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Constituents of *Vigna angularis* and their *in vitro* anti-inflammatory activity

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ABSTRACT

Nine non-phenolic compounds, including four furanylmethyl glycosides, angularides A–D, one *ent*-kaurane diterpene glycoside, angularin A, and four triterpenoid saponins, angulasaponins A–D, were isolated from seeds of *Vigna angularis*, together with eight known compounds. Their structures were elucidated on the basis of extensive 1D and 2D NMR spectroscopic analysis as well as chemical methods. Angularin A, angulasaponins A–C, and azukisaponins III and VI showed inhibition of nitric oxide production in LPS-activated RAW264.7 macrophages, with IC_{50} values ranging from 13 μM to 24 μM.

1. Introduction

The seeds of *Vigna angularis* (Willd.) Ohwi & H. Ohashi (*Phaseolus angularis* (Willd.) W. Wight, Leguminosae), also called Xiaodou (red beans) in Chinese, or Azuki beans in Japanese, are one of the commonly used food materials for preparation of porridges, soups, or confections in the Orient. They are also widely used as a traditional Chinese medicine for their diuretic action, detoxification properties, and to promote the drainage of pus (Chinese Pharmacopoeia Commission, 2010). Previous investigations on this plant have led to the isolation of 3-furanylmethyl β-D-glucopyranoside, flavonoids, saponins, dimeric proanthocyanidins, and glyceroglycolipids (Ariga and Asao, 1981; Kitagawa et al., 1983a,b,c; Kojima et al., 1991; Yumiko et al., 2009). The polyphenolic constituents of *V. angularis* are associated with many bioactivities, such as antioxidant, renal cortex protective, hypoglycemic, anti-hypotensive, and hepatoprotective effects (Han et al., 2004; Itoh et al., 2004, 2009; Mukai and Sato, 2009; Shin et al., 2005; Yumiko et al., 2009). Beside these reports, there is little known on the action of other types of components from *V. angularis*. Thus, in order to find non-phenolic bio-active constituents occurring in this plant, a systematical phytochemical analysis of *V. angularis* was performed and the products were subjected to bioassays. Nine new compounds (Fig. 1), including four new furanylmethyl glycosides, angularides A–D (**1**–**4**), one *ent*-kaurane diterpene glycoside, angularin A (**5**), and four new triterpenoid saponins, angulasaponins A–D (**6**–**9**), were thus isolated, along with eight known compounds (**10**–**17**). Herein, the isolation and structural elucidation of these new compounds **1**–**9** are described, as well as their inhibitory effects on the production of nitric oxide (NO) induced by lipopolysaccharide (LPS) in RAW264.7 cells.

2. Results and discussion

The EtOH extract of the seeds of *V. angularis* was suspended in H_2O and partitioned with EtOAc. The aqueous layer was subjected to passage over a XAD-16 resin column chromatography, eluting with aqueous ethanol in a gradient manner. The 20%, 40% and 70% aqueous ethanol eluates, along with the EtOAc extract, were purified using a combination of silica gel, ODS, and Sephadex LH-20 chromatographic steps, and preparative HPLC to give nine new (**1**–**9**), and eight known (**10**–**17**) compounds (Fig. 1). Their structures were determined by 1D and 2D NMR spectroscopic analysis and mass spectrometry.

Angularides A–C (**1**–**3**) were obtained as hygroscopic powders, and determined to have the same $C_{13}H_{18}O_8$ molecular formulae

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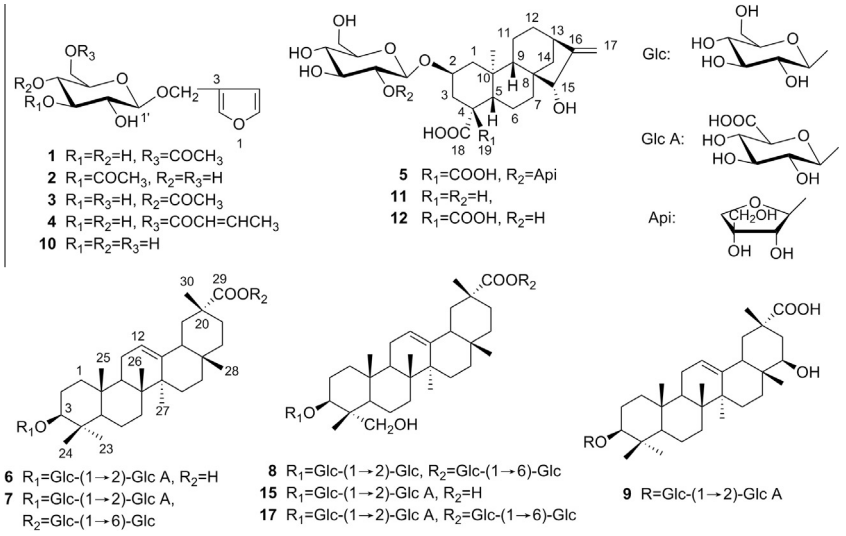

Fig. 1. Structures of compounds **1**–**12**, **15** and **17**.

by HRESIMS (*m*/*z* 325.0894, 325.0885 and 325.0880 $[M+Na]^+$ for **1**, **2** and **3**, respectively). The ^1H and ^{13}C NMR spectra of **1** displayed signals for a 3-substituted furan ring [δ_H 6.49 (1H, d, J = 1.5 Hz), 7.45 (1H, t, J = 1.5 Hz), 7.52 (1H, m), δ_C 111.7, 123.1, 142.5, 144.5], an oxygenated methylene [δ_H 4.56 and 4.71 (2H, d, J = 12.0 Hz); δ_C 63.3], and one anomeric resonance [δ_H 4.34 (1H, d, J = 8.0 Hz), δ_C 103.0], which were very similar to those of **10** (Kitagawa et al., 1983a) except for the presence of an additional acetyl group [δ_H 2.07; δ_C 20.7, 172.8]. Acid hydrolysis of **1** gave D-glucose after HPLC separation and optical rotation measurement. The anomeric configuration was determined to be β-D according to the J_{H1-H2} value (8.0 Hz). The acetylated position was determined by observation of an HMBC correlation between glucose H-6′ (δ_H 4.21 and 4.41) and the acetyl carbonyl (δ_C 172.8). Thus, **1** was determined to be 3-furanylmethyl-6′-*O*-acetyl β-D-glucopyranoside. The acetylation positions of **2** and **3** were determined by the observation of obvious downfield shifts of acetylated positions (1.58 ppm for **2**, and 1.46 ppm for **3**) through comparison with **10**, which are same with the results reported in the literature (Jansson et al., 1987). Thus, **2** was elucidated as 3-furanylmethyl-3′-*O*-acetyl β-D-glucopyranoside, and **3** was deduced as 3-furanylmethyl-4′-*O*-acetyl β-D-glucopyranoside.

