Tributelosides A–D: Four Undescribed Spirostan Glycosides Isolated from the Branches and Leaves of *Tribulus terrestris* with Their NO Production Inhibitory Activity in LPS Activated RAW 264.7 Cells

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Four undescribed spirostan glycosides, (25*S*)-5α-spirostan- 12-one-2α,3β-diol-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**1**), (25*S*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-galatopyranosyl-(1→2)-β-D-glucopyranosyl- (1→4)-β-D-galactopyranoside (**2**), (25*S*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**3**), and hecogenin 3-*O*-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (**4**), together with eleven known compounds (**5**–**15**) were isolated from the branches and leaves of *Tribulus terrestris*. Their chemical structures were established through spectroscopic methods, including HR-ESI-MS, 1D-, and 2D-NMR spectra. Preliminary biological evaluation on NO production inhibitory activity in LPS activated RAW 264.7 cells showed that compounds **1**–**3**, **5**, and **6** had significant inhibitory effects with IC_{50} values ranging from 2.4 to 18.3 μM, compared to that of the positive control compound, dexamethazone (IC_{50} 13.6 μM).

Introduction

The plant *Tribulus terrestris* L. (Zygophyllaceae) is widely distributed in the south of Vietnam such as Quang Binh, Quang Tri, Thua Thien Hue provinces. In Vietnamese folk medicine, this plant is used to treat eye pain, kidney tonic, back pain, acne, red throat, and dysentery.[1] The main components of this plant are steroidal saponins, flavone glycosides, lignanamides, and other phenolic compounds.[2–7] Some of them showed anti-inflammatory, anticancer, antibacterial, diuretic, aphrodisiac, antiurolithic, hypolipidemic, antidiabetic, absorption enhancing, mmunomodulatory, cardiotonic, hepatoprotective, central nervous system, analgesic, antispasmodic, larvicidal anthelmintic, and anticariogenic activities.[2–7] Results of the screening NO inhibitory activity of some selected Vietnamese medicinal plants showed that the MeOH extract of *T. terrestris* exhibited significant effect and was selected for further study. This paper reported four undescribed spirostan glycosides and eleven known compounds from the branches and leaves of this plant and their NO production inhibition activity in LPS activated RAW 264.7 cells.

Results and Discussion

From the MeOH extract of the branches and leaves of *T. terrestris*, fifteen compounds including four undescribed spirostan glycosides (**1**–**4**) were isolated by combined chromatographic methods. The known compounds were identified to be neohecogenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**5**),[8] neohecogenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**6**),[9] terrestrosin E (**7**),[9] 4 hecogenin 3-*O*-β-D-galactopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyra-noside (**8**),[9] tribulosin (**9**),[10] neohecogenin 3-*O*-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (**10**),[11] rutin (**11**),[12] isorhamnetin 3-*O*-(6′′-*O*-(*E*)-*p*-coumaroyl)-β-D-glucopyranoside (**12**),[13] isorhamnetin 3-*O*-(6′′-*O*-(*Z*)-*p*-coumaroyl)-β-D-glucopyranoside (**13**),[14] (6*R*,9*S*)-3-oxo-α-ionol-9-*O*-β-D-glucopyranoside (**14**),[15] and benzyl glycoside (**15**),[16] by 1D- and 2D-NMR spectral data in comparison with those reported in the literature. The absolute configuration of compound **14** was determined by ECD spectrum. Compounds **5**–**15** have been isolated from some species of the genus *Tribulus*, however, this

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is the first report of these compounds from *T. terrestris* growing in Vietnam.

Compound **1** was isolated as a white amorphous powder (Figure 1). Its IR spectrum revealed the presence of hydroxy (3397 cm^{-1}), carbonyl (1703 cm^{-1}), and C–*O*–C (1071 cm^{-1}) functionalities. The HR-ESI-MS of **1** exhibited the quasi molecular ion peaks at *m/z* 769.4021 $[M–H]^-$ (calcd. for $[C_{39}H_{61}O_{15}]^-$: 769.40216) and *m/z* 805.3786 $[M+Cl]^-$ (calcd. for $[C_{39}H_{62}O_{15}Cl]^-$: 805.3783), determining the molecular formula of $C_{39}H_{62}O_{15}$ and nine degrees of unsaturation. The ^{1}H-NMR spectrum of **1** showed two methyl singlets (δ_H 0.86 and 0.97), two methyl doublets [δ_H 0.98 (J = 7.2 Hz) and 1.01 (J = 7.2 Hz)], two anomeric protons [δ_H 4.21 (d, J = 7.8 Hz) and 4.29 (d, J = 7.8 Hz), and three sp^3 methine carbinol protons at δ_H 3.44 (ddd, J = 13.5, 10.0, 2.2 Hz, H-2), 3.30 (ddd, J = 10.0, 9.0, 3.0 Hz, H-3), and 4.20 (m, H-16) suggesting a spirostan diglycoside, the main components from *Tribulus* plants.[7,8] The ^{13}C NMR and HSQC spectra of **1** supported 39 carbons, including 12 of two sugar moieties and 27 of a spirostan aglycone (Tables 1 and 2). In the aglycone part of **1**, four methyl groups [δ_C 15.5 (C-18), 12.5 (C-19), 13.2 (C-21), and 15.9 (C-27)], one ketone carbon [δ_C 212.5 (C-12)], three sp^3 oxygenated methine carbons [δ_C 69.0 (C-2), 82.7 (C-3), 78.8 (C-16)], a sp^3 oxygenated quaternary carbon [δ_C 108.9 (C-22)], and a sp^3 oxygenated methylene group [δ_C 64.3 (C-26)] were identified.[8,17] The ketone group at C-12 determined from HMBC correlations of H_3-18 (δ_H 0.97) to C-12 (δc 212.5), two methine carbinol groups at C-2 and C-3 as indicated from COSY correlation of H-1/H-2/H-3/H-4./H-5 and from HMBC correlations from H-2 to C-2 and C-3 (Figure 2). The orientation of H-2 and H-3 were anpha/a*xial* and beta/a*xial*, respectively, as

