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Steroidal saponins with anti-inflammatory activity from *Tribulus terrestris* L.

Miaojie Yang[1,2], Mahmood Brobbey Oppong[3], Jingrui Di[1,2], Qing Yuan[1], Yanxu Chang[1,2], Miaomiao Jiang[1], Shijie Cao[1], Pengzhi Dong[1], Lin Li[1], Yan Xie[1], Xiaoying Luan[1], Limin Hu[1], Lijuan Chai[1], Peng Zhang[1,2,*]

Abstract

Objective: *Tribulus terrestris* L. (*T. terrestris*) is a highly valuable traditional Chinese medicine used to treat stroke, inflammation, pulmonary fibrosis, liver cancer, and urolithiasis. To identify the basic substance responsible for the anti-inflammatory effect of TST (total saponins of *Tribulus*), its chemical composition was systematically studied, and its effect of inhibiting nitric oxide generation and the expression of related inflammatory factors were determined.

Methods: To separate chemical constituents from *T. terrestris* by column chromatography. Spectroscopic methods, including 1D and 2D nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) techniques, were used to elucidate the isolated compounds. The anti-inflammatory activities of TST and several compounds were evaluated *in vitro*.

Results: Fifteen steroidal saponins, including 9 furostanol steroidal saponins (1, 2, 3, 4, 5, 6, 7, 8, and 15) and 6 isospirostanol steroidal saponins (9, 10, 11, 12, 13, and 14), were isolated from *T. terrestris*. TST significantly decreased the expression of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in RAW 264.7 cells stimulated by lipopolysaccharides. Compounds 13 and 15 evidently reduced TNF-α expression. Compounds 6, 10, 12, 13, and 15 markedly reduced IL-6 expression.

Conclusions: Compounds 1 was a novel furostanol steroidal saponin, named 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-12-carbonyl-20(22)-en-3*β*, 26-diol-3-*O*-{*β*-D-xylopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside}. Compounds 2 was isolated from the family Zygophyllaceae for the first time, and 5 and 6 were isolated from the *Tribulus* genus. TST and compounds 6, 10, 12, 13, and 15 exerts anti-inflammatory activity.

Keywords: Interleukin-6, Nitric oxide (NO) inhibition, Steroidal saponins, *Tribulus terrestris* L., Tumor necrosis factor-α

Introduction

Tribulus terrestris L. (*T. terrestris*) is a traditional Asiatic medicinal plant, which is used for various medicinal purposes in the form of whole dried herbs and ripe fruits[1]. It consists of numerous chemical components, and is especially rich in steroidal saponins[2–7]. Modern pharmacological research has demonstrated that *T. terrestris* has neuroprotection[8], anti-inflammatory, anti-fibrosis[9], anti-cancer[10], and anti-lithiatic[11] effects, which highlight its significant impacts on the treatment of ischemic stroke[12], inflammation, pulmonary fibrosis[9], liver cancer[10], and urolithiasis[11].

Inflammation, which commonly causes atherosclerosis and triggers thrombotic plaque complications, plays an important role in vascular diseases[13]. As an inflammatory mediator, nitric oxide (NO) regulates the occurrence and development of inflammation[14]. The inhibitory effect of a compound on NO production reflects its anti-inflammatory activity[15]. Meanwhile, NO overproduction is related to an increase in pro-inflammatory cytokines TNF-α and IL-6, which are also involved in both the induction and progression of inflammatory diseases. TNF-α is a predominant inflammatory cytokine responsible for modulation of the immune system and plays a critical role in most inflammatory disorders[16]. IL-6 is a multifunctional cytokine that plays a key role in chronic inflammatory diseases and immune responses. High concentrations of IL-6 can induce various pathological states, leading to inflammatory-related diseases[17].

A previous study demonstrated that saponins from *T. terrestris* have anti-inflammatory effects in a rat model of neuropathic pain caused by vincristine[18]. The *T. terrestris* extract also showed anti-inflammatory effects in rats with formalin-induced inflammation[19]. The ethanol extract of *T. terrestris* showed dose-dependent inhibition of NO production in lipopolysaccharides (LPS)-stimulated RAW 264.7 cells[20]. Previous studies have suggested that chemical components of *T. terrestris* could exert certain anti-inflammatory activities, but it is

Miaojie Yang and Mahmood Brobbey Oppong contributed equally to this work.