Angularide D (**4**) was obtained as a hygroscopic powder, and its molecular formula was deduced to be $C_{15}H_{20}O_8$ according to HRESIMS (molecular ion at *m*/*z* 351.1042 $[M+Na]^+$, calcd. 351.1050). The ^1H and ^{13}C NMR spectroscopic data of **4** were similar to those of **1**, except that the acetyl group was replaced by a crotonyl group [δ_H 1.90 (3H, dd, J = 7.0, 1.5 Hz), 5.92 (1H, dq, J = 15.5, 1.5 Hz) and 7.04 (1H, dq, J = 15.5, 7.0 Hz); δ_C 18.0, 123.3, 146.8, 168.0]. The HMBC correlations of glucose H-6′ (δ_H 4.28 and 4.46) with the crotonyl carbonyl signal (δ_C 168.0) proved the linkage position of the crotonyl group. Thus, compound **4** was deduced to be 3-furanylmethyl-6′-crotonyl-β-D-glucopyranoside.

Angularin A (**5**) was isolated as an amorphous powder, and its molecular formula was determined to be $C_{31}H_{46}O_{15}$ from the molecular ion peak $[M+NH_4]^+$ at *m*/*z* 676.3194 (calcd 676.3175) in the positive-ion HRESIMS. The ^1H NMR spectrum showed one tertiary methyl singlet at δ_H 1.02, two exocyclic methylene protons at δ_H 5.08 (1H, br s) and 5.19 (1H, br s), and two anomeric protons at δ_H 4.48 (1H, d, J = 8.0 Hz) and 5.36 (1H, d, J = 1.5 Hz). The ^{13}C NMR spectrum showed the presence of signals for two carbonyls (δ_C 175.4 and 175.8), two olefinic carbons (δ_C 109.0 and 160.4), two oxygenated carbons (δ_C 73.1 and 83.5), one methyl (δ_C 17.8), and two anomeric carbons (δ_C 101.4 and 110.8). Thorough analysis of these data with the help of 2D NMR experiments allowed identification of the aglycone as carboxyatractyligenin, an *ent*-kaurane characterized by two acid functions at C-4 (Lang et al., 2013; Piacente et al., 1996). Acid hydrolysis of **5** offered glucose (Glc) and apiose (Api), characterized as D according to optical rotation measurement after HPLC separation. The J value of the anomeric proton of glucose was characteristic for a β-D configuration in the pyranosyl form, and the comparison of ^{13}C NMR spectroscopic data with literature values was in agreement with a β-D apiose in its furanosyl form (Çaliş et al., 1993). Compound **5** and the known 2-*O*-β-D-glucopyranosyl-carboxyatractyligenin (**12**) (Lang et al., 2013) differed by the presence of a supplementary apiofuranosyl at the C-2 position of glucopyranosyl, demonstrated by HMBC correlation of δ_H 5.36 (H-1 of Api) with δ_C 79.1 (C-2 of Glc) and NOESY correlation of δ_H 5.36 (H-1 of Api) with δ_H 3.31 (1H, overlapped, H-2 of Glc). The relative configuration of **5** was determined by observation of NOESY correlations (Fig. 2), in accordance with those observed for carboxyatractyligenin and its glycoside (Konopleva et al., 2006; Piacente et al., 1996). The isolation of *ent*-kauranes **11** and **12** of known absolute configuration from this plant suggested that **5** belonged to the same series, thus being: 2 β-[(β-D-apiofuranosyl(1→2)-β-D-glucopyranosyl)oxy]-15α-hydroxy-*ent*-kaur-l6-en-18,19-dioic acid.

Compounds **6**–**9** were obtained as white amorphous powders. Angulasaponin A (**6**) was assigned a $C_{42}H_{66}O_{14}$ molecular formula from its HRESIMS (*m*/*z* 793.4370 $[M-H]^-$). The ^1H NMR spectrum of **6** showed signals for seven tertiary methyls at δ_H 0.90, 0.97, 0.99, 1.15, 1.27, 1.31 and 1.50, an olefinic proton at δ_H 5.30 (triplet like), and two anomeric protons at δ_H 5.02 (1H, d, J = 8.0 Hz) and 5.42 (1H, d, J = 8.0 Hz). The ^{13}C NMR spectrum presented two carbonyl resonances at δ_C 173.2 and 181.8, two olefinic carbon signals at δ_C 123.1 and 145.2, and two anomeric carbon resonances at δ_C 106.7 and 105.9. The ^1H and ^{13}C NMR spectroscopic data suggested that **6** was a pentacyclic triterpene substituted by two sugars. In the HMBC spectrum, the methyl proton at δ_H 1.50 had correlations with signals at δ_C 30.5 (C-21), 42.1 (C-19), 43.5 (C-20), and 181.8 (C=O), suggesting that one of the methyls at C-20 was oxidized into a carboxylic acid. The characteristic ^{13}C NMR chemical shifts of the methyl and carboxyl (Mahato and Kumdo, 1994) groups favored the C-29 position for the carboxylic acid, indicating 3-epikatonic acid as the aglycone (Silva et al., 2002). Acid hydrolysis of **6** yielded D-glucose and D-glucuronic acid characterized by optical

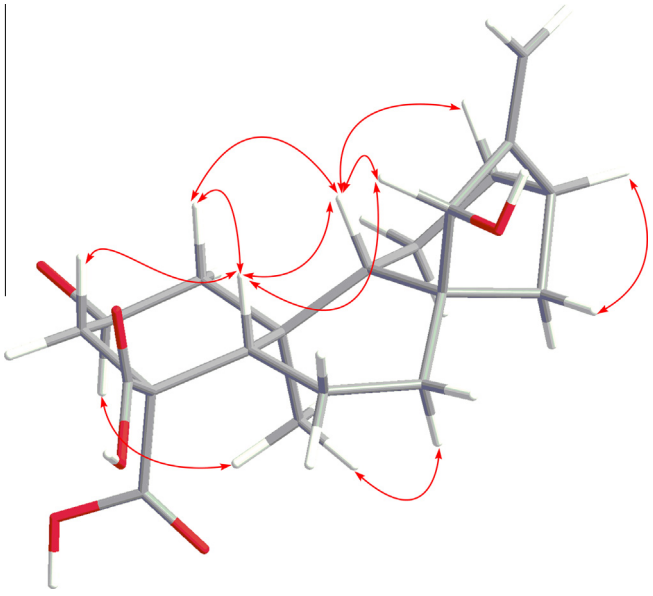

Fig. 2. Key NOESY correlations of **5** (geometry-optimized conformation).

rotation measurement after HPLC separation. 1D and 2D NMR spectroscopic data analysis showed that, in the intact saponin, the sugars were in the pyranose form and in a β-D configuration with J_{H1-H2} = 8.0 Hz for glucose and 8.0 Hz for glucuronic acid (Glc A). The sequence of the sugars was established by an HMBC experiment which showed correlations between the glucuronic acid anomeric proton (δ_H 5.42) and C-3 of the aglycone (δ_C 89.7), and between the Glc anomeric proton (δ_H 5.02) and C-2 of the Glc A (δ_C 83.6). Thus, the structure of **6** was elucidated as 3-epikatonic acid 3-*O*-β-D-glucopyranosyl (1→2)-β-D-glucuronopyranoside.