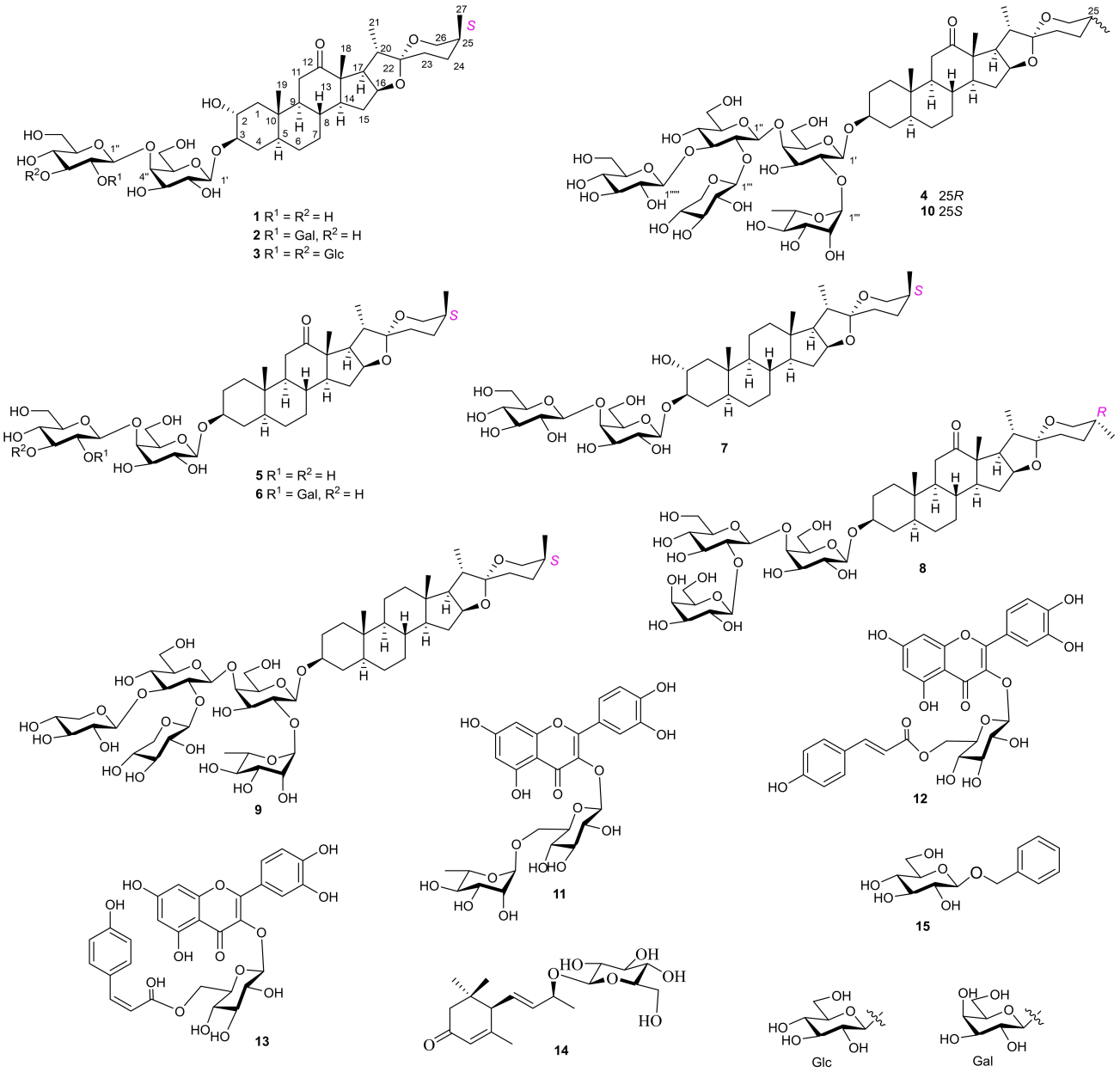

Figure 1. Chemical structures of compounds 1–15.

Table 1. ^{1}H and ^{13}C NMR spectral data for aglycones of compounds **1**–**4** in DMSO-d_6.