[1] Tianjin State Key Laboratory of Component-based Chinese Medicine, Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin, China; [2] Tianjin Key Laboratory of Phytochemistry and Pharmaceutical Analysis, Tianjin University of Traditional Chinese Medicine, Tianjin, China; [3] Department of Pharmaceutical Chemistry, School of Pharmacy, College of Health Sciences, University of Ghana, Ghana

[*] Corresponding author. Peng Zhang, Tianjin State Key Laboratory of Component-based Chinese Medicine, Institute of Traditional Chinese Medicine, Tianjin University of Traditional Chinese Medicine; Tianjin Key Laboratory of Phytochemistry and Pharmaceutical Analysis, Tianjin 301617, China, E-mail: zhangpeng@tjutcm.edu.cn.

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still unclear which exact compound exerts anti-inflammatory activity and whether it is through the NO production inhibition pathway.

In our continued effort to discover the therapeutic basis of *T. terrestris*, we used spectroscopic methods, including 1D and 2D nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) techniques, to elucidate a novel compound with 14 known sub-compounds. To evaluate their anti-inflammatory activities, the inhibitory effects of the isolated compounds on LPS-induced NO production were examined in RAW 264.7 cells.

Materials and methods

General experimental procedures

1D- and 2D-NMR experiments were performed on a Bruker AVANCE spectrometer with tetramethylsilane as an internal standard. Mass spectra were recorded using a Waters Synapt G2 mass spectrometer. Silica gel (200–300 μm; branch of Qingdao Haiyang Chemical Co., Ltd., Qingdao, P.R. China), RP-18 (ODS-A-HG; 5–50 μm; YMC Co., Ltd., Japan), and dextran gel (Sephadex LH-20, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were used for column chromatography. Thin-layer chromatography (TLC) was performed using Merck silica gel GF254. An Agilent 1260 HPLC system (Agilent Corp., USA) was used to perform HPLC separation, including a UV detector, a YMC Pack ODS-A column (250 mm × 10 mm, 5 μm particle size, YMC Co., Ltd., Japan), and a ZORBAX SB-C_{18} Prep HT column (4.6 mm × 150 mm, 5 μm particle size, Agilent Corp., USA). All other reagents used in this study were of chromatographic grade.

Material

T. terrestris extract (total saponin) was obtained from Jilin Aodong Pharmaceutical Group Co. Ltd. (Jilin, China). A voucher specimen (No. 20161104) was deposited in the Tianjin State Key Laboratory of Component-based Chinese Medicine.

Extraction and isolation

T. terrestris extract (70 g) was dissolved in 100 mL of 40% acetonitrile-H_2O. The solution was subjected to an ODS silica gel column and eluted with CH_3CN-H_2O (5:100 to 100:0), yielding six major fractions based on TLC analysis. Fraction 2 (1.5 g) was subsequently subjected to silica gel column elution with CH_2Cl_2-MeOH (100:3 to 100:20) and CH_2Cl_2-MeOH-H_2O (10:3:1 to 5:3:1) to yield four sub-fractions (Fr. 2-1 to 2-4). Fraction 2-1 (7.0 g) was further purified by preparative HPLC using CH_3CN-H_2O (22:78) to yield compounds 3 (40.0 mg, t_R = 47.3 min) and 7 (16 mg, t_R = 58.6 min). Fr. 2-2 was separated over Sephadex LH-20 (MeOH-H_2O) and combined with preparative HPLC with CH_3CN-H_2O (20:80) to generate compounds 1 (20.3 mg, t_R = 50.5 min) and 8 (165.1 mg, t_R = 29.0 min). Fr. 2-3 was subjected to Sephadex LH-20 (MeOH-H_2O) and preparative HPLC with CH_3CN-H_2O (25:75) to yield compound 5 (160.2 mg, t_R = 33.5 min). Fraction 3 (2 g) was isolated using silica gel column chromatography with CH_2Cl_2-MeOH-H_2O (10:3:1 to 4:3:1) to obtain two sub-fractions (Fr. 3-1 to 3-2). Fr. 3-1 was subjected to Sephadex LH-20 (MeOH-H_2O) and combined with preparative HPLC with (CH_3CN-H_2O, 28:72) to yield compound 6 (15 mg, t_R = 61.6 min). Fr. 3-2 was separated by Sephadex LH-20 (CH_2Cl_2-MeOH) and preparative HPLC with CH_3CN-H_2O (30:70) to generate compound 15 (20 mg, t_R = 40.0 min). Fraction 4 (5.2 g) was divided into three sub-fractions (Fr. 4-1 to Fr. 4-3) using silica gel column chromatography with CH_2Cl_2-MeOH (100:3 to 100:35). Fr. 4-1 was subjected to a silica gel column (CH_2Cl_2-MeOH, 100:0 to 100:4) to yield compounds 14 (13 mg) and 11 (10 mg). Fr. 4-2 was separated using preparative HPLC (CH_3CN-H_2O, 30:70) to obtain compound 4 (15 mg, t_R=21.8 min). Fr. 4-3 was subjected to Sephadex LH-20 (MeOH-H_2O) and preparative HPLC with CH_3CN-H_2O (30:70) to yield compound 9 (7.5 mg, t_R = 35.6 min). Fraction 5 (1.5 g) was isolated *via* silica gel column chromatography using CH_2Cl_2-MeOH (100:0 to 0:100) to yield compounds 2 (20.4 mg) and 14 (31.8 mg). Fraction 6 (3 g) was separated on a silica gel column using CH_2Cl_2-MeOH (100:0 to 100:4), resulting in the isolation of compound 13 (20 mg).

