [M + Na]<sup>+</sup> at m/z 615. After

losing CH<sub>3</sub>OH and HCOOH or methylated rhamnose units, it produced [M + H-HCOOH-CH<sub>3</sub>OH]<sup>+</sup> at m/z 515 and [M + H-C<sub>6</sub>H<sub>16</sub>O<sub>5</sub>-H<sub>2</sub>O-CH<sub>3</sub>OH]<sup>+</sup> at m/z 351. Peak 20 showed [M + Na]<sup>+</sup> at m/z 629. Lost a series of units, it produced [M + H + H<sub>2</sub>O-C<sub>6</sub>H<sub>16</sub>O<sub>5</sub>-CH<sub>3</sub>OH-OCH<sub>3</sub>-OH]<sup>+</sup> at m/z 353.

Peak 15, 16, 17, 18, 19 and 20 were only inferred by fragment ions

Fig. 3. Twenty compounds from *S. asper* identified by developed HPLC-Q-TOF-MS.

**Table 2**  
IC<sub>50</sub> values of compounds on Na<sup>+</sup>,K<sup>+</sup>-ATPase.

Compounds	IC <sub>50</sub> (Mean ± SD, μM) <sup>a</sup>
1	7.55 ± 1.09
2	11.12 ± 1.04
4	10.16 ± 1.60
5	> 50
8	13.60 ± 1.24
11	> 50
Ouabain <sup>b</sup>	7.09 ± 0.04

<sup>a</sup> IC<sub>50</sub> value was obtained on the basis of triplicate assay results.

<sup>b</sup> Reference compound.

and the principle of producing fragment ions, without standards or literature. The index names were not yet assigned, which should be confirmed in later research.

### 2.3. Inhibitory effects of cardiac glycosides on Na<sup>+</sup>,K<sup>+</sup>-ATPase

Na<sup>+</sup>,K<sup>+</sup>-ATPase, in parallel to its transport function, serves as a receptor in the ouabain signaling pathway. The inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity could activate Src protein and indirectly decreased the expression of Akt protein, as well as influencing the apoptotic protein such as caspases and Bcl-2 with NF-κB transcription factor. Therefore, it could induce apoptosis and act as a tumor suppressor [27]. The chemical structure of cardiac glycosides from *Streblus asper* are similar to that of other cardiac glycosides, such as ouabain, although it possesses a relatively unusual formyl group at C-10. So, the inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by cardiac glycosides were determined, and ouabain

was used as a reference compound.