No.	1		2		3		4	
	δ_C[a]	δ_H (mult., *J* in Hz)[b]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[b]
1	44.4	0.83 (dd, 13.5, 13.5) 1.69 (dd, 13.5, 2.4)	44.4	0.83 (dd, 12.0, 12.0) 1.68 (dd, 12.0, 3.5)	44.3	0.84 (dd, 13.5, 13.5) 1.69 (dd, 13.5, 2.4)	35.8	0.91–9.92 (m) 1.48–1.49 (m)
2	69.0	3.44 (m)	69.0	3.45 (m)	69.0	3.46 (m)	28.7	1.33*/1.70–1.72 (m)
3	82.7	3.30 (m)	82.8	3.30 (m)	82.2	3.32 (m)	75.3	3.53–3.54 (m)
4	33.0	1.26*/1.65*	32.9	1.26*/1.65*	32.9	1.27*/1.65*	33.0	1.20–1.22 (m)/1.68*
5	43.6	1.12–1.13 (m)	43.6	1.12–1.14 (m)	43.5	1.12–1.14 (m)	43.7	1.02–1.03 (m)
6	27.2	1.24*/1.32–1.34 (m)	27.2	1.24*/1.32–1.35 (m)	27.2	1.23*/1.32–1.34 (m)	28.0	1.27–1.28 (m)
7	30.6	1.38*/2.00–2.02 (m)	30.6	1.38*/2.00–2.02 (m)	30.6	1.39*/2.01–2.02 (m)	32.0	1.40*/1.98–2.00 (m)
8	33.0	1.85–1.87 (m)	32.9	1.85–1.86 (m)	32.9	1.84–1.85 (m)	33.2	1.85–1.87 (m)
9	54.8	1.38–1.40 (m)	54.8	1.39–1.41 (m)	54.8	1.40–1.41 (m)	54.9	1.04–1.06 (m)
10	36.7	–	36.7	–	36.7	–	35.8	–
11	37.5	2.45 (dd, 13.8, 13.8) 2.02–2.03 (m)	37.5	2.45 (dd, 13.8, 13.8) 2.02–2.04 (m)	37.5	2.45 (dd, 13.8, 13.8) 2.02–2.03 (m)	37.2	2.45 (dd, 13.8, 13.8) 2.02 (dd, 12.0, 4.2)
12	212.5	–	212.4	–	212.4	–	212.8	–
13	54.4	–	54.4	–	54.4	–	55.1	–
14	54.5	1.10–1.12 (m)	54.5	1.10–1.12 (m)	54.4	1.10–1.12 (m)	53.6	1.27–1.28 (m)
15	30.9	0.90–0.92 (m)/1.69*	30.9	0.90–0.92 (m)/1.69*	30.9	0.90–0.92 (m)/1.69*	31.2	0.90–0.92 (m)/1.69*
16	78.8	4.19–4.21 (m)	78.8	4.20–4.22 (m)	78.8	4.21–4.22 (m)	79.5	4.24–4.25 (m)
17	53.0	2.31 (dd, 8.4, 7.2)	53.0	2.31 (dd, 8.4, 7.2)	53.0	2.31 (dd, 8.4, 6.6)	52.7	2.36 (dd, 9.0, 4.8)
18	15.5	0.97 (s)	15.5	0.97 (s)	15.5	0.97 (s)	16.1	1.09 (s)
19	12.5	0.86 (s)	12.5	0.86 (s)	12.5	0.86 (s)	11.3	0.83 (s)
20	42.0	1.65*	42.0	1.65*	42.0	1.65–1.66 (m)	41.8	1.91–1.92 (m)
21	13.2	0.98 (d, 7.2)	13.2	0.98 (d, 7.2)	13.2	0.98 (d, 7.2)	15.9	0.93 (d, 6.0)
22	108.9	–	108.9	–	108.8	–	109.6	–
23	25.3	1.34*/1.88–1.89 (m)	25.3	1.34*/1.88–1.89 (m)	25.3	1.34*/1.89–1.90 (m)	28.3	1.35*/1.58*
24	25.5	1.27*/1.79–1.81 (m)	25.5	1.27*/1.79–1.81 (m)	25.5	1.27*/1.80–1.81 (m)	27.7	1.30*/1.58*
25	26.4	1.64*	26.4	1.64*	26.4	1.64–1.65 (m)	29.8	1.58*
26	64.3	3.77 (dd, 10.8, 2.4) 3.23 (br d, 10.8)	64.3	3.77 (br d, 9.0) 3.23 (br d, 9.0)	64.3	3.77 (dd, 9.0, 1.8) 3.23*	67.9	3.47 (dd, 10.8, 9.6) 3.30 (dd, 10.8, 6.6)
27	15.9	1.01 (d, 7.2)	15.9	1.01 (d, 7.2)	15.9	1.01 (d, 7.2)	17.0	0.73 (d, 6.0)

[*] Overlapped signals, [a] Recorded in 150 MHz, [b] Recorded in 600 MHz.

determined from the NOESY cross peaks between H-3 (δ_H 3.30) and H-5 (δ_H 1.12) and between H_3-19 (δ_H 0.86) and H-2 (δ_H 3.44) (Figure 3).[17] Protons H_2-26 appeared at δ_H 3.23 (br d, J= 10.8 Hz) and 3.77 (dd, J=10.8, 2.4 Hz) indicating small $^{3}J_{25,26}$ value (0~2.4 Hz). This evidence suggested 25*S* with *equatorial* orientation of H-25.[8] The NMR data of sugar moieties of **1** were closely resembling those of neohecogenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**5**),[8] suggested the glucose (δ_C 105.0, 74.2, 76.8, 70.4, 76.7, and 61.4) linked to C-4′ of galactose (δ_C 101.4, 70.9, 73.2, 78.3, 74.1, and 59.6), which linked to C-3. These sugar linkages were further confirmed by HMBC correlations from H-1′′ to C-4′, and from H-1′ to C-3 (Figure 2). Furthermore, the coupling constants (J=7.5 Hz) observed for the anomeric protons at 4.21 and 4.29 in the ^{1}H-NMR spectrum suggested the β-glycoside linkages. In addition, compound **1** gave D-glucose and D-galactose with the acid hydrolysis test, identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[18] Thus, compound **1** was determined to be (25*S*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, undescribed compound and named as tributeloside A.

The IR spectrum of **2** suggested the presence of hydroxy (3405 cm^{-1}), carbonyl (1705 cm^{-1}), and C–*O*–C (1067 cm^{-1}) functionalities. The HR-ESI-MS of **2** exhibited the quasi molec-

Table 2. ^{1}H and ^{13}C NMR spectral data for sugar moieties of compounds **1**–**4** in DMSO-d_6.