^{1}H-NMR (400 MHz, C_5D_5N), ^{13}C-NMR (100 MHz, C_5D_5N), HSQC, HSBC, and TOCSY spectra of compound 1 are shown in Table 1, Figure S1, Figure S2, Figure S3, Figure S4, and Figure S5 in the Supplementary Materials, http://links.lww.com/AHM/A8 respectively.

Biological assays

Cell culture

RAW 264.7 cell line obtained from the Cell Bank of Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, Logan, UT, USA) in a fully humidified incubator containing 5% CO_2. Lipopolysaccharides (Escherichia coli 055: B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay

Cell viability was assessed using a CCK8 assay. Briefly, 100 μL cells (1×10^5 cells/mL) were plated in 96-well plates, incubated overnight, and further treated with different concentrations of the tested compounds. DMEM was used as the vehicle control group and DMEM was used as a blank group. After 24 h of incubation, 10 μL of CCK8 reagent was added to 100 μL of medium in each well and incubated for 2 h in a CO_2 incubator at 37°C. Absorbance was measured at a wavelength of 490 nm. Cell viability was calculated using the following formula: cell viability (%) = (OD_{490} (treated cell culture) − OD_{490} (blank))/[OD_{490} (control cell culture) − OD_{490} (blank)] × 100.

Determination of NO production

Griess reaction was used to measure nitrite production, an indicator of NO synthesis, in the supernatant of cultured macrophages RAW 264.7. Briefly, 100 μL of the cell suspension (2×10^5 cells/mL) was seeded into 96-well plates, incubated for 24 h, and further stimulated with 6.25 ng/mL of LPS for 24 h in the presence or absence of the test compounds. The cell culture supernatant (50 μL)

Table 1

NMR spectroscopic data for compound 1 (C_5D_5N)

No.	δ_H (400 MHz)	δ_C (125 MHz)	HMBC
1	1.36 (1H, m)	37.1	
	0.72 (1H, m)		
2	1.57 (1H, m)	30.0	
	1.93 (1H, m)		
3	3.85 (1H, m)	77.0	Gal-1″
4	1.71 (1H, m)	34.5	
	1.93 (1H, m)		
5	1.19 (1H, m)	44.8	
6	1.18 (2H, m)	29.0	C-4, 8, 10
7	1.57 (2H, m)	32.2	
8	1.67 (1H, m)	34.5	
9	0.84 (1H, m)	55.9	C-1
10		36.7	
11	2.27 (1H, m)	38.6	C-12, 13
	2.39 (1H, t, J=13.2 Hz)		
12		213.2	
13		57.9	
14	1.16 (1H, m)	54.5	
15	2.17 (2H, m)	34.2	
16	4.74 (1H, m)	83.4	
17	3.42 (1H, d, J=10.4 Hz)	56.6	C-12, 14, 20
18	0.93 (3H, s, H-18)	14.5	C-12, 13, 14, 17
19	0.88 (3H, s, H-19)	12.2	C-1, 5, 9
20		103.6	
21	1.77 (3H, s, H-21)	12.0	C-17, 20, 22
22		153.5	
23	2.25 (2H, m)	24.1	
24	0.76 (1H, m)	31.8	
25		33.9	C-27
26	3.62 (1H, m)	75.3	C-24, 25, 27
	3.89 (1H, m)		
27	1.02 (3H, d, J=6.8 Hz)	17.7	C-24, 25, 26
26-O-Glc-1′	4.84 (1H, d, J=7.6 Hz)	105.3	
2′		75.6	
3′		79.0	
4′		72.1	
5′		78.9	
6′		63.2	
3-O-Gal-1′	4.86 (1H, d, J=7.6 Hz)	100.5	C-3
2″		76.9	Gal-1″
3″		77.0	
4″		81.7	Glc-1‴
5″		76.2	
6″		60.7	
Glc-1‴	5.00 (1H, d, J=7.6 Hz)	105.4	Gal-4″
2‴		81.9	Xly′-1⁗
3‴		88.0	Xly″-1⁗′
4‴		70.8	
5‴		78.1	
6‴		63.2	
Rha-1⁗	6.21 (1H, brs)	102.3	Gal-2″
2⁗		72.8	
3⁗		73.1	
4⁗		74.4	
5⁗		69.7	
6⁗	1.72 (3H, d, J=6.0 Hz)	18.8	
Xly′-1⁗′	5.44 (1H, d, J=7.6 Hz)	106.2	Glc-2‴
2⁗′		75.5	
3⁗′		79.4	
4⁗′		71.2	
5⁗′		68.0	
Xly"-1⁗′′	5.26 (1H, d, J=7.6 Hz)	105.7	Glc-3‴
2⁗′′		75.4	
3⁗′′		79.1	
4⁗′′		71.1	
5⁗′′		67.7	

