Three new flavonoid glycosides from the aerial parts of Allium sativum L. and their anti-platelet aggregation assessment

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ABSTRACT

Three new flavonoid glycosides, Dasuanxinoside F–H (1–3), were isolated from the aerial parts of Allium sativum, together with eight known compounds which were firstly reported in this plant, including three flavonoid glycosides (4–6) and five phenylethanoid glycosides (7–11). Their structures were identified by UV-vis, IR, 1D and 2D NMR spectra, as well as HR-ESI-MS analyses. The inhibitory effect of the isolated compounds on platelet aggregation induced by adenosine diphosphate (ADP) was evaluated \textit{in vitro}. The results showed that most compounds displayed different degrees of inhibition. Among them, 2, 5, 8 and 9 exhibited the strongest activity on platelet aggregation.

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ARTICLE HISTORY
Received 17 October 2021
Accepted 22 February 2022

KEYWORDS
Allium sativum; the aerial parts; flavonoid glycosides; phenylethanoid glycosides; platelet aggregation

Supplemental data for this article can be accessed online at https://doi.org/10.1080/14786419.2022.2047045.
1. Introduction

*Allium sativum* L. is a perennial herb of *Liliaceae* family, whose underground bulbs are well known as garlic, and used as traditional Chinese medicine (TCM), Dasuan. It is recorded in Chinese pharmacopoeia (2020 edt), with the functions of detoxification, detumescence, insecticide and dysentery control (Chinese Pharmacopoeia Commission 2020, 25–26). Scholars in the field of medicine and food (Bianchi 2015) have done a lot of research work on garlic and more than 130 compounds have been isolated from garlic bulbs since the 1970s, mainly including organic sulfur compounds (Tan et al. 2015; Woo et al. 2015; Ono et al. 2017; Kim et al. 2018; Nakamoto et al. 2018; Nohara et al. 2018), steroidal saponins (Matsuura et al. 1988; Lanzotti et al. 2012), flavonoids (Kim et al. 2005; Li et al. 2015), pyranone derivatives (Kodera et al. 2002), cerebrosides, phenols, vitamins, amino acids, polysaccharides, protein and volatile oil. Clinically, the bulbs of *A. sativum* are commonly used in the treatment of cardiovascular diseases, infections, digestive system and urinary system diseases, etc. (Karuppiah and Rajaram 2012; Ried and Fakler 2014; Bin et al. 2020; Mai et al. 2021). *A. sativum* is an indispensable dual-purpose plant for medicine and food. The huge market demand has led to the cultivation of garlic all over the world. However, most people only harvest bulbs, and a large number of aboveground parts are discarded directly. This has resulted in a huge waste of resources.

In this study, the chemical isolation and purification were carried out for the aerial parts of *A. sativum*, and three new (1–3) along with eight known (5–11) compounds were obtained. Their structures were identified by ultraviolet visible (UV-vis), infra-red (IR), nuclear magnetic resonance (NMR) spectra and high resolution electrospray ionisation mass spectrometry (HR-ESI-MS), as well as comparing the data with the literature. The anti-platelet aggregation activities of compounds were also studied in vitro. As a result, nine ingredients were active, revealing the pharmacodynamic material basis of the aerial parts of *A. sativum* on platelet aggregation. This study provided a potential for decreasing resources waste, further deeply exploiting and utilizing the aerial parts.

2. Results and discussion

2.1. Isolation and analysis of the compounds

The aerial parts of *A. sativum* (30.0 Kg) were extracted with 70% ethanol. The 70% ethanol extract (2723 g) was further separated by different organic solvent extraction, various column chromatography (CC) and preparative HPLC to obtain compounds 1–11 (Figure 1). Their structures were identified by UV-visible, IR, NMR spectra and HR-ESI-MS analyses.