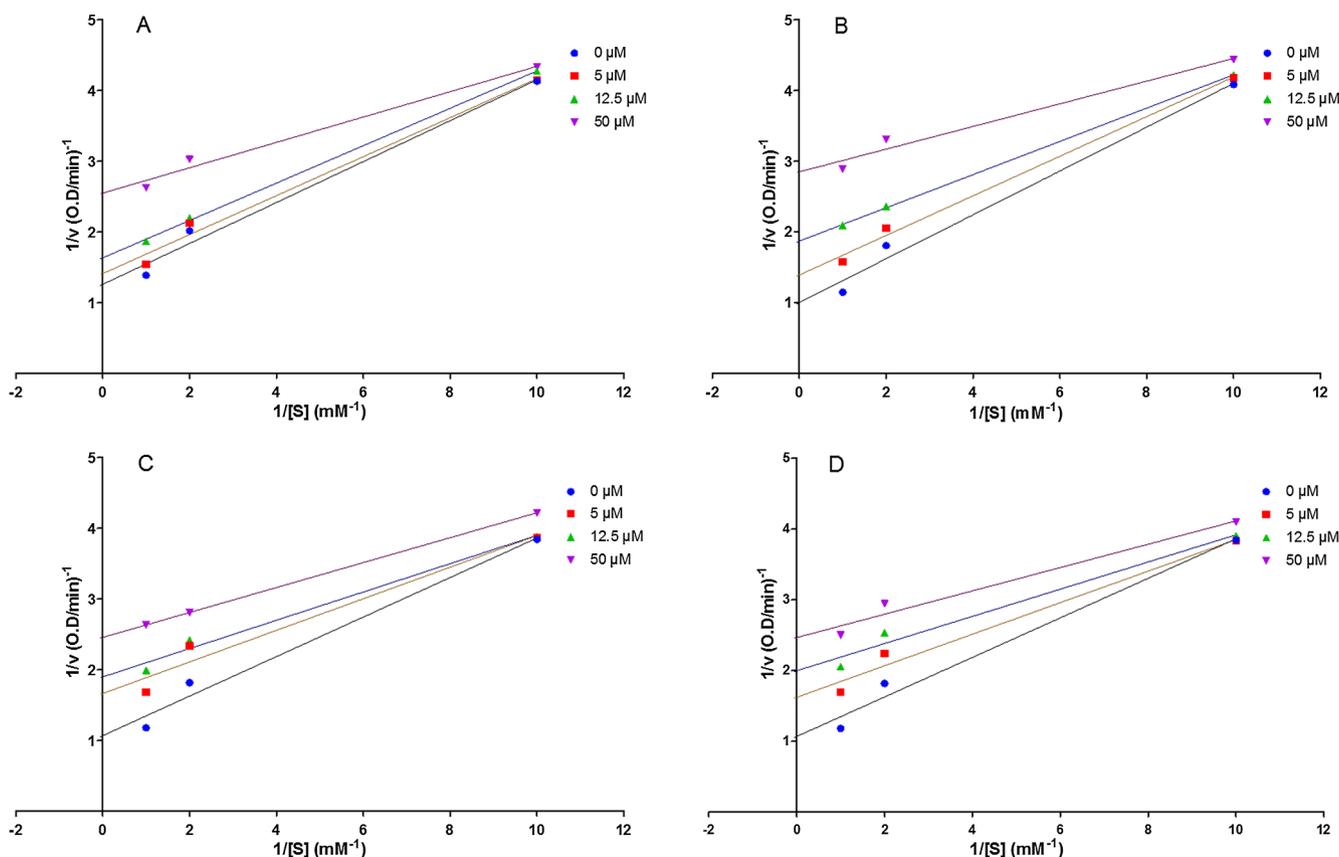
Compounds 1, 2, 4, 5, 8 and 11 were isolated from *S. asper*, which were chosen to determine the inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. As shown in Table 2, compounds 1, 2, 4 and 8 could inhibit Na<sup>+</sup>,K<sup>+</sup>-ATPase activity efficiently and also be chosen in quantitative determination.

### 2.4. Kinetic analysis of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibition

Kinetic assays were repeated in the presence of different concentrations of compounds 1, 2, 4 and 8 to further characterize inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase. The Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitory properties of isolated compounds were also modelled using double reciprocal plots, Lineweaver-Burk plots. This analysis showed that V<sub>max</sub> decreased with the decreasing of K<sub>m</sub> in presence of increasing concentrations of all inhibitors (1, 2, 4 and 8), shown in Fig. 4 and Table 3. Whereas 1/V<sub>max</sub> increased, the x-intercept (1/K<sub>m</sub>) was also affected by inhibitor concentrations, which increased significantly. These features indicated that compounds 1, 2, 4 and 8 exhibited anticompetitive inhibitory characteristics towards Na<sup>+</sup>,K<sup>+</sup>-ATPase, in which the inhibitors combined with the enzyme-substrate complex but not with the enzyme. In this study, the inhibitors were compound 1, 2, 4 and 8, the enzyme was Na<sup>+</sup>,K<sup>+</sup>-ATPase and the substrate was ATP, which exhibited anticompetitive inhibitory characteristics.

### 2.5. Molecular docking studies

Na<sup>+</sup>,K<sup>+</sup>-ATPase is a receptor in the ouabain signaling pathway. The inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity could activate Src protein and decreased the expression of Akt protein. Meanwhile, the apoptotic protein such as caspases and Bcl-2 with NF-κB transcription factor were also influenced [27]. Compound 4 had the potent of inhibiting Na<sup>+</sup>,K<sup>+</sup>-



**Fig. 4.** Lineweaver-Burk plots of different compounds for the inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase. (A–D) Lineweaver-Burk plots of compounds 1, 2, 4 and 8 for the inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase.