or standard material (50 μL) was added to the 96-well plates, then reacted with 50 μL of Griess reagent I and 50 μL of Griess reagent II, and incubated for 15 min. Absorbance was measured at a wavelength of 540 nm. NO production was calculated using a $NaNO_2$ standard curve.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were stimulated with LPS (6.25 ng/mL) and combined with different concentrations (1 μM) of test compounds for 24 h. After 24 h of stimulation, 100 μL of the sample medium was collected for ELISA. The levels of TNF-α (CME0004, 4A Biotech, China) and IL-6 (CME0006, 4A Biotech, China) were measured using an ELISA kit. All procedures were performed in accordance with the manufacturer's instructions. Absorbance was measured at 450 nm using a Tecan microplate reader (Spark, TECAN, Switzerland). The concentration of compound 8 was 0.1 μM.

Results

Structural elucidation of isolated compounds

Compound 1 was obtained as a white, amorphous powder. The solution of compound 1 turned red with Ehrlich's reagent, indicating furostan saponin. The molecular formula was established as $C_{61}H_{98}O_{31}$ based on high resolution-electron spray ionization-mass spectrometry [(m/z 1,327.6177 $[M+H]^+$), calculated. 1,326.6092]. The positive mass spectrum exhibited ion peaks at m/z 593.3699 $[M+H\text{-}132\times2\text{-}162\times2\text{-}146]^+$ and 431.3144 $[M+H\text{-}132\times2\text{-}162\times3\text{-}146]^+$, indicating the presence of three hexoses, two pentoses, and one rhamnose.

The ^{1}H-NMR spectrum of compound 1 displayed signals characteristic of three methyl groups at δ_H 0.93 (3H, s, CH_3-18), 0.88 (3H, s, CH_3-19), and 1.77 (3H, s, CH_3-21), one methyl doublet at δ_H 1.02 (3H, d, J=6.8 Hz, CH_3-27), and signals for five anomeric protons at δ_H 6.21(1H, brs), 4.84 (1H, d, J=7.6 Hz), 4.86 (1H, d, J=7.6 Hz), 5.00 (1H, d, J=7.6 Hz), and 5.26 (1H, d, J=7.6 Hz). The C-25 configuration was inferred to be *R* because of the different chemical shifts of the geminal protons at CH_3-26 ($\Delta\delta ab$=0.27 ppm)[21].

In the ^{13}C-NMR spectrum recorded for C_5D_5N, one carbonyl signal at δ_C 213.2 (C-12), two olefinic carbon signals at δ_C 103.6 (C-20) and 153.5 (C-22), and C-26 at δ_C 75.3 suggested that the F ring is an open loop in this structure. The five anomeric carbon signals at δ_C 106.2, 105.7, 105.4, 105.3, and 100.5, together with the coupling constant of proton signals, explained the β-D orientations of these sugars.

In the HMBC spectrum (Figure 2 and Figure S4, http://links.lww.com/AHM/A8), the methyl protons at δ_H 1.02 (CH_3-27) revealed correlations with the carbons at δ_C 33.9 (C-25), 75.3 (C-26), and 31.8 (C-24). The methyl protons at δ_H 0.93 (CH_3-18) revealed a long-range correlation with the carbons at δ_C 213.2 (C-12), 57.9 (C-13), 54.5 (C-14), and 56.6 (C-17), explaining the carbonyl group at C-12. In the HMBC spectrum, the cross peak from proton signal at δ_H 4.86 (H-1″, galactose) to the carbon at δ_C 77.0 (C-3, aglycone) from δ_H 4.84 (H-1′, glucose) to δ_C 75.3 (C-26) were displayed, showing the glycosylation of the aglycone at C-3 and C-26. Furthermore, glucose is linked at C-4″ of the galactose, the