2.1.1. Structure elucidation of new compound 1

Compound 1 was isolated as a yellow amorphous powder. HR-ESI-MS ([M - H]– m/z 947.2448, calc. for 947.2463) established the molecular formula of 1 as C₄₃H₄₈O₂₄. Acid hydrolysis experiment and GC analysis of 1 gave D-glucose. In the ¹H-NMR spectrum of 1 (Supplementary material, Table S1) exhibited the signals at δH 12.47 (1H, s), 6.13
(1H, s), 6.24 (1H, s), 8.06 (2H, d, J = 8.8 Hz) and 7.15 (2H, d, J = 8.8 Hz). Combined with the \( ^{13} \)C-NMR spectrum (Supplementary material, Table S2), HSQC and DEPT of 1 showed 15 carbon signals at \( \delta_{C} 155.1, 133.5, 177.0, 161.0, 99.4, 166.6, 94.1, 156.6, 103.1, 123.9, 130.5, 103.1, 123.9, 130.5 \times 2, 115.8 \times 2 \) and 158.9. The above information showed that compound 1 contains kaempferol structure. The \( ^{1} \)H-NMR spectrum of 1 also gave signals at \( \delta_{H} 6.72 \) (1H, d, J = 8.0 Hz), 7.09 (1H, dd, J = 8.0, 1.6 Hz) and 7.29 (1H, d, J = 1.6 Hz) belong to ABX coupling system for a 1,3,4-trisubstituted benzene ring, and two trans-coupled alkene protons at \( \delta_{H} 7.55 \) (1H, d, J = 15.8 Hz) and 6.47 (1H, d, J = 15.8 Hz), together with a typical signal for a methoxy group at \( \delta_{H} 3.76 \) (3H, s). \( ^{13} \)C-NMR spectrum of 1 showed 10 carbon signals, including carbon signals of a 1,3,4-trisubstituted benzene ring at \( \delta_{C} 125.1, 111.0, 148.2, 150.1, 115.9 \) and 123.3, a pair of alkene carbon signals at \( \delta_{C} 145.3 \) and 113.9, a carbonyl carbon signal at \( \delta_{C} 166.6 \), along with one methoxy carbon at \( \delta_{C} 55.6 \). Combined with the \( ^{1} \)H-NMR spectrum, it is speculated that the compound 1 has feruloyl structure. The three anemic proton signals which occurred at \( \delta_{H} 5.48 \) (1H, d, J = 7.6 Hz), 4.27 (1H, d, J = 7.8 Hz) and 5.09 (1H, d, J = 7.2 Hz) along with 18 carbon signals (\( \delta_{C} 100.8, 73.9, 74.7, 80.0, 70.0, 61.0, 103.1, 73.2, 76.8, 73.8, 76.3, 63.0, 99.9, 73.2, 76.4, 69.6, 75.3 \) and 60.1) were attributed to three glucose moieties. And the three anemic proton coupling constants indicated that the glycoside bonds are all \( \beta \)-Configuration.
The HMBC cross-peaks (Supplementary material, Figure S1) between H-1’’’’ (δ_H 5.09) and C-4’ (δ_C 158.9), H-1’’’’ (δ_H 5.48) and C-3 (δ_C 133.5) defined C-4’ and C-3 of kaempferol as sites of O-glucosylation. Moreover, interglucosidic linkage of the disaccharide moiety was also determined by the HMBC correlations between H-1’’’’ (δ_H 4.27) and C-4’’’’ (δ_C 80.0). Similarly, the feruloyl group was linked to C-7 based on the correlations of the H-6 (δ_H 6.13)/H-8 (δ_H 6.24) with ester carbonyl carbon C-9’’ (δ_C 166.6). Assignments of all groups of compound 1 were achieved by COSY, HSQC, TOCSY and HMBC (Supplementary material, Figure S1). Thus, the structure of compound 1 was established as kaempferol-3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-7-O-feruloyl-4’-O-β-D-glucopyranoside, named Dasuanxinoside F.