**Table 3**  
Effect of different concentrations of different compounds on  $V_{\max}$  and  $K_m$ .

Compounds	Concentration ( $\mu\text{M}$ )	$V_{\max}^a$	$K_m^b$
1	0	1.43	0.68
	5.0	0.77	0.25
	12.5	0.54	0.13
	50.0	0.35	0.06
2	0	0.95	0.37
	5.0	0.79	0.27
	12.5	0.62	0.17
	50.0	0.40	0.07
4	0	1.25	0.53
	5.0	0.66	0.19
	12.5	0.54	0.12
	50.0	0.41	0.07
8	0	1.25	0.53
	5.0	0.66	0.17
	12.5	0.52	0.11
	50.0	0.41	0.07

<sup>a</sup>  $V_{\max}$  value was obtained on the basis of triplicate assay results.

<sup>b</sup>  $K_m$  value was obtained on the basis of triplicate assay results.

ATPase, and the molecular docking studies showed it could reasonably bind to the active sites of  $\text{Na}^+, \text{K}^+$ -ATPase with binding energy  $-8.15 \text{ kcal}\cdot\text{mol}^{-1}$ . The details were depicted in Fig. 5, the hydroxyl and carbonyl of compound 4 inserted into the catalytic domains of  $\text{Na}^+, \text{K}^+$ -ATPase, Trp891 and Gly29, respectively. The binding modes indicated that the hydroxyl group at C-17 and the carbonyl group at C-23 played important roles in the binding of the ligand to the protein. Overall, compound 4 could be used as a potential ligand for  $\text{Na}^+, \text{K}^+$ -ATPase in the energy view.

## 2.6. Method validation

The method validation was carried out as shown in Table 4, the proposed HPLC method for quantification of four compounds (1, 2, 4 and 8) was validated. Specificity was evaluated by comparing chromatogram of blank solvent with chromatograms of mixed standard solution and sample test solution, shown in Supplementary Data. Under the developed conditions, blank solvent did not interfere with the

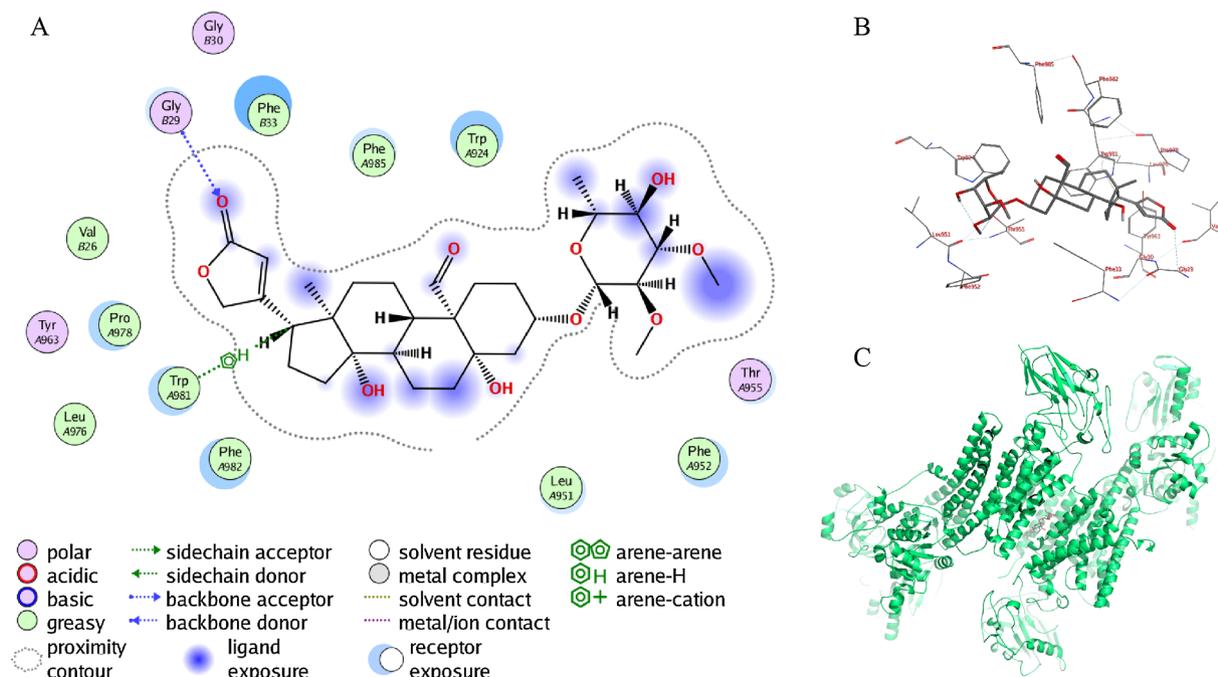
determination of these four compounds in the samples. A series of mixed standard solutions with five appropriate concentrations were prepared for the assessment of linearity. All the calibration curves showed good linearity ( $r^2 \geq 0.999$ ) within the test range. The LOD and LOQ were determined at S/N ratios of 3 and 10, respectively. The LODs and LOQs of these four compounds were in the range of 0.06–2.50 and 0.13–12.50  $\mu\text{g mL}^{-1}$ , respectively. The method precision was based on analysis of one sample solution for six replicates respectively. Six independently prepared samples were assessed for the repeatability test. A single sample solution was analyzed at 0, 2, 4, 8, 12 and 24 h to assess the stability. The RSD values precision, repeatability and stability of the four compounds were all lower than 3% (Table 4). The recovery was evaluated by assessing the accuracy of this developed method. The accurate amounts of three standard solutions were added into 0.25 g of *S. asper* powder and then processed and analyzed by the developed method. An unspiked sample (0.25 g) was also prepared and analyzed simultaneously for comparison. Six replicates were prepared and the mean recoveries were calculated. As shown in Table 4, all the mean recoveries were between 90% and 110% and the RSD values were all less than 2%. The results indicated that the developed method was validated and applicable for *S. asper* sample analysis. All tests described above were carried out on one sample from Thailand.