No.	1		2		3		4	
	δ_C[a]	δ_H (mult., *J* in Hz)[b]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[b]
		3-*O*-gal		3-*O*-gal		3-*O*-gal		3-*O*-gal
1′	101.4	4.21 (d, 7.8)	101.4	4.22 (d, 7.8)	101.3	4.22 (d, 7.8)	98.1	4.32 (d, 7.8)
2′	70.9	3.30 (dd, 9.0, 7.8)	71.1	3.30 (dd, 9.0, 7.8) 4.82 (br s, OH)	70.9	3.36 (dd, 9.0, 7.8) 4.90 (br s, OH)	74.9	3.42 (dd, 9.0, 7.8)
3′	73.2	3.41 (dd, 9.0, 3.0)	73.5	3.38 (dd, 9.0, 3.0)	73.4	3.37 (dd, 9.0, 3.0)	74.6	3.53 (dd, 9.0, 3.0)
4′	78.3	3.81 (br d, 3.0)	78.9	3.77 (br d, 3.0)	78.9	3.80 (br d, 3.0)	80.2	3.74 (br d, 3.0)
5′	74.1	3.65–3.66 (m)	74.0	3.35*	74.2	3.40*	73.2	3.37–3.38 (m)
6′	59.6	3.31*/3.64–3.65 (m)	59.2	3.35*/3.72–3.73 (m)	59.4	3.40*/3.73–3.74 (m)	59.1	3.38*3.72–3.73 (m)
		4′-*O*-glc		4′-*O*-glc		4′-*O*-glc		4′-*O*-glc
1″	105.0	4.29 (d, 7.8)	103.3	4.37 (d, 7.8)	103.1	4.43 (d, 7.8)	103.3	4.38 (d, 7.8)
2″	74.2	3.04 (dd, 9.0, 7.8)	83.7	3.31 (dd, 9.0, 7.8)	79.3	3.55 (dd, 9.0, 7.8)	79.8	3.47 (dd, 9.0, 7.8)
3″	76.8	3.12–3.13 (m) 5.82 (br s, OH)	76.4	3.41–3.43 (m) 5.82 (br s, OH)	86.9	3.58–3.59 (m) 5.82 (br s, OH)	87.2	3.61 (t, 9.0)
4″	70.4	3.00 (t, 9.0)	70.3	3.02 (t, 9.0)	70.1	3.15 (t, 9.0)	69.1	3.12 (t, 9.0)
5″	76.7	3.16–3.18 (m)	76.3	3.19–3.20 (m)	75.9	3.24–3.25 (m)	75.9	3.24–3.25 (m)
6″	61.4	3.36 (dd, 12.0, 5.4) 3.69 (dd, 12.0, 1.8)	61.6	3.32 (dd, 12.0, 5.4) 3.72 (dd, 12.0, 1.8)	61.3	3.38 (dd, 12.0, 5.4) 3.71 (dd, 12.0, 1.8)	61.4	3.36 (dd, 12.0, 5.4) 3.72 (dd, 12.0, 1.8)
				2″-*O*-gal		2″-*O*-glc		2′-*O*-rha
1‴			105.4	4.41 (d, 7.8)	102.4	4.73 (d, 7.8)	100.3	4.99 (br s)
2‴			72.3	3.42 (dd, 9.0, 7.8), 5.58 (br s, OH)	74.2	2.95 (dd, 9.0, 7.8) 5.26 (br s, OH)	70.6	3.62 (br d, 3.0)
3‴			72.5	3.35 (dd, 9.0, 3.0)	76.1	3.15 (m)	70.6	3.41–3.42 (m)
4‴			68.1	3.68 (br d, 3.0)	69.9	3.07 (t, 9.0)	71.9	3.20 (t, 9.0)
5‴			75.9	3.43–3.44 (m)	76.8	3.18 (m)	67.8	3.98–3.99 (m)
6‴			60.1	3.57 (dd, 12.0, 5.4) 3.65 (dd, 12.0, 1.8)	60.9	3.39–3.40 (m) 3.71–3.73 (m)	17.5	1.08 (d, 6.0)
						3″-*O*-glc		2″-*O*-Xyl
1⁗					102.9	4.45 (d, 7.8)	103.6	4.67 (d, 7.8)
2⁗					73.6	3.05 (dd, 9.0, 7.8) 5.62 (br s, OH)	73.9	2.87 (dd, 9.0, 7.8)
3⁗					76.5	3.15*	76.9	3.08 (dd, 9.0, 3.0)
4⁗					68.9	3.15*	69.4	3.35 (br d, 3.0)
5⁗					76.9	3.20–3.22 (m)	65.7	3.02* 3.91 (dd, 10.8, 5.4)
6⁗					60.9	3.39–3.40 (m) 3.71–3.72 (m)		
							3″-*O*-glc	

Table 2. continued

No.	1		2		3		4	
	δ_C[a]	δ_H (mult., *J* in Hz)[b]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[a]	δ_C[a]	δ_H (mult., *J* in Hz)[b]
1′′′′′							102.7	4.46 (d, 7.8)
2′′′′′							73.6	3.04 (dd, 9.0, 7.8) 5.70 (br s, OH)
3′′′′′							76.6	3.13 (t, 9.0)
4′′′′′							70.1	3.07 (t, 9.0)
5′′′′′							76.8	3.19–3.20 (m)
6′′′′′							60.9	3.39*/3.71–3.72 (m)

[*] Overlapped signals, [a] Recorded in 150 MHz, [b] Recorded in 600 MHz.