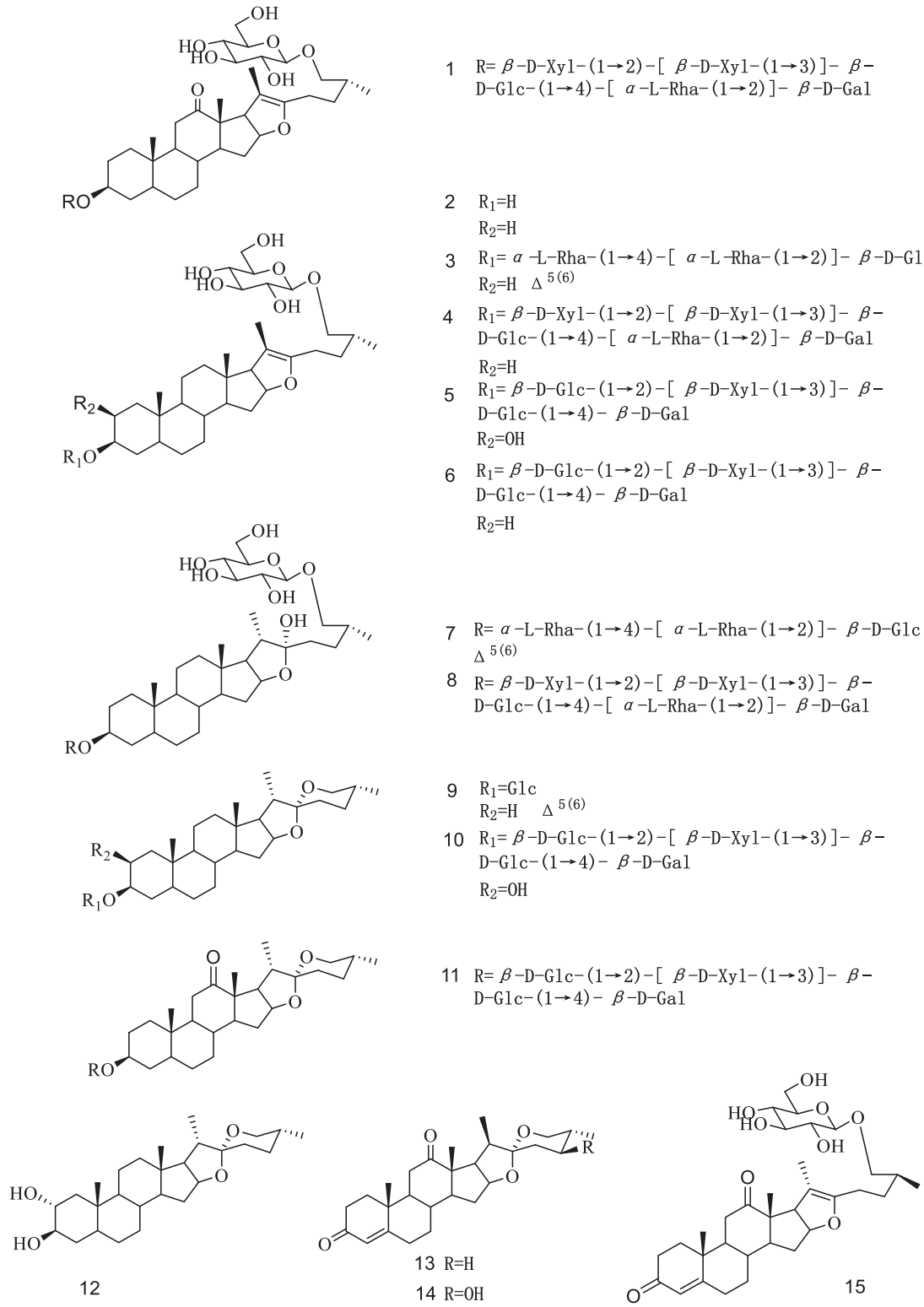

Figure 1. Structure of compounds isolated from *T. terrestris*.

rhamnose linked at C-2″ of galactose, the xylose linked at C-3‴ of the inner glucose, and another xylose linked at C-2‴ of glucose was detected by the cross peak in the HMBC spectrum. Thus, the structure of compound 1 was identified as 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-12-carbonyl-20(22)-en-3*β*,26-diol-3-*O*-{*β*-D-xylopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside}.