## 2.7. Quantitative application

The results in Table 5 showed that samples collected from different parts of *S. asper* had different contents of these four compounds, which would indicate various qualities and bioactivities of this herb. Compound 2 showed a high content in the heartwood of *S. asper*. Compound 1 showed a significant high content in the root of *S. asper*. As shown in Table 5, compound 4 was also the predominant cardiac glycoside, which was sufficient in root, stem and heartwood. Compound 8 was also a typical compound, which was abundant in root and stem. As a result, four compounds could be undoubtedly determined to comprehensively evaluate the quality of *S. asper*.

## 2.8. Similarity analysis (SA)

As revealed by Fig. 6 and Table 6, samples from different parts of *S.*



**Fig. 5.** Molecular docking binding mode of compound 4 in  $\text{Na}^+, \text{K}^+$ -ATPase. (A) 2D docking mode. (B) Analysis of binding interaction. (C) 3D docking mode.

**Table 4**  
Method validation for quantification of four compounds in *S. asper*.

Compounds	Calibration curves <sup>a</sup>	Linear range (mg·mL <sup>-1</sup> )	r <sup>2</sup>	LOD (µg·mL <sup>-1</sup> )	LOQ (µg·mL <sup>-1</sup> )	Precision (RSD, %; n = 6)	Repeatability (RSD, %; n = 6)	Stability (RSD, %; n = 6)	Recovery (%; n = 6)		RSD (%)		
									Spiked (mg)	Original (mg)		Detected (mg)	Recovery <sup>b</sup> (%)
1	Y = 2 × 10 <sup>7</sup> X - 140.7	0.03–0.25	0.9996	0.06	0.13	1.48	2.20	2.12	0.0619	0.0620	0.1237	99.68	1.35
2	Y = 2 × 10 <sup>7</sup> X - 1368.9	0.01–0.10	0.9995	0.15	0.45	1.05	1.08	0.95	0.0312	0.0310	0.0620	99.35	1.07
4	Y = 3 × 10 <sup>7</sup> X + 55104	0.05–0.36	0.9997	0.45	0.90	1.37	1.22	1.56	0.0738	0.0740	0.1476	99.73	1.72
8	Y = 3 × 10 <sup>7</sup> X - 14,547	0.01–0.10	0.9991	2.50	12.50	1.32	0.63	0.77	0.0171	0.0170	0.0326	91.23	1.59

<sup>a</sup> Y, peak area; X, concentration of each compound (mg·mL<sup>-1</sup>).

<sup>b</sup> Recovery (%) = 100 × (Detected - Original)/Spiked.

**Table 5**Contents of four compounds in different parts of *S. asper*.

Sample	Content (mg/g) <sup>a</sup>			
	1	2	4	8
Leaf	0.03	0.01	0.04	0.05
Root	1.44	0.03	0.26	0.56
Stem	0.15	0.05	0.09	0.29
Heartwood	0.25	0.12	0.30	0.25

<sup>a</sup> Content value was obtained on the basis of triplicate assay results.