ular ion peaks at *m/z* 931.4517 $[M–H]^-$ (calcd. for $[C_{45}H_{71}O_{20}]^-$: 931.4544) and *m/z* 967.4303 $[M+Cl]^-$ (calcd. for $[C_{45}H_{72}O_{20}Cl]^-$: 967.4311) determining the molecular formula of $C_{45}H_{72}O_{20}$ and ten degrees of unsaturation. The NMR spectra of **2** were similar to those of **1** except for the additional signals due to a sugar moiety, suggesting a spirostan-12-one-2α,3β-diol glycoside.[8] The ketone and two methine carbinol groups were identified at δ_C 212.4 (C-12), 69.0 (C-2), and 82.8 (C-3), respectively, and further confirmed by COSY and HMBC correlations as shown in Figure 2. The matching of NMR data in the F ring between compounds **1** and **2** (Table 1), together with the small $^3J_{25,26}$ value (~0 Hz) indicated the *equatorial* orientation of H-25. The NOESY cross peaks between H-3 and H-5, and between H-2 and H_3-19 indicated β and α-orientaion of H-2 and H-3, respectively.[8] Comparing the sugar moiety NMR data between compounds **1** and **2** (Table 2) suggested the additional was a galatose (δ_C/δ_H: 105.4/4.22, 72.3/3.30, 72.5/3.38, 68.1/3.68, 75.9/3.43, 60.1/3.65 and 3.75), similar to that of terrestrosin A isolated from *T. terrestris*.[9] The broad singlet signal at 3.68 of H-4′′′ suggested *equatorial* orientaion of the galatose moiety.[8,9] The

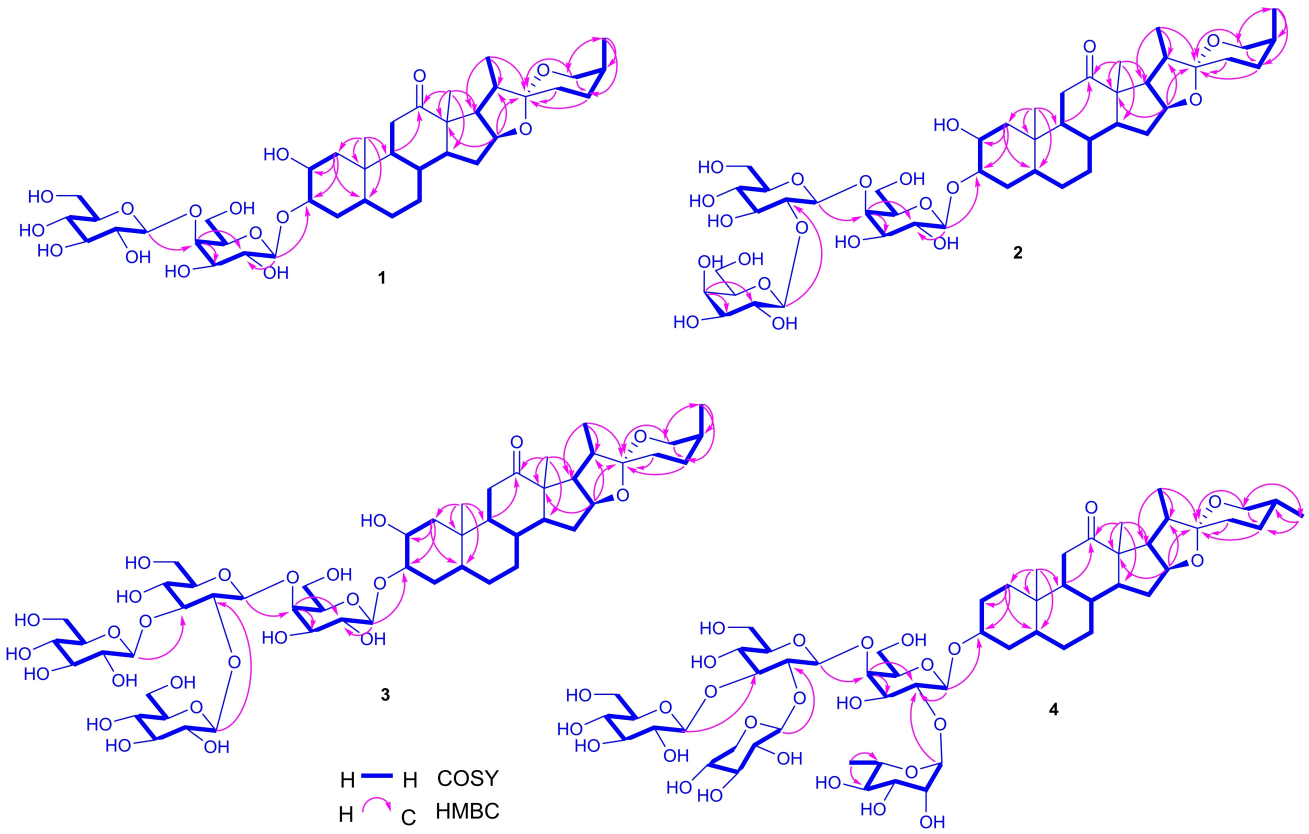

Figure 2. The key HMBC and COSY correlations of compounds 1–4.