The molecular formula of angulasaponin B (**7**) was deduced to be $C_{54}H_{86}O_{24}$ from the quasi-molecular ion at *m/z* 1117.5436 $[M-H]^-$ in the HRESIMS. The ^{1}H NMR spectrum showed signals for seven tertiary methyl groups at δ_H 0.89 × 2, 0.95, 1.14, 1.15, 1.31 and 1.39, an olefinic proton at δ_H 5.18 (triplet like), and four anomeric protons at δ_H 5.01 (1H, d, *J* = 8.0 Hz), 5.04 (1H, d, *J* = 7.5 Hz), 5.42 (1H, d, *J* = 8.0 Hz) and 6.31 (1H, d, *J* = 8.0 Hz). The ^{13}C NMR spectrum presented two carbonyl resonances at δ_C 172.8 and 178.2, two olefinic carbons at δ_C 123.5 and 144.7, and four anomeric carbons at δ_C 96.4, 105.7 × 2 and 106.5. The ^{1}H and ^{13}C NMR spectra of **6** and **7** were almost superimposable, except that the carbonyl signal (C-29) showed an upfield shift from δ_C 181.8 to 178.2, and two additional glucopyranosyl resonances (δ_H 5.01, δ_C 105.7; δ_H 6.31, δ_C 106.5) were observed (Table 2). The deshielded chemical shift of the anomeric proton at δ 6.31 suggested that it corresponded to a sugar linked by an ester function, therefore glycosidation took place at C-29 of the aglycone. Acid hydrolysis of **7** gave D-glucose and D-glucuronic acid, whose configurations were determined in the same way as in **6**. The attachment of sugars to the aglycone and their sequences were established by observation of HMBC correlations and in particular, between H-1 (δ_H 6.31) of inner Glc′ and C-29 (δ_C 178.2) of the aglycone, and between H-1 (δ_H 5.01) of the terminal Glc″ and C-6 (δ_C 69.9) of the inner Glc′. Therefore, structure of **7** was elucidated to be 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-3-epikatonic acid-29-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.

The molecular formula of angulasaponin C (**8**) was determined to be $C_{54}H_{88}O_{24}$ from its quasi-molecular ion at *m/z* 1119.5588 $[M-H]^-$ in the HRESIMS. The ^{1}H NMR spectrum showed six tertiary methyl groups at δ_H 0.88, 0.97, 0.97, 1.09, 1.13 and 1.38 and an olefinic proton at δ_H 5.17 (triplet like). The ^{13}C NMR spectrum presented signals for one carbonyl at δ_C 178.1, two olefinic carbons at δ_C 123.4 and 144.7, one oxygenated methine carbon at δ_C 83.1, and one oxygenated methylene carbon at δ_C 65.3. The ^{1}H and ^{13}C NMR spectroscopic data of **8** were reminiscent of those of 3β, 23-dihydroxyolean-12-en-29-oic acid (Van Le et al., 2009), except that C-3 and C-29 showed obvious glycosidation shifts. The HMQC spectrum of **8** showed correlations corresponding to four anomeric signals [δ_H 5.01 (1H, d, *J* = 8.0 Hz), δ_C 105.6; δ_H 5.22 (1H, d, *J* = 6.0 Hz), δ_C 104.6; δ_H 5.43 (1H, d, *J* = 8.0 Hz), δ_C 106.3; δ_H 6.30 (1H, d, *J* = 8.0 Hz), δ_C 96.3]. After acid hydrolysis of **8**, the detection of hydrolysate only gave glucose, which was in the β-D-configuration according to the *J* values of the anomeric protons and optical rotation. The linkage positions and sequence of the sugar moieties were established by observation of HMBC correlations between H-1 (δ_H 5.22) of Glc and C-3 (δ_C 83.1) of the aglycone, between H-1 (δ_H 5.43) of Glc′ and C-2 (δ_C 83.9) of Glc, between H-1 (δ_H 6.30) of Glc″ and C-29 (δ_C 178.1) of the aglycone, and between H-1 (δ_H 5.01) of Glc‴ and C-6 (δ_C 69.9) of Glc″. The reverse HMBC correlations of these signals further proved the linkage positions. Therefore, structure of **8** was elucidated as 3-*O*-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-3β,23-dihydroxyolean-12-en-29-oic acid-29-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside.

The HRESIMS of angulasaponin D (**9**) presented a quasi-molecular ion at *m/z* 809.4356 $[M-H]^-$, in accordance with a $C_{42}H_{66}O_{15}$ molecular formula. The ^{1}H NMR spectrum of **9** showed seven tertiary methyl groups at δ_H 0.92, 1.05, 1.16, 1.29, 1.31 × 2, and 1.85, and an olefinic proton at δ_H 5.42 (triplet like); its ^{13}C NMR spectrum presented signals for one carbonyl at δ_C 181.8, two olefinic carbons at δ_C 123.4 and 144.7, and two oxygenated methines at δ_C 89.6 (C-3) and 75.8 (C-22). The ^{1}H and ^{13}C NMR spectroscopic data of the aglycone moiety of **9** were almost superimposable with those of 3β, 22β-dihydroxyolean-12-en-29-oic acid (Kutney et al., 1992), except that C-3 showed obvious glycosidation shift ($\Delta\delta_C$ + 11.7 ppm). Configuration of the hydroxyl at C-22 position was established as β to account for the coupling constant of H-22 (1H, dd, *J* = 7.0, 2.0 Hz); in the opposite configuration, a much larger *trans* diaxial coupling constant would have been observed. The HMQC spectrum of **9** showed correlations corresponding to two anomeric signals [δ_H 5.01 (1H, d, *J* = 7.5 Hz), δ_C 105.6; δ_H 5.41 (1H, d, *J* = 8.0 Hz), δ_C 106.3]. Just as the above compounds, the sugars were identified as β-D-glucose and β-D-glucuronic acid on the basis of acid hydrolysis and analysis of the NMR spectroscopic data. The sequence of the sugars was established by observation of HMBC correlations between H-1 of Glc A (δ_H 5.01) and C-3 (δ_C 89.6) and between H-1 of Glc (δ_H 5.41) and C-2 of Glc A (δ_C 83.2). The reverse HMBC correlations of these signals further proved the linkage positions. Therefore, **9** was elucidated as 3-*O*-[β-D-glucopyranosyl (1→2)-β-D-glucuronopyranosyl]-3β, 22β-dihydroxyolean-12-en-29-oic acid.