*asper* had relatively low similarities (less than 0.8), which indicated that quality consistency of *S. asper* produced was different among different parts.

### 2.9. Principle compounds analysis (PCA)

Fig. 7 showed PCA scree plot of different parts of *S. asper*. The first two PCs, PC1 (compound 1) and PC2 (compound 4) with 94.25% of cumulative variance were major contributors to discrimination. The twenty-four samples of four different parts of *S. asper* can be classified into four groups. Group 1 contained leaf of *S. asper*. Group 2 contained heartwood of *S. asper*. Group 3 contained stem of *S. asper*. Group 4 contained root of *S. asper*. Above all, results of SA and PCA were consistent.

### 3. Conclusions

In this study, the enzyme kinetics and molecular docking approaches were used to explore the inhibitory activity and combination modes of structurally related cardiac glycosides on Na<sup>+</sup>,K<sup>+</sup>-ATPase. Among which, the contents of compounds 1, 2, 4 and 8 which exhibited obvious activities of inhibiting Na<sup>+</sup>,K<sup>+</sup>-ATPase were extremely different in four parts of *S. asper*, providing a reference for determination of effective part and administered dosage, as well as further study of pharmacological activity. Furthermore, enzyme kinetics showed anti-competitive inhibitory characteristics towards Na<sup>+</sup>,K<sup>+</sup>-ATPase, in which the inhibitors combined with the enzyme-substrate complex but not with the enzyme. Meanwhile, molecular docking studies showed that cardiac glycosides could appropriately bind to the active sites of Na<sup>+</sup>,K<sup>+</sup>-ATPase with hydroxyl at C-17 and carbonyl at C-23 of compound 4 inserting into the catalytic domains of Na<sup>+</sup>,K<sup>+</sup>-ATPase, Trp891 and Gly29, respectively, which have potential for the development of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibitor drug candidate.

### 4. Materials and methods

#### 4.1. Chemicals and reagents

HPLC-grade methanol was purchased from Tedia (Fairfield, OH). Other reagents with analytical grade were provided from Nanjing Chemical Reagent Corp. (Nanjing, China). High purity water for HPLC was purified using a Milli Q-Plus system (Millipore, Bedford, MA).

Standards of strophanthidin-3-O- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  4)-6-deoxy- $\beta$ -D-allopyranoside (1), glucostreblsode (2), streblsode (4), mansonin-19-carboxylic acid (5), mansonin (8) and kamaloslode (11) were obtained from *S. asper* (Supplementary Data). The purities of these standards were all over 98%.

#### 4.2. Plant materials and preparation of sample solutions

The root, stem, leaf and heartwood of *S. asper* were collected from Chiang Mai, Thailand in October of 2015, which were identified by Prof. Dr Jie Zhang. A voucher specimen (No. 20150060) was deposited in the Department of Natural Medicinal Chemistry, China