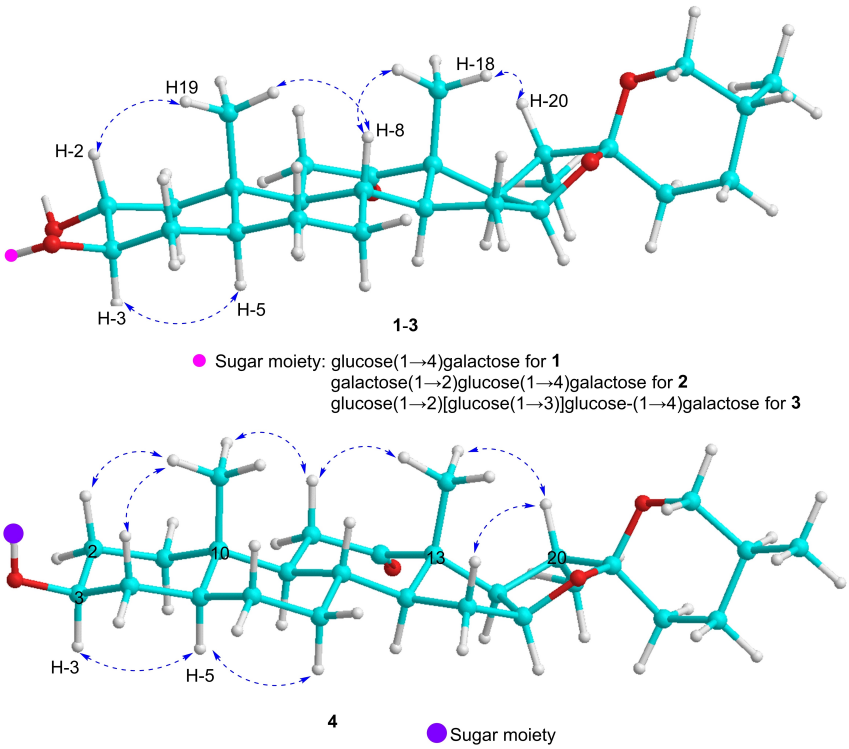

Figure 3. The key NOESY correlations of aglycones of compounds 1–4.

HMBC correlation from H-4‴ to C-2″, from H-1″ to C-4′, and from H-1′ to C-3 were observed (Figure 2) confirming the sugar moiety as 3-*O*-galactopyranosyl-(1→2)-glucopyranosyl-(1→4)-β-galactopyrano-side.[9] The large *J* value of the anomeric protons at 4.22 (H-1′), 4.37 (H-1″), and 4.41 (H-1‴) suggested β-form of these glycosidic linkages. Acid hydrolysis of **2** gave D-glucose and D-galactose identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[18] From the above evidence, compound **2** was determined to be (25*S*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-galatopyranosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside, undescribed compound and named as tributeloside B.

The IR spectrum of compound **3** indicated the presence of OH, C=O, and C–*O*–C functional groups. The molecular formular of **3** was $C_{51}H_{82}O_{25}$ as determined from the HR-ESI-MS (found *m/z* 1093.5039 $[M-H]^-$, calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5073 and *m/z* 1129.4838 [M + Cl], calcd. for $[C_{51}H_{82}O_{25}Cl]^-$: 1129.4839), indicating eleven degrees of unsaturation. The NMR data of the aglycone of **3** were perfectly match those of compounds **1** and **2** (Table 1) suggesting they have the same aglycone, which were further confirmed by COSY, HSQC, HMBC, and NOESY spectra. In the sugar moieties, four anomeric protons at δ_H 4.22, 4.43, 4.73, and 4.45 showed HSQC correlations with carbonds at δ_C 101.3, 103.1, 102.4, and 102.9, respectively, suggesting four sugar units. Detail analyzing the NMR data of sugar moieties suggested that the sugar moiries of **3** were coincident with that of (25*R*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside.[19] This was further indicating by COSY, HSQC, and HMBC correlations (Figure 2). In addition, the small $^3J_{25,26}$ value (1.8 Hz) of protons H-25 and H-26 suggested the *equatorial* orientation of H-25. All the sugar linkages must be in the β-form as indicated by the large *J* value (7.8 Hz) of all the anomeric protons. The anomeric protons δ_H 4.22 (H-1′), 4.43 (H-1″), 4.73 (H-1‴), and 4.45 (H-1⁗) showed HMBC correlations with C-3 (δ_C 82.2), C-4′ (δ_C 78.9), C-2″ (δ_C 79.3), and C-3″ (δ_C 86.9), respectively, revealed the above glycosidic linkages. Acid hydrolysis of **3** gave D-glucose and D-galactose identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[18] From the above evidence, compound **3** was determined to be (25*S*)-5α-spirostan-12-one-2α,3β-diol-3-*O*-β-D-glucopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside undescribed compound and named as tributeloside C.[19]

Compound **4** was obtained as a white amorphous powder. Its IR spectrum supported the presence of hydroxy (3394 cm^{-1}), ketone (1702 cm^{-1}), and C–*O*–C (1070 cm^{-1}) functionalities. The molecular formula of **4** was determined to be $C_{56}H_{90}O_{27}$ by HR-ESI-MS ion peaks at *m/z* 1129.4838 $[M+Cl]^-$ (calcd. for $[C_{56}H_{90}O_{27}Cl]^-$: 1129.4839) and m/z 11093.5039 $[M-H]^-$ (calcd. for $[C_{56}H_{89}O_{27}]^-$: 1093.5072), indicating 12 degrees of unsaturation. The NMR data of **4** were similar to those of compounds **1**–**3** suggesting a spirostan glycoside. The ketone group was evident at δ_C 212.8 (C-12), four methoxy groups at δ_C/δ_H 16.1/1.09 (s), 11.3/0.83 (s), 15.9/0.93 (d, *J* = 6.0 Hz), and 17.0/0.73 (d, *J* = 6.0 Hz), and one methine carbinol group at δ_C 75.3 (C-3)/δ_H 3.53 (m). The large $^3J_{25,26}$ value (9.6 Hz) of H_2-26 protons at δ_H 3.47 suggested *axial* orientation for H-25, corresponding to 25*R* configuration.[8,9] In the NOESY spectrum, H-3 (δ_H 3.53) showed

cross peak with H-5 (δ_H 1.02) suggested *alpha/axial* orientation for H-3. The NMR data of aglycone of **4** were coincident with those of hecogenin.[20] The sugar moiety NMR data of **4** matched those of neohecogenin 3-*O*-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyrano-syl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (**10**), a compound previously isolated from *Tribulus cistoides*.[11] The sugar linkages were further confirmed by COSY, HSQC, and HMBC correlations as shown in Figure 2. The small $^3J_{1,2}$ value (~0 Hz) of the rhamnose anomeric proton at δ_H 4.99 suggested α-form, meanwhile the large $^3J_{1,2}$ values (7.8 Hz) of remaining anomeric protons indicated β-form of the glycosidic linkages. Acid hydrolysis of **4** obtained D-glucose and D-galactose identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[18] Therefore, compound **4** was elucidated as hecogenin 3-*O*-β-D-glucopyranosyl-(1→3)-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyrano-syl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside, undescribed compound and named as tributeloside D.[11]