Further to these, eight known compounds were identified through comparison of their spectroscopic data with those reported in the literature: 3-furanylmethyl β-D-glucopyranoside (**10**) (Kitagawa et al., 1983a), 2 β-*O*-β-D-glucopyranosyl-15α-hydroxykaur-l6-en-18-carboxylic acid (**11**) (Lang et al., 2013; Vargas et al., 1988), 2β-*O*-β-D-glucopyranosyl-15α-hydroxykaur-l6-en-18,19-dicarboxylic acid (**12**) (Lang et al., 2013; Piacente et al., 1996), and azukisaponins I–III, V and VI (**13**–**17**) (Kitagawa et al., 1983a,b,c).

Among the isolated compounds, compounds **1**–**3**, are the acetylated products of 3-furanylmethylβ-D-glucopyranoside (**10**) at the different positions of glucose, except position C-2. Concerned by the possibility that they could be artefacts produced by random migration of the acetyl under storage or under extraction conditions, a rapid extraction was performed anew and the extract was swiftly examined by LC/MS, which unambiguously showed

the presence of such compounds in *V. angularis* (see Supplementary material for experimental conditions). Compounds **5**, **11** and **12** are three *ent*-kaurane diterpenoids, with atractyligenin or carboxyatractyligenin as aglycone, which are isolated for the first time from the *Vigna* genus. The sulfates and esters of **11** and **12**, atractyloside and carboxyatractyloside were reported to be toxic to kidney or liver through inhibition of the adenine nucleotide translocase (ANT) in mitochondria (Stewart and Steenkamp, 2000; Vignais et al., 1978). Moreover, compound **12** isolated from raw coffee beans was recently reported to present a similar toxic activity, although this can be suppressed by degradation during roasting. Even so, there is a recommendation to control the content of **12** in raw coffee extracts when being used as the additives for food products (Lang et al., 2013). The seeds of *V. angularis* are one kind of widely consumed edible beans in Asia, and as a consequence, the presence of carboxyatractyligenin-glycosides in this plant deserves notice and precaution. Since the toxic carboxyatractyligenin-glycoside **12** decarboxylated to nontoxic **11** during coffee-roasting (Lang et al., 2013), it would be worth investigating the cooking procedure for the detoxification of red beans.

NO, one of the important pro-inflammatory mediators, is involved in some inflammatory disorders, such as rheumatoid arthritis, chronic hepatitis, and pulmonary fibrosis (Isomaki and Punnonen, 1997; Kanwar et al., 2009; Tilg et al., 1992). Suppression of the release of NO and other inflammatory mediators plays a critical role in the treatment of such diseases. Therefore, the *in vitro* NO production inhibitory model has been widely used for screening bioactive components with potential anti-inflammatory effect from natural medicines (Hsu et al., 2012; Li et al., 2012; Wang et al., 2013). Yu et al. (2011) reported that *V. angularis* ethanol extract dose-dependently suppressed the release of PGE_2 and NO in LPS-, poly(I:C)-, and pam3CSK activated macrophages. This plant was also incorporated as part of an anti-inflammatory diet to prevent and reduce risk factors for heart disease (Sato et al., 2008). However, the anti-inflammatory constituents are still unknown. Thus, in order to search for the potential anti-inflammatory components from this plant, the compounds isolated here were assessed for their inhibitory effects against LPS-induced NO production in RAW264.7 macrophages. Compounds **5**–**8**, **15** and **17** exhibited the most potent inhibitory activities with IC_{50} values ranging from 13 μM to 24 μM and the diterpenoids and saponins showed much better NO inhibition effects than the furanylmethyl glycosides (Table 3). In the screened diterpenoids, the diglycoside (compound **5**) showed a stronger inhibitory effect than the mono-glycoside (compound **12**), which suggests that the sugar moiety is related to the activity of the diterpenoids. While for saponins, not only the sugar moiety but also the aglycone has influence on the activity, and that of the carboxyl at C-29 may be prominent. When the C-29 carboxyl was reduced to a methyl group, the activity decreased, for example, the IC_{50} values for **13**, **14** and **16** were all larger than 50 μM.

3. Conclusions

Seventeen compounds, including four new furanylmethyl glycosides, angularides A –D (**1**–**4**), one new *ent*-kaurane diterpene glycoside, angularin A (**5**), and four new triterpenoid saponins, angulasaponins A–D (**6**–**9**), together with eight known compounds (**10**–**17**), were obtained from the 96% ethanol extract of the seeds of *V. angularis*. Compounds **5**–**8**, **15** and **17** showed potent NO production inhibitory effects in LPS-activated macrophages, with IC_{50} values ranging from 13 μM to 24 μM. These data suggest that not only the phenolic constituents, but also some non-phenolic compounds, such as saponins and diterpenoids are the active components of the red beans. Moreover, the quantity variance of the relatively toxic carboxyatractyligenin-glycosides in *V. angularis* during the processing deserves further study.

4. Experimental

4.1. General experimental procedures

Column chromatography (CC) was performed on silica gel 60 (40–63 μm, Merck) with Buchi pump manager C-615, pump module C-601 and Buchi fraction collector C-660. Preparative RP-18 CC was performed on a prefilled column for packing stand NM 50 with Lichrospher 100 RP-18 (12 μm, Merck), controlled by a La Prep-system, including fraction valve P 206, P110 pump and P311 detector (VWR International). Amberlite XAD-16 and Sephadex LH-20 (25–100 μm) were purchased from Sigma. TLC was carried out on pre-coated silica gel 60 GF_{254} plates (Merck), and detected by spraying with 1% vanillin H_2SO_4 ethanol solution, and then heating at 110 °C for 5 min. UV spectra were measured on a Lambda Perkin Elmer UV/vis spectrometer. Preparative HPLC (prep. HPLC) was performed on a Merck Hitachi chromatograph with a UV–vis detector L-7420, an interface D-7000, an autosampler L-7200 and a pump L-7150N. The column used for prep. HPLC is a Waters symmetry shield RP-18 column (7 μm, 19 × 300 mm). Lyophilization was run on a Christ lyophilizator (Germany). Optical rotations were measured on an Autopol-IV polarimeter (Rudolph Research, USA). FT-IR spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. NMR spectra were recorded on a Bruker Avance II-500 NMR spectrometer. ^1H–^1H COSY, HMQC, HMBC and NOESY NMR spectra were obtained. MS and HRESIMS were measured on a Bruker Daltonics Apex IV FT mass spectrometer or a Waters Xevo G2 Q Tof mass spectrometer.