2.1.2. Structure elucidation of new compound 2

Compound 2 was isolated as a yellow amorphous powder. HR-ESI-MS ([M - H]⁻ m/z 947.2457, calc. for 947.2463) determined the molecular formula of 2 as C_{43}H_{48}O_{24}. Acid hydrolysis experiment and GC analysis of 2 gave D-glucose. The ¹H-NMR (Supplementary material, Table S1) and ¹³C-NMR (Supplementary material, Table S2) spectra of 2 which were assigned by various NMR experiments including COSY, HSQC, TOCSY and HMBC spectra, showed signals assignable to a kaempferol part [δ_H 12.55 (1H, s, 5-OH), 6.24 (1H, d, J = 2.0 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8), 8.09 (2H, d, J = 8.8 Hz, H-2’,6’)] and 7.18 (2H, d, J = 8.8 Hz, H-3’,5’)], a feruloyl group [δ_H 6.73 (1H, d, J = 8.1 Hz, H-5’’), 7.09 (1H, dd, J = 8.1, 2.0 Hz, H-6’’), 7.31 (1H, d, J = 2.0 Hz, H-2’’’), 7.54 (1H, d, J = 15.9 Hz, H-7’’’), 6.49 (1H, d, J = 15.9 Hz, H-8’’’), 3.78 (3H, s, 3’’’-OCH₃)], together with three β-D-glucopyranosyl moieties [δ_H 5.51 (1H, d, J = 7.6 Hz, H-1’’’’), 4.29 (1H, d, J = 7.6 Hz, H-1’’’’)] and 5.13 (1H, d, J = 7.2 Hz, H-1’’’’)]. Furthermore, the HMBC analysis (Supplementary material, Figure S1) of compound 2, long-range correlations were observed between H-1’’’’ [δ_H 5.13 (1H, d, J = 7.2 Hz)] and C-4’ (δ_C 159.0), H-1’’’’/[δ_H 5.51 (1H, d, J = 7.6 Hz)] and C-3 (δ_C 133.6), H-1’’’’/[δ_H 4.29 (1H, d, J = 7.6 Hz)] and C-4’’’ (δ_C 80.0), H-6’’’’ [δ_H 4.25 (1H, m)] and the ester carbonyl carbon (δ_C 166.6, C-9’’). Compounds 2 and 1 are isomers. The ¹H-NMR chemical shifts of 6-, 8-positions in compound 2 [6.24 (1H, d, J = 2.0 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8)] are downfield compared with 1 [6.13 (1H, s, H-6), 6.24 (1H, s, H-8)], and the ¹³C-NMR chemical shifts of 7-position in 2 (δ_C 164.3) is upfield compared with 1 (δ_C 166.6). Comparing compounds 1 and 2 with compound 4, combined with other spectral data, it was further confirmed that the 7-position of compound 1 was associated with a feruloyl group, while the 6’’’’-position of compound 2 was replaced by a feruloyl structure. From the above data, the structure of compound 2 was established as kaempferol-3-O-[6-O-feruloyl]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-4’-O-β-D-glucopyranoside, named Dasuanxinoside G.

2.1.3. Structure elucidation of new compound 3

Compound 3 was isolated as a yellow amorphous powder. Its molecular formula was determined by HR-ESI-MS ([M - H]⁻ m/z 947.2470, calc. for 947.2463) to be C_{43}H_{48}O_{24}. The glycosyl moieties of 3 were determined as D-glucose with the same method as the above-mentioned compounds. The ¹H-NMR (Supplementary material, Table S1) and ¹³C-NMR (Supplementary material, Table S2) spectrum of 3 which were assigned
by various NMR experiments including COSY, HSQC, TOCSY and HMBC spectra, showed signals assignable to a kaempferol skeleton [δ₃H 12.56 (1H, s, 5-OH), 6.24 (1H, d, J = 1.9 Hz, H-6), 6.41 (1H, d, J = 1.9 Hz, H-8), 8.14 (2H, d, J = 8.8 Hz, H-2′,6′) and 7.22 (2H, d, J = 8.8 Hz, H-3′,5′)], a feruloyl group [δ₃H 6.82 (1H, d, J = 8.2 Hz, H-5′0), 7.13 (1H, dd, J = 8.2, 1.6 Hz, H-6), 7.34 (1H, d, J = 1.6 Hz, H-2′′), 7.56 (1H, d, J = 15.8 Hz, H-7′′), 6.53 (1H, d, J = 15.8 Hz, H-8′′) and 3.84 (3H, s, 3′-OCH₃)], together with three β-D-glucopyranosyl moieties [δ₃H 5.52 (1H, d, J = 7.8 Hz, H-1′000), 4.29 (1H, d, J = 7.6 Hz, H-1′0000), 5.28 (1H, d, J = 7.8 Hz, H-1′00000) and 5.28 (1H, d, J = 7.8 Hz, H-1′00000)]. Moreover, the HMBC analysis (Supplementary material, Figure S1) of compound 3, long-range correlations were observed between H-1′000 [δ₃H 5.52 (1H, d, J = 7.8 Hz)] and C-3 (δC 133.6), H-1′0000 [δ₃H 4.29 (1H, d, J = 7.6 Hz)] and C-4′000 (δC 80.0), H-1′00000 [δ₃H 5.28 (1H, d, J = 7.8 Hz)] and C-4′ (δC 158.9), H-4′00000 [δ₃H 4.86 (1H, m)] and the ester carbonyl carbon (δC 165.8, C-9′). Compounds 3, 2 and 1 are isomers. The connection positions of kaempferol and three glucopyranosyl moieties are the same, but the differences are the substitution positions of the feruloyl group. Compared with compound 1, the chemical shifts at 7-, 4′′00000-positions of compound 3 are significantly different. Combined with other spectral data, it can be determined that the feruloyl group of compound 3 is connected at 4′00000-position. Finally, the structure of compound 3 was identified as kaempferol-3-O-(4′-O-feruloyl)-β-D-glucopyranoside, named Dasuanxinoside H.