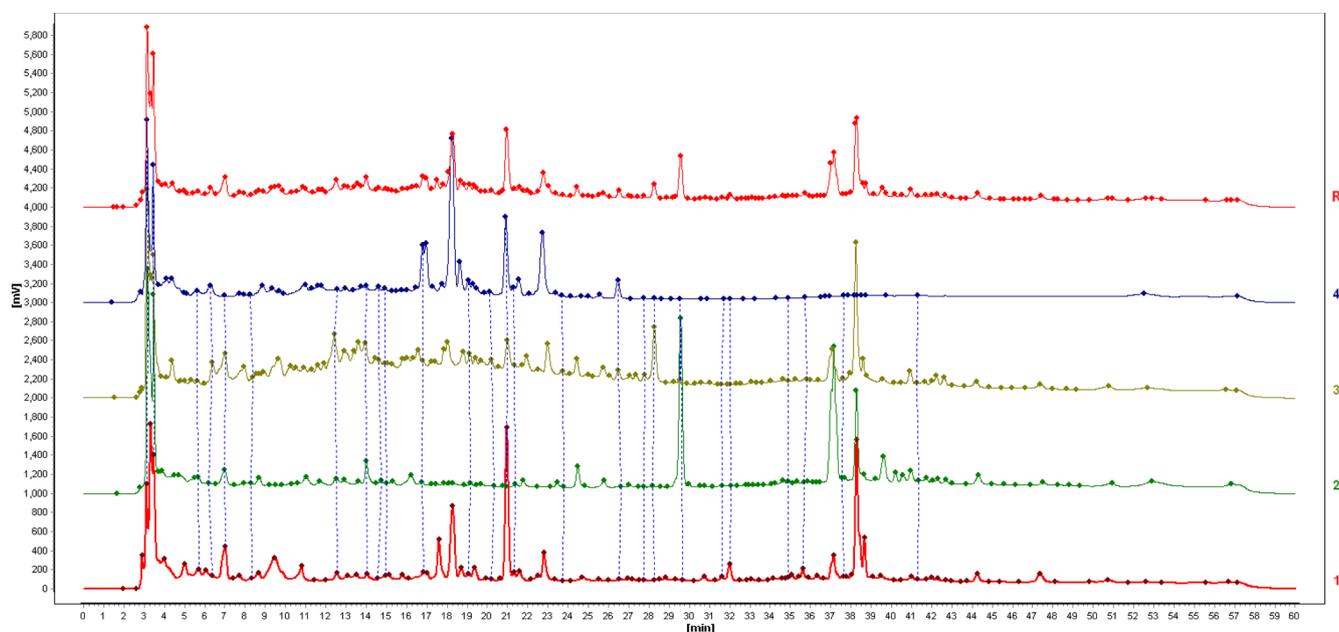


Fig. 6. The chromatographic fingerprints for the four parts of *S. asper*. Reference (R) was produced by the median of four chromatograms (1-heartwood, 2-leaf, 3-stem, 4-root, R-reference).

**Table 6**  
Similarity analysis of different parts of *S. asper*.

	Heartwood	Leaf	Stem	Root	Reference
Heartwood	1				
Leaf	0.43	1			
Stem	0.43	0.47	1		
Root	0.57	0.40	0.44	1	
Reference	0.76	0.75	0.81	0.74	1

Pharmaceutical University, Nanjing, China.

All parts were washed, air-dried and pulverized, then extracted with methanol ultrasonically for four times, two hours each. The methanol extracts were completely dried under reduced pressure (0.8 MPa) to get defatted methanol extracts (yield: 6.7%, 19.0%, 20.0% and 7.2%, respectively, w/w).

Accurately weighed the extracts of root, stem, leaf and heartwood,

then extracted ultrasonically with methanol at room temperature for 30 min. Each extracted solution was then centrifuged at 16,000 rpm for 5 min and each supernatant solution was filtered through a 0.22  $\mu\text{m}$  membrane before HPLC analysis. Each concentration was 0.50  $\text{g}\cdot\text{mL}^{-1}$ , calculated as the original plant.

Each accurately weighed reference compounds (1, 2, 4 and 8) was dissolved and diluted with methanol to obtain a series of stock standard solutions, each terminal concentration was 1.00  $\text{mg}\cdot\text{mL}^{-1}$ . A mixed stock standard solution containing four standards was prepared by adding and diluting each stock standard solution with methanol to a working standard solution, the terminal concentration of each was 0.13, 0.06, 0.18 and 0.05  $\text{mg}\cdot\text{mL}^{-1}$ , respectively.

#### 4.3. Chromatographic conditions

HPLC analysis was acquired on a Shimadzu series 2010 HPLC instrument (Shimadzu Corp., Kyoto, Japan) equipped with a quaternary pump, an online vacuum degasser, a column compartment, an