4.2. Plant material

The seeds of *V. angularis* were purchased from Zizhou County, Shaanxi Province, China, in May 2009, and identified by one of us (YJ). A herbarium specimen is deposited in the Herbarium of the Pierre Fabre Research Institute (CBPF, Soual, France) under reference V600273.

4.3. Extraction and isolation

The seeds of *V. angularis* (4.0 kg) were extracted with EtOH: H_2O (7.5 L × 4, 96:4, v/v) under reflux conditions, each time for 1.5 h, and a residue (161.6 g) was produced. This extract was suspended in H_2O and partitioned with EtOAc to give the EtOAc extract (37.2 g). The aqueous layer was subjected to XAD-16 resin CC, and eluted with a gradient of H_2O and EtOH to give an aqueous eluate (89.5 g), a 20% aqueous EtOH eluate (5.5 g), 40% aqueous EtOH eluate (1.9 g) and 70% aqueous ethanol eluate (4.5 g).

Part of the EtOAc extract (32.1 g) was subjected to silica gel CC, and eluted with *n*-hexane–EtOAc (10:1 → 0:1) to obtain nine fractions. Fr. 9 (8.2 g) was further separated on silica gel CC, with $CHCl_3$–MeOH–H_2O (80:20:2 and 70:30:5) as eluents to afford five subfractions. Subfraction 2 (0.35 g) was purified by silica gel CC [eluting with $CHCl_3$-acetone (6:4)] and prep. HPLC [eluting with MeCN-0.05% TFA aqueous solution (12:88)] to furnish **2** (5.7 mg) and **3** (6.4 mg).

The 20% aqueous EtOH eluate (5.2 g) was submitted to silica gel CC, and eluted with $CHCl_3$–MeOH–H_2O gradients (70:30:10 and 65:35:10, lower phase) to give five fractions. Fr. 1 (0.82 g) was purified by Sephadex LH-20 CC (eluting with EtOH: H_2O, 8:2) to afford three subfractions. Subfraction 2 (0.21 g) was separated on silica gel CC (eluting with $CHCl_3$-acetone from 6:4 to 2:8) and prep. HPLC [eluting with MeCN–H_2O (25:75)] to yield **4** (1.6 mg) and **1**

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Table 1
NMR spectroscopic data of compounds **1**–**4** (in CD_3OD, 500 MHz for ^1H and 125 MHz for ^{13}C, δ in ppm and J in Hz).

No.	**1**		**2**		**3**		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
2	7.52 (m)	142.5	7.53 (m)	142.5	7.53 (m)	142.5	7.51 (m)	142.5
3		123.1		123.1		123.1		123.1
4	6.49 (d, 1.5)	111.7	6.49 (d, 1.5)	111.6	6.50 (d, 1.5)	111.7	6.49 (d, 1.5)	111.7
5	7.45 (t, 1.5)	144.5	7.45 (t, 1.5)	144.5	7.45 (t, 1.5)	144.5	7.44 (t, 1.5)	144.5
6	4.71 (d, 12.0) 4.56 (d, 12.0)	63.3	4.78 (d, 12.0) 4.59 (d, 12.0)	63.3	4.78 (d, 12.0) 4.60 (d, 12.0)	63.3	4.69 (d, 12.0) 4.56 (d, 12.0)	63.3
1′	4.34 (d, 8.0)	103.0	4.42 (d, 9.0)	102.8	4.37 (d, 8.0)	103.0	4.34 (d, 8.0)	103.0
2′	3.21 (dd, 8.0, 9.0)	75.3	3.46 (t, 9.0)	73.3	3.30 (o) [a]	75.0	3.21 (t, 8.0)	75.0
3′	3.35 (t, 9.0)	77.9	4.91 (t, 9.0)	79.1	3.53 (t, 9.5)	77.9	3.35 (t, 8.0)	77.9
4′	3.32 (o) [a]	71.7	3.35 (m)	69.9	4.75 (t, 9.5)	71.7	3.31 (o) [a]	71.7
5′	3.44 (m)	75.0	3.33 (m)	77.8	3.43 (m)	75.4	3.47 (m)	75.4
6′	4.41 (dd, 2.0, 12.0) 4.21 (dd, 6.0, 12.0)	64.8	3.91 (dd, 2.0, 12.0) 3.70 (dd, 5.5, 12.0)	62.5	3.63 (dd, 2.5, 12.0) 3.54 (dd, 6.0, 12.0)	64.6	4.46 (dd, 2.0, 12.0) 4.28 (dd, 6.0, 12.0)	64.6
7′						168.0		168.0
8′						123.3	5.92 (dq, 15.5, 1.5)	123.3
9′						146.8	7.04 (dq, 15.5, 7.0)	146.8
10′						18.0	1.90 (dd, 1.5, 7.0)	18.0
$\underline{CH_3}CO$-	2.07 (s)	20.7	2.10 (s)	21.1	2.09 (s)			
$CH_3\underline{CO}$-		172.8		172.7				

[a] Overlapped signal.

(37.1 mg). Fr. 2 (1.82 g) was recrystallized with MeOH to produce **10** (1.28 g). Fr. 5 (0.54 g) was first separated on Sephadex LH-20 CC (eluting with MeOH), and then on RP-18 CC (eluting with MeCN:0.05%TFA aqueous solution from 10:90 to 100:0), and finally on prep. HPLC [eluting with MeCN:H_2O (25:75)] to afford **5** (7.7 mg).

The 40% aqueous EtOH eluate (1.9 g) was purified using Sephadex LH-20 (MeOH: H_2O, 80:20, as eluent) and silica gel CC (eluting with $CHCl_3$–MeOH–H_2O, 80:20:2, 70:30:5 and 60:40:10) to give 5 fractions. Fr.1 (0.42 g) was further isolated on Sephadex LH-20 CC (MeOH: H_2O, 80:20, as eluent), and prep. HPLC (MeOH:0.05%TFA, 40:60) to give **11** (118.8 mg). Fr. 5 (0.23 g) was separated on Sephadex LH-20 CC (MeOH: H_2O, 80:20, as eluent) to furnish **12** (16.2 mg).