2.1.4. The known compounds

Nine known compounds were firstly reported in A. sativum, identified as kaempferol 3,4′-di-O-β-D-glucopyranoside (4) (Cui et al. 1993), ombuin-3-O-β-D-glucopyranoside (5) (Malek et al. 2013), isorhamnetin-3-O-β-D-glucopyranoside (6) (Zhang et al. 2012), rhodioloside (7) (Song et al. 2000), 2-phenylethyl-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (8) (Yan et al. 2017), heterodontoside (9) (Yousef et al. 2006), benzylethyl-O-β-D-xylopyranosyl-(1→2)-O-β-D-glucopyranoside (10) (Nishimura et al. 1990) and 2-phenethyl-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (11) (Fang et al. 2018) by comparing their physico-chemical constants and spectroscopic data with those reported in literatures (Figure 1).

2.2. Anti-platelet aggregation activity of the isolated compounds

Isolated compounds 1–9 were evaluated for anti-platelet aggregation activity using aspirin as a positive control. The results showed that compounds 2, 5, 8 and 9 had strong anti-platelet aggregation activity, which was equivalent to positive drug. Compounds 1, 3 and 7 showed weak activity. Compounds 4 and 6 were almost inactive. The data were shown in Table S3 (Supplementary material).

3. Experimental

3.1. General methods

Multiple column chromatographies, such as silica gel (800–100/200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), and octadecyl silica (ODS) gel (ODS-A-
HG, 12 nm, S-50 μm, YMC Ltd., Kyoto, Japan), were used for isolations. Preparative HPLC (2535-2489-2414, Waters, Milford MA, USA) was performed with Hypersil GOLD C18 (5 μm, 21.2 × 250 mm, Thermo). Bruker DPX 400 MHz NMR instrument (Bruker SpectroSpin, Karlsruhe, Germany) with TMS as an internal standard was used to measure the NMR spectra, including 1D-NMR and 2D-NMR spectra. The HR-ESI-MS analyses were carried out on ORBITRAP FUSION MS spectrometer (Thermo, Waltham, MA, USA). The GC (TRACE1300-ISQ, Thermo, Waltham, MA, USA) instrument was used to analyze the sugar derivatives from compounds. All the solvents (Tianjindamao Company Ltd., Tianjin, China) were of analytical grade. Adenosine diphosphate (ADP), sodium citrate and dimethyl sulfoxide (DMSO) for the study of anti-platelet aggregation activity were purchased from Macklin (Shanghai Macklin Biochemical Co., Ltd., Shanghai, China). Aspirin (Bayer, Leverkusen, Germany) was a positive drug for the determination of anti-platelet aggregation activity.

3.2. Plant materials

The aerial parts of *Allium sativum* L. were collected from Heilongjiang province of China in July 2017 and identified by Zhenyue Wang of Heilongjiang University of Chinese Medicine. The voucher specimen (No. 20170575) is deposited at the Herbarium of Heilongjiang University of Chinese Medicine, Heilongjiang, China.