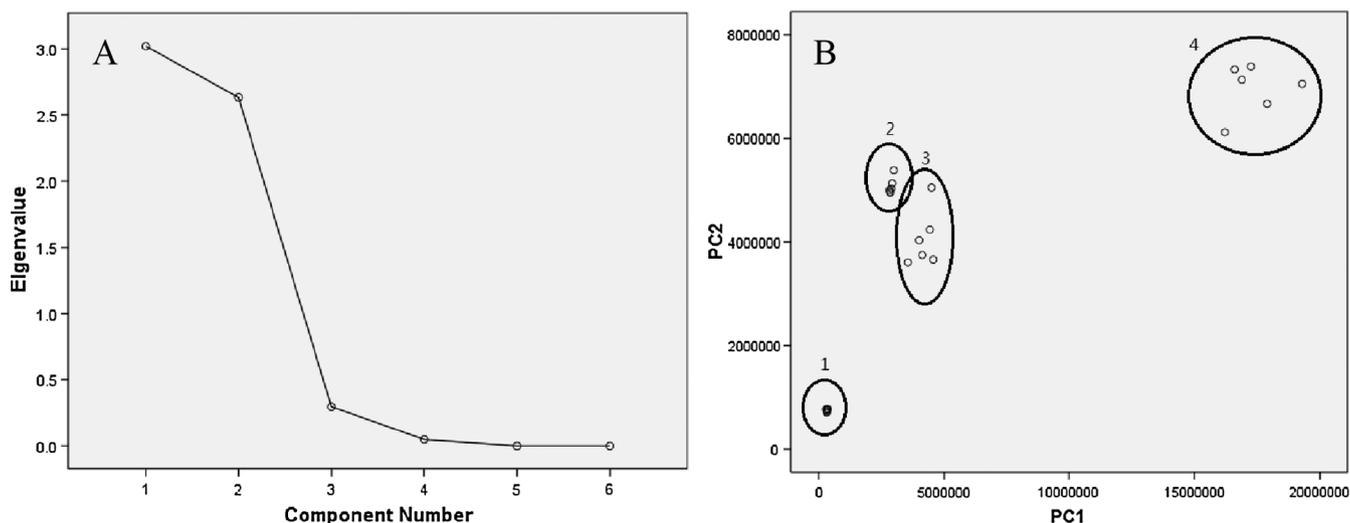


Fig. 7. Principle compounds analysis. (A) Scree plot. (B) PCA for the different parts of *S. asper* (1-leaf, 2-heartwood, 3-stem, 4-root).

autosampler and a UV detector. All separations were conducted on Inertsil ODS-SP column (4.6 mm × 250 mm, 5 μm) from Jiangsu Hanbon Sci. & Tech. Corp. (Huaian, China) in gradient elution mode with a mobile phase consisted of methanol (A) and water (B). The flow rate was maintained at 1.0 mL·min<sup>-1</sup>. The gradient elution program for *S. asper* was carried out as follows: 0–10 min, 20–45% A; 10–13 min, 45–50% A; 13–28 min, 50–70% A; 28–34 min, 70–85% A; 34–55 min, isocratic 85% A. The column temperature was maintained at 40 °C, the injection volume was 20 μL and the UV detector was set at 220 nm. The data were processed with Shimadzu 2.0 ChemStation software (Shimadzu Corp., Kyoto, Japan).

#### 4.4. HPLC-Q-TOF-MS conditions

HPLC-Q-TOF-MS analysis for qualitative analysis was performed on an Agilent 6520 Q-TOF mass spectrometer equipped with a diode array detector (DAD) and electrospray interface (ESI) (Agilent Technologies, Santa Clara, CA). The MS system was operated in positive ionization modes with the mass spectra scan range set at *m/z* 50–1000. The typical ionization source conditions were as follows: nebulizer pressure 40 psi, drying gas temperature 325 °C, drying gas flow rate 8.0 L·min<sup>-1</sup>, sheath gas temperature 400 °C, sheath gas flow rate 10 L·min<sup>-1</sup>, fragmentor voltage 100 V, capillary voltage 4000 V (+ESI), skimmer voltage 65 V and collision energy 35 eV. Data acquisition and analysis were processed by Agilent Mass Hunter Workstation Data Acquisition Software Version B.04.00 (Agilent Technologies, Santa Clara, CA).

#### 4.5. Inhibitory effects of cardiac glycosides on Na<sup>+</sup>,K<sup>+</sup>-ATPase

The Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined by measuring the amount of inorganic phosphate (Pi) liberated from ATP [28–30]. A commercial Na<sup>+</sup>,K<sup>+</sup>-ATPase from the porcine cerebral cortex (Sigma, USA; 0.5 units/mg) was incorporated into 4 mL buffer. The buffer contained 1 mmol·L<sup>-1</sup> EGTA, 5 mmol·L<sup>-1</sup> NaN<sub>3</sub>, 1 mmol·L<sup>-1</sup> MgCl<sub>2</sub>, 100 mmol·L<sup>-1</sup> NaCl, 20 mmol·L<sup>-1</sup> KCl and 20 mmol·L<sup>-1</sup> Tris, added HCl to adjust to pH 7.8.