Based on the experience used in traditional medicine and the bioactivity screening results, the isolated compounds were screened for were further evaluated for their NO production inhibition. Compounds **1**–**15** did not show significant cytotoxic activity at a concentration of 100 μM in the MTT assay (Table S1). Therefore, these compounds were further screened for their NO production effects in LPS stimulated RAW 264.7 cells. As shown in Table 3, compounds **1**, **2**, **5**, **6** showed significant effects with IC_{50} value of 7.0, 2.4, 9.8, and 13.2 μM, respectively, lower than that of the positive control compound, dexamethasone, which showed IC_{50} value of 13.6 μM. Compounds **3**, **4**, **8**, **10**–**13** showed weaker effects with IC_{50} values ranging from 18.3 to 75.8 μM. Compounds **7**, **9**, **14**, and **15** were inactive with IC_{50} values over 100 μM (Figure S58). These results suggested that the ketone group at C-12 may play important role in the NO production inhibitory activity of the spirostan glycoside compounds.

Table 3. NO inhibitory effects in LPS-activated RAW 264.7 cells of the isolated compounds.

Compounds	NO inhibition (IC_{50}, μM)
1	7.0 ± 0.7
2	2.4 ± 0.2
3	18.3 ± 1.2
4	31.2 ± 1.3
5	9.8 ± 0.6
6	13.2 ± 1.6
7	> 100
8	24.7 ± 1.5
9	> 100
10	25.9 ± 1.1
11	75.8 ± 1.4
12	68.3 ± 1.2
13	71.5 ± 2.1
14	> 100
15	> 100
Dexamethasone*	13.6 ± 1.1

[*] positive control compound.

Conclusions

Phytochemical study on the methanol extract of branches and leaves of *Tribulus terrestris* using combination of various chromatoghraphic methods led to the isolation of four undescribed spirostan glycosides (**1**–**4**) and eleven known compounds (**5**–**15**). Their chemical structures were elucidated by IR, HR-ESI-MS, 1D- and 2D NMR spectra in comparison with the reported data. In the activity screening for their inhibition of NO production in LPS stimulated RAW 264.7 cells, spirostan glycosides **1**, **2**, **5**, and **6** showed significant effects with IC_{50} value ranging from 2.4 to 13.2 μM, while compounds **3**, **4**, **8**, **10**–**13** showed weaker activity with IC_{50} values ranging from 18.3 to 75.8 μM. The remaining compounds were inactive with IC_{50} values over 100 μM.

Experimental Section

General

The optical rotations were measured on a Jasco P2000 polarimeter. The infrared spectra (IR) were recorded on a Spectrum Two FT-IR spectrometer. The high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was acquired on an Agilent 6530 Accurate Mass Q-TOF LC/MS. The NMR spectra were recorded on a Bruker 600 MHz spectrometer. Semi-preparative high-performance liquid chromatography (HPLC) were run on an Agilent 1260 system including binary pump, autosampler, DAD detector, and semi-preparative HPLC column YMC J'sphere ODS–H80 (4 μm, 20×250 mm). Isocratic mobile phase with the flow rate of 2.5 mL/min was used in Semi-prep-HPLC. The compound was monitored at wavelengths of 205, 230, 254, and 280 nm. Flash column chromatography was performed using silica gel, reversed phase C-18, and diaion HP-20 resins as stationary phase. Thin layer chromatography was carried out on pre-coated silica gel 60 F_{254} and RP-18 F_{254S} plates. The spots were detected by spraying with aqueous solution of H_2SO_4 5% followed by heating with a heat gun.

Plant Material

The branches and leaves of *Tribulus terrestris* L., were in Nha Trang, Khanh Hoa, Vietnam, in September 2022 and identified by Dr Nguyen The Cuong, Institute of Institute of Ecology and Biological Resources. A voucher specimen (NCCT–P107) was deposited at the Institute of Marine Biochemistry, VAST.

Extraction and Isolation

The dried branches and leaves (3.8 kg) of *T. terrestris* were minced and ultrasonic extracted with MeOH to obtain the residue (TT1, 288 g) after removal the solvent. This was suspended in water and then partitioned with EtOAc to give the extract (TT2, 15.0 g) and water layer (TT3). The EtOAc extract was chromatographed on a