3.3. Extraction and isolation

The dried aerial parts of *A. sativum* (30.0 Kg) were extracted 2 times (each time for 2 hours) with 70% EtOH (300 L) and then concentrated under vacuum to give 70% ethanol extract (2723 g). The 70% ethanol extract was suspended in distilled water (24 L), and then extracted with petroleum ether (60–90 °C), ethyl acetate and n-butanol, respectively. Solvents were removed under vacuum to obtain extracts of petroleum ether (275 g), ethyl acetate (117 g), n-butanol (396 g) and remained water (1935 g). The n-butanol fraction (194 g) was subjected to silica gel column chromatography and eluted with a gradient of CH2Cl2/MeOH (20:1 to 0:1) to yield 10 fractions (Fr.1-Fr.10). Fr.4 (16.6 g) was further chromatographed on silica gel column and eluted with CH2Cl2/MeOH (40:1 to 1:1) to yield 9 sub-fractions (Fr.4.1-Fr.4.9). Fr.4.4 (1.19 g) was subjected to open reversed phase (ODS) column chromatography and eluted with H2O/MeOH (3:5 to 0:1) to yield 5 fractions (Fr.4.4.1- Fr.4.4.5). Fr.4.4.2 was purified by preparative HPLC using MeOH/H2O (60%, flow rate 3 mL/min) to give compound 5 (4.9 mg). Fr.5 (3.77 g) was subjected to open reversed phase (ODS) column chromatography and eluted with H2O/MeOH (4:5 to 0:1) to yield 6 sub-fractions (Fr.5.1-Fr.5.6). Fr.5.2 was purified by preparative HPLC using MeOH/H2O (18%, flow rate 6 mL/min) to give compound 7 (12 mg). Fr.6 (16.3 g) was subjected to open reversed phase (ODS) column chromatography and eluted with H2O/MeOH (9:1 to 0:1) to yield 10 sub-fractions (Fr.6.1-Fr.6.10). Fr.6.4 was purified by preparative HPLC using MeOH/H2O (35%, flow rate 6 mL/min) to give compounds 8 (5.0 mg), 9 (16.1 mg), 10 (5.6 mg) and 11 (6.1 mg). Fr.6.7 was purified by preparative HPLC using MeOH/H2O (48%, flow rate 6 mL/min) to give compound 4 (21.4 mg). Fr.6.9 was purified by preparative HPLC...
using MeOH/H₂O (48%, flow rate 6 mL/min) to give compound 6 (3.2 mg). Fr.8 (15 g) was subjected to open reversed phase (ODS) column chromatography and eluted with H₂O/MeOH (9:1 to 0:1) to yield 5 sub-fractions (Fr.8.1-Fr.8.5). Fr.8.4 (785 mg) was further chromatographed on reversed phase (ODS) column and eluted with H₂O/MeOH (7:3 to 0:1) to afford compounds 2 (30.1 mg) and 3 (34.9 mg). The sub-fraction of Fr.8.4 was purified by preparative HPLC using MeOH/H₂O (48%, flow rate 6 mL/min) to give compound 1 (12.9 mg).

3.3.1. **Kaempferol-3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-7-O-feruloyl-4′-O-β-D-glucopyranoside (Dasuanxinoside F) (1)**

Yellow amorphous powder; [α]_{D}^22 D = 25.4 (c 0.21, MeOH); UV λ_{max} (MeOH): 247.3, 267.4, 324.6 nm; IR (KBr) ν_{max}: 3411, 3185, 1694, 1654, 1604, 1509, 1399, 1251, 1175, 1074, 844 cm⁻¹; HR-ESI-MS [M - H]⁻ m/z 947.2448, calcd. for C₄₃H₄₇O₂₄⁻ ([M - H]⁻) 947.2463; ¹H- and ¹³C-NMR data, see Tables S1 and S2 (Supplementary material).

3.3.2. **Kaempferol-3-O-[(6-O-feruloyl)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside]-4′-O-β-D-glucopyranoside (Dasuanxinoside G) (2)**

Yellow amorphous powder; [α]_{D}^22 D = 22.7 (c 0.17, MeOH); UV λ_{max} (MeOH): 246.1, 267.4, 324.6 nm; IR (KBr) ν_{max}: 3407, 3187, 1698, 1655, 1604, 1509, 1398, 1251, 1170, 1074, 809 cm⁻¹; HR-ESI-MS [M - H]⁻ m/z 947.2457, calcd. for C₄₃H₄₇O₂₄⁻ ([M - H]⁻) 947.2463; ¹H- and ¹³C-NMR data, see Tables S1 and S2 (Supplementary material).