The 70% aqueous EtOH eluate (6.0 g) was subjected to silica gel CC, and eluted with $CHCl_3$–MeOH–H_2O (70:30:5 and 60:40:10) to give 5 fractions. Fr. 2 (0.28 g) was isolated by repeated prep. HPLC (MeCN:0.05%TFA, 29:71) to give **6** (3.5 mg), **9** (1.7 mg), **13** (8.7 mg), **14** (18.5 mg), **15** (7.2 mg) and **16** (13.9 mg), respectively. Fr. 4 (1.21 g) was separated on prep. HPLC (MeCN:0.05%TFA, 29:71) to give **7** (48.5 mg), **8** (26.8 mg) and **17** (372.7 mg).

Angularide A (**1**): hygroscopic powder, $[\alpha]_D^{18}$ −45 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.68) nm; IR (KBr) ν_{max} 3396, 2889, 1735, 1250, 1082, 1040 cm^{-1}; For ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Table 1; positive ESIMS *m/z* 325 $[M+Na]^+$; HRESIMS *m/z* 325.0884 $[M+Na]^+$ (calcd for $C_{13}H_{18}O_8Na$, 325.0894).

Angularide B (**2**): hygroscopic powder; $[\alpha]_D^{18}$ −42 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.45) nm; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Table 1; positive ESIMS *m/z* 325 $[M+Na]^+$, 627 $[2M+Na]^+$; HRESIMS *m/z* 325.0885 $[M+Na]^+$ (calcd for $C_{13}H_{18}O_8Na$, 325.0894).

Angularide C (**3**): hygroscopic powder; $[\alpha]_D^{18}$ −44 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.62) nm; for ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Table 1; positive ESIMS *m/z* 325 $[M+Na]^+$, 627 $[2M+Na]^+$; HRESIMS *m/z* 325.0880 $[M+Na]^+$ (calcd for $C_{13}H_{18}O_8Na$, 325.0894).

Angularide D (**4**): hygroscopic powder; $[\alpha]_D^{18}$ −56 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.81) nm; For ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 125 MHz) spectroscopic data, see Table 1; positive ESIMS *m/z* 351 $[M+Na]^+$, 679 $[2M+Na]^+$; negative ESIMS *m/z* 327 $[M-H]^-$, 655 $[2M-H]^-$; HRESIMS *m/z* 351.1042 $[M+Na]^+$ (calcd for $C_{15}H_{20}O_8Na$, 351.1050).

Angularin A (**5**): amorphous powder; $[\alpha]_D^{18}$ −91 (*c* 0.01, MeOH); IR (KBr) ν_{max} 3401, 2932, 1657, 1059 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz), δ 5.36 (1H, d, *J* = 1.5 Hz, Api-1), 5.19 (1H, br s, H-17a), 5.08 (1H, br s, H-17b), 4.48 (1H, d, *J* = 8.0 Hz, Glc-1), 4.21 (1H, m, H-2), 4.01 (1H, d, *J* = 9.5 Hz, Api-4a), 3.93 (1H, d, *J* = 1.5 Hz, Api-2), 3.84 (1H, dd, *J* = 12.0, 2.0 Hz, Glc-6a), 3.79 (1H, s, H-15), 3.72 (1H, d, *J* = 9.5 Hz, Api-4b), 3.67 (1H, dd, *J* = 12.0, 5.5 Hz, Glc-6b), 3.62 (2H, d, *J* = 2.0 Hz, Api-5), 3.48 (1H, t, *J* = 9.0 Hz, Glc-3), 3.31 (1H, overlapped, Glc-2), 3.29 (1H, overlapped, Glc-4), 3.27 (1H, m, Glc-5), 2.73 (1H, m, H-3a), 2.72 (1H, m, H-13), 2.36 (1H, dd, *J* = 12.0, 3.5 Hz, H-1a), 1.88 (1H, d, *J* = 11.0 Hz, H-14a), 1.87 (1H, m, H-6a), 1.86 (1H, m, H-5), 1.69 (1H, m, H-11a), 1.68 (1H, m, H-6b), 1.66 (1H, m, H-7a), 1.64 (1H, m, H-12a), 1.51 (1H, m, H-11a), 1.48 (1H, m, H-12b), 1.46 (1H, m, H-7b), 1.42 (1H, m, H-3b), 1.40 (1H, dd, *J* = 11.0, 5.0 Hz, 14b), 1.15 (1H, d, *J* = 7.5 Hz, H-9), 1.02 (3H, s, H-20), 0.88 (1H, t, *J* = 12.0 Hz, H-1b). ^{13}C NMR (CD_3OD, 125 MHz), δ 175.8 (C-18), 175.4 (C-19), 160.4 (C-16), 110.8 (Api-1), 109.0 (C-17), 101.4 (Glc-1), 83.5 (C-15), 80.7 (Api-3), 79.1 (Glc-2), 78.7 (Glc-3), 78.2 (Api-2), 77.7 (Glc-5), 75.4 (Api-4), 73.1 (C-2), 71.7 (Glc-4), 66.3 (Api-5), 62.7 (Glc-6), 59.3 (C-4), 55.0 (C-9), 52.3 (C-5), ~49.0 (C-1, C-8, overlapped with signal of CD_3OD), 43.7 (C-13), 41.4 (C-10), 41.0 (C-3), 37.3 (C-14), 36.3 (C-7), 33.7 (C-12), 24.2 (C-6), 19.3 (C-11), 17.8 (C-20); positive ESIMS *m/z* 681 $[M+Na]^+$; negative ESIMS *m/z* 657 $[M-H]^-$, 613 $[M-H-COO]^-$; HRESIMS *m/z* 676.3196 $[M+NH_4]^+$ (calcd for $C_{31}H_{50}NO_{15}$, 676.3175).

Angulasaponin A (**6**): amorphous powder; $[\alpha]_D^{18}$ −15 (*c* 0.08, MeOH); IR (KBr) ν_{max} 3391, 2927, 1686, 1204 cm^{-1}; for ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) spectroscopic data, see Table 2; negative ESIMS *m/z* 793 $[M-H]^-$; HRESIMS *m/z* 793.4370 $[M-H]^-$ (calcd for $C_{42}H_{65}O_{14}$, 793.4374).