Furthermore, comparing physical and spectroscopic data with those reported in the literature, the chemical structures of the known compounds (Figure 1) were determined as 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-20(22)-en-3*β*,26-diol (2)[22], pseudoprotodioscin (3)[23], 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furostan-20(22)-en-3*β*,26-diol-3-*O*-{*β*-D-xylopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside}

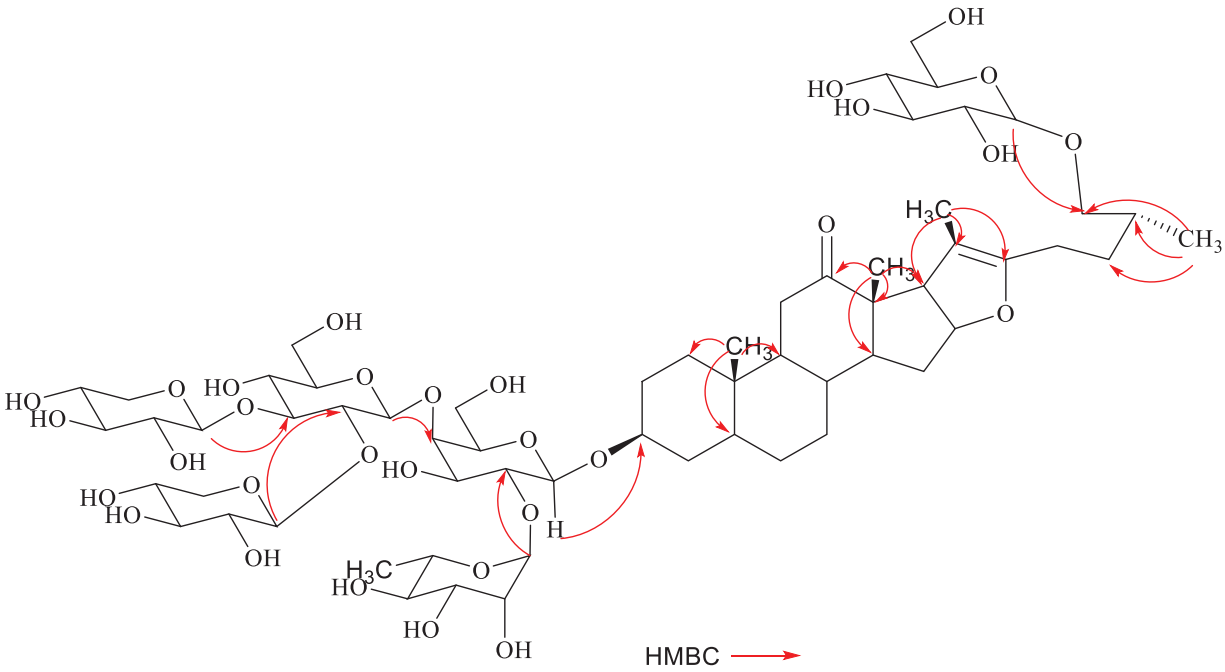

Figure 2. Key correlations observed in the HMBC spectrum of compound 1.

(4)[24], 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*α*-furost-20(22)-en-2*α*, 3*β*,26-triol-3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (5)[25], 26-*O*-*β*-D-glucopyranosyl-(25*R*)-5*β*-furostan-20(22)-en-3*β*, 26-diol-3-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-xylopyranosyl (1→3)]-*β*-D-glucopyranosyl (1→4)-*β*-D-galactopyranoside (6)[26], protodioscin (7)[27], (25*R*)-26-*O*-*β*-D-glucopyranosyl-5*α*-furostan-3*β*,22*α*, 26-triol-3-*O*-{*β*-D-xylopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-[*α*-L-rhamnopyranosyl-(1→2)]-*β*-D-galactopyranoside} (8)[28], tibllin (9)[29], (25*R*)-5*α*-spirostan-2*α*, 3*β*-diol-3-*O*-*β*-D-glucopyranosyl-(1→2)-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (10)[30], hecogenin-3-*O*-*β*-D-glucopyranosyl (1→2)-[*β*-D-xylopyranosyl (1→3)]-*β*-D-glucopyranosyl (1→4)-*β*-D-galactopyranoside (11)[25], gitogenin (12)[31], (25*R*)-spirostan-4-en-24*β*-hydroxy-3, 12-dione (13)[32], (25*R*)-spirostan-4-en-3, 12-dione (14)[32], and terrestrinin A (15)[33].

Biological activity

Overall, when the concentration of the compounds was 1 μM, the test compounds, except compound 8, had no cytotoxic effects on RAW 264.7 cells. Therefore, compound 8 was tested at 0.1 μM in the following assay.