silica gel column eluting with CH2Cl2/MeOH (50/1, 20/1, 5/1, 2/1, v/v) to obtain four fractions TT2 A-TT2D, respectively. Fraction TT2 C was isolated an YMC RP18 column eluting with MeOH/H_2O (1.5/1) to get four fractions, TT2 C1-TT2 C4. Fraction TT2 C1 was chromatographed on a silica gel column eluting with acetone/H_2O (1/2.5) and then purified by a semi-preparative HPLC eluting with 16% acetonitrile (ACN) in water to yield compound **15** (5.8 mg, t_R 31.2 min). Fraction TT2 C3 was isolated on a silica gel column eluting with acetone/H_2O (1/1.3) to get four fractions, TT2 C3 A-TT2 C3D. Fraction TT2 C3 A was purified on the HPLC eluting with 27% ACN to get compound **14** (6.0 mg, t_R 29.5 min). Fraction TT2 C3 C was purified on the HPLC eluting with 30% ACN to get compounds **12** (5.4 mg, t_R 44.4 min) and **13** (4.3 mg, t_R 50.2 min). The water layer was chromatographed on a Diaion HP20 eluting with MeOH/H_2O (1/4, 1/1, 4/1, 100% MeOH) to get four fractions, TT3 A-TT3D. Fraction TT3B was chromatographed on a silica gel column eluting acetone/H_2O (1/2) to get four fractions, TT3B1-TT3B4. Fraction TT3B2 was purified on the HPLC eluting with 18% ACN to get compound **11** (4.7 mg, t_R 47.9 min). Fraction TT3D was chromatographed on a silica gel column eluting CH_2Cl_2/MeOH (50/1, 5/1, 2.5/1, 1/1, v/v) to obtain four fractions, TT3D1-TT3D4. Fraction TT3D2 was isolated on an YMC RP18 column eluting MeOH/H_2O (2.6/1) to get four fractions, TT3D2 A-TT3D2D. Fraction TT3D2 A was purified on the HPLC eluting with 40% ACN to get compound **2** (12.9 mg, t_R 47.0 min). Fraction TT3D2B was purified on the HPLC eluting with 34% ACN to get compounds **1** (15.8 mg, t_R 32.0 min) and **8** (6.1 mg, t_R 34.0 min). Fraction TT3D2 C was purified on a silica gel column eluting with CH_2Cl_2/MeOH (7/1) to get compounds **5** (15.2 mg) and **6** (9.9 mg). Fraction TT3D2D was purified on a silica gel column eluting with CH_2Cl_2/MeOH (10/1) to get compound **7** (7.6 mg). Fraction TT3D3 was isolated an YMC RP18 column eluting with acetone/H_2O (1.7/1) to get compound **9** (4.4 mg). Fraction TT3D4 was isolated an YMC RP18 column eluting with MeOH/H_2O (2.5/1 to give four fractions, TT3D4 A-TT3D4D. Fraction TT3D4B was chromatographed on a silica gel column eluting with CH_2Cl_2/MeOH/H_2O (3/1/0.15) and further isolated on the HPLC eluting with 45% ACN to get compounds **3** (15.1 mg, t_R 24.8 min), **4** (14.9 mg, t_R 32.6 min), **10** (14.6 mg, t_R 49.0 min).

Tributeloside A (1)

A white amorphous powder; $[\alpha]_D^{25}$: −43.2 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3397, 2928, 2881, 1703, 1449, 1380, 1071. HR-ESI-MS *m/z* 769.4021 $[M-H]^-$, calcd. for $[C_{39}H_{61}O_{15}]^-$: 769.40216 (Δ = +0.6 ppm); m/z 805.3786 $[M+Cl]^-$, calcd. for $[C_{39}H_{62}O_{15}Cl]^-$: 805.3783 (Δ = +0.4 ppm); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in the Table 1 (Figures S1–S12).

Tributeloside B (2)

A white amorphous powder; $[\alpha]_D^{25}$: −45.7 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3405, 2931, 2869, 1705, 1449, 1373, 1067. HR-ESI-MS *m/z* 931.4517 $[M-H]^-$, calcd. for $[C_{45}H_{71}O_{20}]^-$: 931.4544 (Δ = −2.9 ppm, m/z 967.4303 $[M+Cl]^-$, calcd. for $[C_{45}H_{72}O_{20}Cl]^-$: 967.4311 (Δ = −0.8 ppm); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in the Table 1 (Figures S13–S23).

Tributeloside C (3)

A white amorphous powder; $[\alpha]_D^{25}$: +4.8 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3392, 2929, 2881. 1703, 1448, 1378, 1069; HR-ESI-MS *m/z* 1093.5039 $[M-H]^-$, calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5073 (Δ = −3.0 ppm), *m/z* 1129.4838 $[M+Cl]^-$, calcd. for $[C_{51}H_{82}O_{25}Cl]^-$: 1129.4839 (Δ = −0.1 ppm); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in the Table 1 (Figures S24–S34).

Tributeloside D (4)

A white amorphous powder; $[\alpha]_D^{25}$: −51.9 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3394, 2929, 2881, 1702, 1448, 1378, 1070; HR-ESI-MS *m/z* 1129.4838 $[M+Cl]^-$, calcd. for $[C_{56}H_{90}O_{27}Cl]^-$: 1129.4839 (Δ = −0.1 ppm), m/z 11093.5039 $[M-H]^-$, calcd. for $[C_{56}H_{89}O_{27}]^-$: 1093.5072, (Δ = −3.0 ppm); ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in the Table 1 (Figures S35–S46).

The ^{1}H and ^{13}C NMR spectra of the known compounds were shown in Supplementary information (Figures S47–S57).

Acid Hydrolysis of Compounds 1–4

Acid hydrolysis of compounds **1**–**4** were the same as described in previous work[18,21,22] referred to Supplementary information.

Nitric Oxide Assay

The NO assay protocol is the same as described in previous papers[22–24] referred to Supplementary information.

Supplementary Material

Additional references cited within the Supporting Information.[18,21–24]

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Author Contributions

Kiem PV, Tai BH, Yen PH, Giang LD designed experiments, elucidated chemical structures and wrote the paper. Quoc NV, Hoang NH, Cuc NT, Huong PTT, Dung DT, Trang DT extracted and isolated compounds and prepared sample for bioassay.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Zygophyllaceae · NO inhibitory activity · spirostan glycoside · tributeloside · *Tribulus terrestris*

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