3.3.3. **Kaempferol-3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-4′-O-(4-O-feruloyl)-β-D-glucopyranoside (Dasuanxinoside H) (3)**

Yellow amorphous powder; [α]_{D}^22 D = 20.1 (c 0.15, MeOH); UV λ_{max} (MeOH): 266.2, 328.2 nm; IR (KBr) ν_{max}: 3415, 3175, 1706, 1655, 1605, 1510, 1399, 1250, 1171, 1073, 812 cm⁻¹; HR-ESI-MS [M - H]⁻ m/z 947.2470, calcd. for C₄₃H₄₇O₂₄⁻ ([M - H]⁻) 947.2463; ¹H- and ¹³C-NMR data, see Tables S1 and S2 (Supplementary material).

3.4. **Acid hydrolysis of 1–3**

Acid hydrolysis experiments were carried out by using the methods in the literature (Li et al. 2012). In short, the sugar residues were gained by hydrolyzing of compounds 1–3 (2.0 mg) with H₂SO₄ (2 mol/L, 2.0 mL), and then treated with trimethylchlorosilane, respectively. Then the sugar derivants were further analyzed by GC. The results showed that the sugar derivatives of compounds 1–3 were D-glucose (tₚ = 11.32 min).

3.5. **Anti-platelet aggregation activity test**

The platelet aggregation experiment were carried out by using the methods in the literature (Chen et al. 2011). Briefly, SD male rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml·kg⁻¹), and blood was taken from abdominal aorta with syringe containing 3.8% sodium citrate (v/v 3.8% sodium citrate: Blood = 1:9). After 30 minutes of static setting, blood samples were centrifuged for 8 minutes with 1000 rpm at room temperature, and then platelet-rich plasma (PRP) was absorbed...
from the upper layer. The remaining plasma was centrifuged for 10 minutes with 3000 rpm to obtain platelet-poor plasma (PPP), which was used as a reference solution in platelet aggregation assays. The platelets of PRP were adjusted to the proper number (2–2.5 × 10¹¹/L) with PPP for the aggregation assay. The 90 µL PRP and 5 µL sample solution (final concentration: 200 µmol.L⁻¹) were added into 96-well plates and incubated on 37 °C for 10 minutes. After incubation, 5 µL ADP (final concentration: 5 µmol.L⁻¹) was added and 570 nm transmission was monitored with Microplate Reader for every 30 seconds until absorbance (A) became stable. The aggregation rate (AR) = (Aₚₛ𝐀ₛ – Aₚₚ) / (Aₛ – Aₛ) × 100%. The aggregation inhibition rate (AIR) = [1 – (ARₛ/ARₖ)] × 100%.

4. Conclusions

We studied the chemical components from the aerial parts of A. sativum and assessed their activity based on its clinical application of treating cardiovascular diseases. Eleven compounds were obtained, including three new flavonoid glycosides together with eight known compounds. The structures of new compounds were established as kaempferol-3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-7-O-feruloyl-4'-O-β-D-glucopyranoside (Dasuanxinoside F) (1), kaempferol-3-O-[(6-O-feruloyl)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside]-4'-O-β-D-glucopyranoside (Dasuanxinoside G) (2), and kaempferol-3-O-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside-4'-O-(4-O-feruloyl)-β-D-glucopyranoside (Dasuanxinoside H) (3), respectively. The isolated compounds were evaluated for the inhibitory effect of platelet aggregation *in vitro*, as a result, compounds 2, 5, 8 and 9 exhibited strong anti-platelet aggregation activity. The results provided a potential to utilize the abundant aerial parts of A. sativum.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the [Chinese Medicine Science and Technology Plan Project of Jiangxi Province] under Grant [number 2020A0357]; [Starting Fund for Scientific Research of High-level Talents of Gannan Medical University] under Grant [number QD202009]; and [Key special project of “Research on modernization of traditional Chinese medicine” in national key R & D plan of China] under Grant [number 2018YFC1707100].

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