The above results show that the isolated compounds have inhibitory effects on NO production in LPS-induced (6.25 ng/mL) RAW 264.7 cells (Figure 3 and Table S1, http://links.lww.com/AHM/A8). Compared with that in

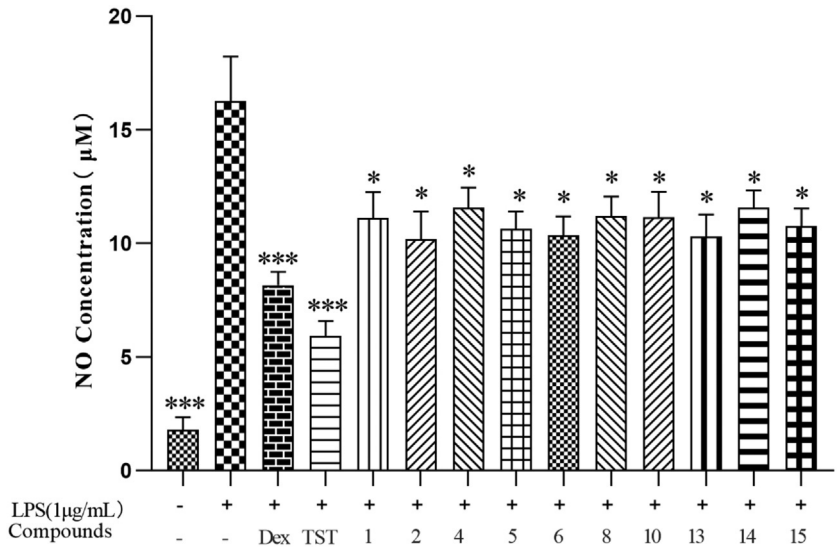

Figure 3. Inhibition of NO production by selected compounds from *T. terrestris*. Values were represented as means ± SEM ($n = 10$); $^{***}P < 0.001$, $^{*}P < 0.05$ *vs.* Model group. NO: Nitric oxide.

the model group, NO production in the tested groups [Dex ($P < 0.001$), TST ($P < 0.001$), and compounds 1, 2, 4, 5, 6, 8, 10, 13, 14, and 15 ($P < 0.05$)] was significantly decreased.

To demonstrate the anti-inflammatory effect of the test compounds in LPS-induced RAW 264.7 cells, we examined the expression of inflammatory cytokines TNF-α and IL-6. TNF-α expression was significantly higher in the LPS group than that in the control group. Compared with LPS, TST and compounds 13 and 15 significantly decreased the expression of TNF-α ($P < 0.05$, Figure 4A). Compared with that in the control group, the expression of IL-6 was significantly higher in the LPS group. In addition, compared with LPS, TST and compounds 6, 10, 12, 13, and 15 significantly decreased the expression of IL-6 ($P < 0.05$, Figure 4B).

Discussion

T. terrestris calms the liver, relieves depression, activates blood, and dispels gas, and it is mostly used clinically to treat cardiovascular and cerebrovascular ischemic diseases, such as atherosclerosis and stroke[34]. Anti-inflammatory effects are crucial in the treatment of cardiovascular and cerebrovascular diseases[35]. To identify the basic substance responsible for the anti-inflammatory effect of TST, its chemical composition was systematically studied, and its effect of inhibiting NO generation and the expression of related inflammatory factors were determined.

In this study, a novel furostanol steroidal saponin (1), along with 14 known compounds, was obtained from *T. terrestris*, and its anti-inflammatory activities were evaluated *in vitro*. Among these, compound 2 was first isolated from the Zygophyllaceae family, and compounds 5 and 6 were first isolated from the genus *Tribulu*s. The structure of compound 1 was elucidated by extensive analysis of 1D and 2D NMR data, including HSQC, HMBC, and TOCSY spectra.

LPS stimulation of RAW 264.7 macrophages leads to the production of NO cytokines. Meanwhile, overproduction of NO leads to an increase in pro-inflammatory cytokines, such as TNF-α and IL-6, which are known to contribute to inflammatory diseases[36]. The anti-inflammatory activities of TST, and the compounds isolated from TST, were evaluated in RAW 264.7 macrophages using LPS. As indicated by biological activity assays, TST showed an anti-inflammatory effect, which significantly inhibited the production of NO (Figure 3) and decreased the expression of TNF-α and IL-6 in LPS-stimulated Raw