Angulasaponin B (**7**): amorphous powder; $[\alpha]_D^{18}$ −22 (*c* 0.09, MeOH); IR (KBr) ν_{max} 3343, 2944, 1739, 1620, 1031 cm^{-1}; For ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) spectroscopic data, see Table 2; positive ESIMS *m/z* 1141 $[M+Na]^+$; negative ESIMS *m/z* 1117 $[M-H]^-$; HRESIMS *m/z* 1117.5436 $[M-H]^-$ (calcd for $C_{54}H_{85}O_{24}$, 1117.5431).

Angulasaponin C (**8**): amorphous powder; $[\alpha]_D^{18}$ −35 (*c* 0.09, MeOH); IR (KBr) ν_{max} 3400, 2932, 1717, 1629, 1032 cm^{-1}; for ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) spectroscopic data, see Table 2; negative ESIMS *m/z* 1119 $[M-H]^-$; HRESIMS *m/z* 1119.5588 $[M-H]^-$ (calcd for $C_{54}H_{88}O_{24}$, 1119.5587).

Angulasaponin D (**9**): amorphous powder; $[\alpha]_D^{18}$ −18 (*c* 0.08, MeOH); IR (KBr) ν_{max} 3393, 2927, 1645, 1025 cm^{-1}; For ^1H NMR

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Table 2
NMR spectroscopic data of compounds **6**–**9** (in pyridine-d_5, 500 MHz for ^{1}H and 125 MHz for ^{13}C, δ in ppm and J in Hz).

No.	**6**		**7**		**8**		**9**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.44 (m) 0.87 (o)	39.4	1.45 (dd, 10.5, 3.0) 0.88 (o)	39.2	1.52 (m) 0.99 (o)	39.2	1.48 (br d, 12.0) 0.88 (o)	39.2
2	2.27 (m) 1.91 (m)	27.2	2.27 (dd, 13.0, 3.0) 1.92 (dd, 13.0, 1.0)	27.0	2.35 (br d, 11.0) 2.04 (o)	26.4	2.27 (dd, 13.0, 4.0) 1.91 (o)	27.0
3	3.30 (dd, 12.0, 4.5)	89.7	3.36 (dd, 12.0, 4.5)	89.6	4.24 (dd, 9.0,4.0)	83.1	3.32 (dd, 11.5, 4.0)	89.6
4		40.2		40.0		43.9		39.9
5	0.74 (d, 11.0)	56.3	0.73 (d, 12.0)	56.1	1.55 (o)	48.3	0.76 (d, 11.0)	56.2
6	1.52 (o)[a] 1.34 (m)	19.1	1.51 (o) 1.33 (o)	18.8	1.55 (o) 1.40 (o)	18.6	1.54 (m) 1.36 (o)	18.9
7	1.47 (m) 1.29 (o)	33.5	1.42 (m) 1.24 (dd,13.0, 3.0)	33.3	1.56 (o) 1.23 (o)	33.0	1.50 (m) 1.35 (o)	33.6
8		40.7		40.5		40.5		40.4
9	1.59 (o)	48.4	1.51 (o)	48.1	1.62 (o)	48.2	1.62 (dd, 8.0, 9.5)	48.3
10		37.4		37.2		37.1		37.2
11	1.87 (m)	24.4	1.81 (m)	24.2	1.82 (m)	24.2	1.89 (o)	24.2
12	5.30 (t-like)	123.1	5.18 (t-like)	123.5	5.17 (t-like)	123.4	5.42 (br s)	123.4
13		145.2		144.7		144.7		144.7
14		42.5		42.2		42.2		43.0
15	1.80 (dd, 13.5, 3.0) 0.98 (o)	27.1	1.72 (dd, 13.0, 4.0) 0.90 (o)	26.7	1.68 (m) 0.82 (m)	26.7	2.04 (td, 12.0, 2.5) 1.48 (br d, 12.0)	29.3
16	2.18 (m) 0.89 (o)	27.9	1.99 (td,13.5, 4.0) 0.78 (d, 13.5)	27.5	1.96 (td, 13.0, 3.0) 0.73 (d, 13.0)	27.4	1.90 (o) 1.06 (o)	26.7
17		33.4		33.0		33.0		38.4
18	2.18 (m)	47.1	2.06 (dd, 13.5, 4.0)	46.7	2.04 (dd, 13.0, 4.0)	46.7	2.54 (br d, 13.5)	45.0
19	2.60 (t, 13.0) 1.74 (br d, 13.0)	42.1	2.48 (t, 13.5) 1.66 (br d, 13.5)	41.0	2.45 (t, 13.0) 1.64 (o)	40.9	2.83 (t, 13.5) 1.72 (br d, 13.5)	41.9
20		43.5		43.5		43.4		42.8
21	2.30 (dd, 13.0, 3.0) 1.74 (br d, 13.0)	30.5	2.14 (td, 13.5, 3.5) 1.64 (br d, 13.5)	29.8	2.10 (td, 13.0, 3.5) 1.62 (o)	29.8	2.66 (dd, 14.0, 2.5) 2.16 (dd, 14.0, 7.0)	38.2
22	1.59 (o) 1.40 (m)	37.1	1.44 (dd, 13.5, 3.0) 1.28 (o)	36.5	1.43 (m) 1.26 (m)	36.5	4.03 (dd, 7.0, 2.0)	75.8
23	1.31 (s)	28.7	1.31 (s)	28.5	3.79 (d, 11.0) 4.38 (d, 11.0)	65.3	1.31 (s)	28.5
24	1.15 (s)	17.4	1.15 (s)	17.2	1.13 (s)	13.8	1.16 (s)	17.2
25	0.90 (s)	16.2	0.89 (s)	16.0	0.97 (s)	16.6	0.92 (s)	16.1
26	0.99 (s)	17.6	0.95 (s)	17.4	0.97 (s)	17.4	1.05 (s)	17.7
27	1.27 (s)	26.7	1.14 (s)	26.5	1.09 (s)	26.5	1.31 (s)	25.9
28	0.97 (s)	29.0	0.89 (s)	28.6	0.88 (s)	28.5	1.29 (s)	21.3
29		181.8		178.2		178.1		181.8
30	1.50 (s)	20.6	1.39 (s)	19.9	1.38 (s)	19.9	1.85 (s)	25.3
Glc A			Glc A		Glc		Glc A	
1	5.02 (d, 8.0)	105.9	5.04 (d, 7.5)	105.7	5.22 (d, 6.0)	104.6	5.01 (d, 7.5)	105.6
2	4.35 (dd, 8.0)	83.6	4.35 (o)	83.4	4.29 (o)	83.9	4.34 (o)	83.2
3	4.40 (t, 8.0)	78.4	4.40 (t, 9.0)	78.1	4.15 (t, 8.0)	78.4	4.40 (t, 9.0)	78.2
4	4.57 (t, 8.0)	73.8	4.57 (t, 9.0)	73.5	4.20 (o)	72.0	4.55 (m)	73.5
5	4.62 (d, 8.0)	77.9	4.61 (d, 9.0)	77.8	4.31 (o)	78.4	4.60 (d, 9.5)	77.7
6		173.2		172.8	4.52 (dd,11.5, 2.5) 4.46 (o)	63.0		173.1
Glc			Glc		Glc′		Glc	
1	5.42 (d, 8.0)	106.7	5.42 (d, 8.0)	106.5	5.43 (d, 8.0)	106.3	5.41 (d, 8.0)	106.3
2	4.14 (t, 8.0)	77.7	4.14 (o)	77.5	4.12 (t, 8.0)	77.2	4.14 (t, 8.0)	77.4
3	4.27 (t, 8.0)	78.6	4.26 (t, 9.0)	78.4	4.24 (o)	78.8	4.26 (t, 8.0)	78.3
4	4.35 (t, 8.0)	72.4	4.34 (o)	72.2	4.19 (t, 7.0)	71.9	4.33 (t, 8.0)	72.2
5	3.95 (m)	78.8	3.95 (m)	78.8	3.94 (m)	78.8	3.95 (m)	78.6
6	4.52 (dd, 12.0, 3.0) 4.48 (dd, 12.0, 5.0)	63.4	4.49 (dd, 12.0, 3.0) 4.34 (o)	63.2	4.46 (o) 4.35 (dd, 12.0, 5.0)	63.1	4.52 (dd, 12.0, 3.0) 4.48 (dd, 12.0, 4.5)	63.2
Glc′			Glc′		Glc″			
1			6.31 (d, 8.0)	96.4	6.30 (d, 8.0)	96.3		
2			4.13 (o)	74.5	4.12 (t, 8.0)	74.5		
3			4.19 (t, 8.0)	79.0	4.20 (o)	79.0		
4			4.31 (t, 8.0)	71.4	4. 31 (o)	71.4		
5			4.26 (m)	78.4	4.30 (o)	78.4		
6			4.72 (dd, 12.0, 3.0) 4.35 (o)	69.9	4.72 (dd, 10.0, 2.0) 4.34 (dd, 11.0, 5.0)	69.9		
Glc″			Glc″		Glc‴			
1			5.01 (d, 8.0)	105.7	5.01 (d, 8.0)	105.6		
2			3.99 (t, 8.0)	75.5	3.99 (t, 8.0)	75.5		
3			4.18 (t, 8.0)	78.6	4.17 (o)	78.8		
4			4.35 (o)	72.0	4.20 (o)	71.9		
5			3.87 (m)	78.8	3.87 (m)	78.7		
6			4.49 (dd, 12.0, 3.0) 4.34 (o)	63.1	4.46 (o) 4.35 (dd, 12.0, 5.0)	63.1		