Adding a series concentrations of standard solutions 10 μL and Na<sup>+</sup>,K<sup>+</sup>-ATPase solution 10 μL into 70 μL buffer, shaken quickly. After incubating 15 min under 37 °C, 10 μL ATP solution (20 mmol·L<sup>-1</sup>) was added. After incubating 25 min under 37 °C, the enzymatic reaction was terminated by adding 17.5 μL of 25% (w/v) trichloroacetic acid. After centrifugation at 15,000 rpm for 3 min, the supernatant was detected by the phosphate assay kit. After 30 min of incubation under 37 °C, the color intensity was measured at 620 nm on a SpectraMax M2 reader (Molecular Devices, Sunnyvale, CA, USA). Sodium pump activity was expressed as μmol Pi liberated from ATP by 1 mg of Na<sup>+</sup>,K<sup>+</sup>-ATPase in 1 h. Lineweaver-Burk plots methods were used to determine kinetic parameters [31,32].

#### 4.6. Kinetic analysis of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibition

Lineweaver-Burk plots were used for the analysis of enzyme's inhibitory kinetics, caused by the tested bioactive compounds, and its comparison was performed with the data collected in the absence of inhibitors [31,32]. Steady-state rates were determined at different inhibitor concentrations and changing concentrations of substrate, then enzyme inhibition mechanism and related kinetic parameters were determined [31,32]. Eq. (1) was used for linear regression analysis.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1)$$

where *v* is the value for reaction rate; *V*<sub>max</sub> is the value for maximum reaction rate; [S] is the value for concentrations of substrate; *K*<sub>m</sub> is the value for Michaelis constant.

#### 4.7. Molecular docking

The structure of the protein was downloaded from RCSB Protein DataBank (<http://www.rcsb.org/pdb>), PDB code for Na<sup>+</sup>,K<sup>+</sup>-ATPase was 4hj. The docking calculations were carried out with Autodock 4.2 program [33]. The substrates were minimized using semiempirical AM1 method with the algorithm Polak-Ribiere in Hyperchem and the protein was considered stiff.

#### 4.8. Quantitative application

The established analytical method in Sections 4.2 and 4.3 were subsequently applied to analyze different parts of cultured *S. asper*. Quantitation of four compounds was performed in triplicate and the results were expressed as means.

#### 4.9. Similarity analysis (SA)

The similarities of these four chromatograms of different parts of *S. asper* were compared with their mean chromatographic fingerprint. Each peak was compared between different parts of *S. asper*, as well as establishing a simulative mean chromatogram as a representative standard fingerprint chromatogram, namely a reference. The software "Similarity Evaluation System for Chromatographic Fingerprint of TCM" was published by Chinese Pharmacopoeia Committee (Version 2004A, Beijing, China) and applied in the SA of chromatographic patterns.

#### 4.10. Principle compounds analysis (PCA)

PCA is one of the typical chemometric tools to monitor the outline of all data. The target of it is to reduce the dimensionality of complex data sets, namely allow projection of data from a higher to a lower dimensional space (defined by principal compounds, PCs) and then reconstruct them without any preliminary assumption about their distribution. The significant peaks were chose to establish scree plot, which is generally required that the cumulative contribution rate of the extracted *n* principal components can reach over 80%. Peaks were selected as the variables and different parts of *S. asper* by PCA using SPSS software (SPSS 22; SPSS, Chicago, IL).

#### 4.11. Statistical analysis

The empirical molecular formula data sets were analyzed by MassHunter. Significant differences of means at *P* < 0.05 were determined by analysis of variance (ANOVA) using SPSS. The data of measurement of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and enzyme kinetics were analyzed by GraphPad Prism (Version 5.01, California, USA).

#### Acknowledgements

The work was supported by the Youth Science Fund Project of National Natural Science Foundation of China (Grant No. 81703383), the Natural Science Foundation of Jiangsu Province (Grant No. BK20170742), the "Double First-Class" University project (Grant No. CPU2018GY34), and National Found for Fostering Talents of Basic Science (Grant No. J1310032).

#### Conflict of interest

The authors have declared no conflict of interest.

#### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2019.03.049>.

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