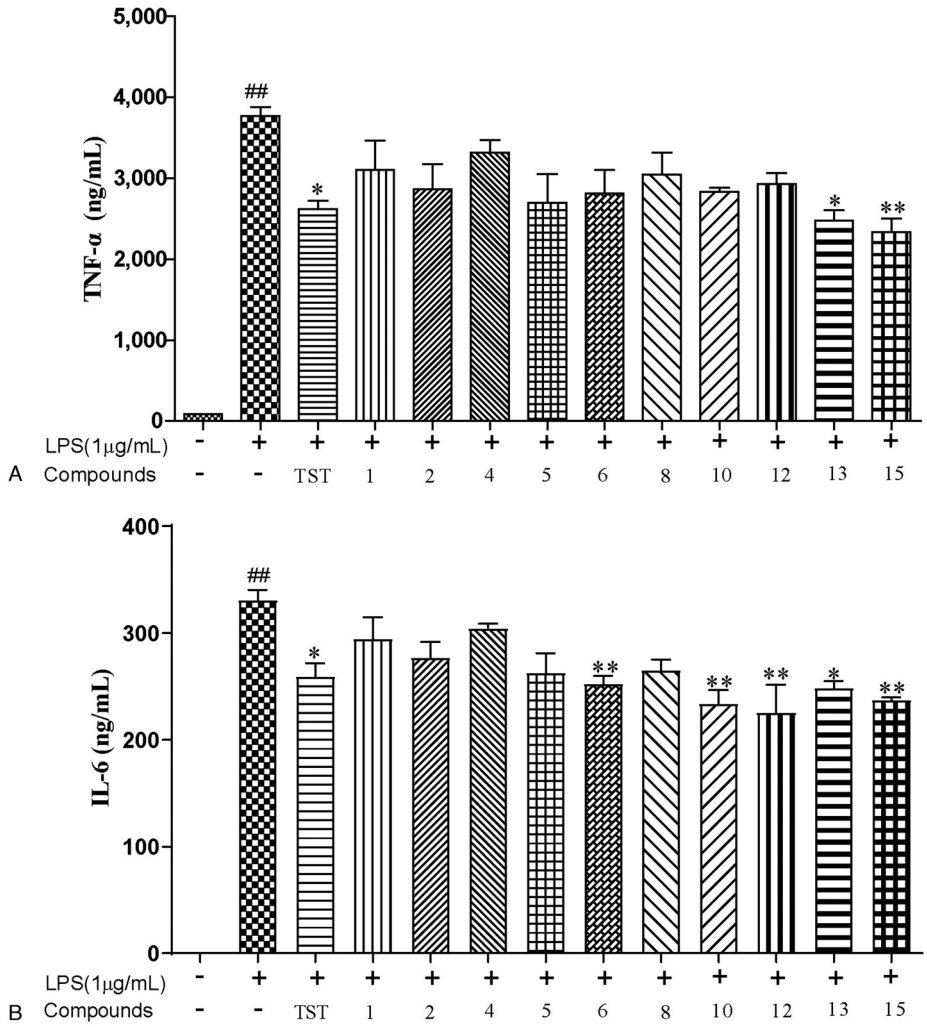

Figure 4. The expression levels of (A) TNF-α and (B) IL-6 were measured by ELISA. Data are expressed as mean ± SEM ($n = 3$). $^{\#\#}P < 0.01$ *vs.* control; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* LPS group. ELISA: Enzyme-linked immunosorbent assay; IL-6: Interleukin-6; LPS: lipopolysaccharides; TNF-α: Tumor necrosis factor-α.

264.7 cells. To identify anti-inflammatory compounds in TST, we tested the effect of the isolated compounds using the same assay. Among the isolated compounds, compounds 13 and 15 significantly decreased the expression of TNF-α. Compared with other compounds, compounds 13 and 15 have a carbonyl group at C-3 and an olefinic bond at C-4. Thus, the presence of the carbonyl group at C-3 and olefinic bond at C-4 affected NO production and decreased the expression of TNF-α. In contrast, compounds 6, 10, 12, 13, and 15 significantly decreased the expression of IL-6. Additionally, the presence of the carbonyl group at C-3 and olefinic bond at C-4 in compounds 13 and 15 similarly decreased the expression of IL-6.

Conflict of interest statement

The authors declare no conflicts of interest.

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Author contributions

Peng Zhang participated in research design. Miaojie Yang and Jingrui Di participated in the writing of the paper. Qing Yuan participated in the activity experiments. Yan Xie and Xiaoying Luan participated in the performance of the research. Jingrui Di, Mahmood Brobbey Oppong, Yanxu Chang, Miaomiao Jiang, Shijie Cao, Pengzhi Dong, Lin Li, Limin Hu, and Lijuan Chai participated in data analysis.

Ethical approval of studies and informed consent

Not applicable-Not required for this study.

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