[a] Overlapped signal.

(pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) spectroscopic data, see Table 2; HRESIMS m/z 809.4356 $[M-H]^-$ (calcd for $C_{42}H_{65}O_{15}$, 809.4323), 833.4294 $[M+Na]^+$ (calcd for $C_{42}H_{66}O_{15}Na$, 833.4299).

4.4. Acid hydrolysis of compounds *1* and *5*–*8*

Compounds **1**, **5**–**8** (2 mg for each compound) were heated in 2 mol/L aqueous CF_3COOH (5 mL) at 110 °C for 6 h in a sealed tube.

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Table 3
NO inhibitory activities of the isolated compounds[a,b].

Compounds	IC_{50}/μM	Compounds	IC_{50}/μM
5	22	**9**	13
6	22	**15**	15
7	14	**17**	14
8	13	Indomethacin [c]	7

[a] Inhibition of the 96% ethanol crude extract of *V. angularis* was only 21.46% at 50 μg/mL.
[b] The data of **1**, **3** and **4** were not reached due to quantity limitation, and the IC_{50} values of **2**, **10**–**14**, and **16** were more than 50 μM.
[c] Positive control.

Then, the sugars and aglycone were separated by liquid–liquid partitioning between $CHCl_3$ and H_2O. The aqueous layer was repeatedly concentrated with MeOH until neutral, and the residue was separated by Sep-Pak C18 cartridge column ($H_2O \rightarrow$ MeOH). The H_2O-eluted fraction was subjected to HPLC analysis under following conditions: HPLC column, Alltech Prevail carbohydrate ES, 5 μm, 4.6 mm i.d. × 250 mm; detection, Chiralyser-mp optical rotation detector (IBZ Messtechnik Company, Germany); mobile phase, CH_3CN–H_2O (90:10, v/v); flow rate 0.7 mL/min. Detection of D-glucose from **1** and **8**, D-glucose and D-apiose from **5**, and D-glucose and D-glucuronic acid from **6** and **7** was carried out by comparison of their retention times and optical rotations with those of the authentic samples (D-glucose, t_R 43.44 min, positive; D-apiose, t_R 12.43 min, positive; D-glucuronic acid, t_R 5.23 min, positive).

4.5. Cell culture and viability assay

Cells were seeded at the density of 5×10^4 cells per well in 48-well culture plate, then treated with various concentrations of each compound and LPS (1.0 μg/mL). The cell viability of the cultured cells was detected by MTT method. Briefly, RAW 264.7 cells were incubated with MTT solution (500 μL, 0.5 mg/mL in medium) for 4 h at 37 °C, and then the supernatants were removed and the residues were dissolved in DMSO (500 μL). The absorbance was detected at 540 nm using a microplate reader. The results were displayed in percentage of control samples. Indomethacin was used as the positive control.

4.6. Measurement of NO production

The NO concentration was detected by the Griess reagent (Green et al., 1982). Briefly, RAW 264.7 cells were treated with LPS (1.0 μg/mL) and compounds for 24 h. After that, culture supernatant (400 μL) was allowed to react with Griess reagent (100 μL, 1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) for 10 min at room temperature in the dark. Then, the optical density (100 μL per well) was measured at 540 nm using a microplate reader. Sodium nitrite in medium was used to calculate a standard curve in the assay. Inhibition (%) = $(A_{LPS\ treated} - A_{\ untreated})/(A_{LPS\ treated} - A_{LPS+sample\ treated}) \times 100$. The experiments were performed in triplicate. Indomethacin was used as the positive control.

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Appendix A. Supplementary data

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