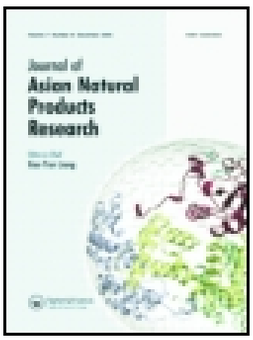

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Isolation of chemical constituents with anti-inflammatory activity from *Reineckia carnea* herbs

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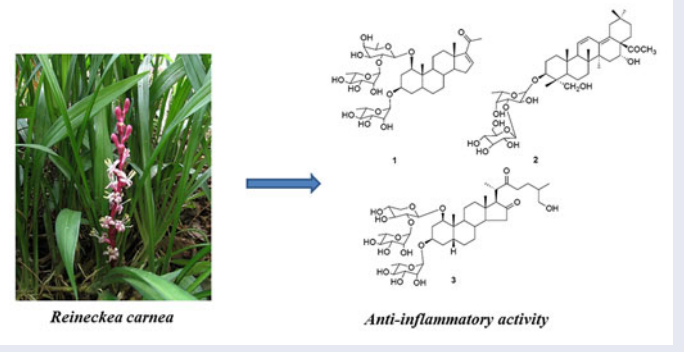
ABSTRACT

Three new saponins (**1–3**), a new natural product (**4**) and six other known compounds (**5–10**) were isolated from the whole *Reineckia carnea* plant. Their structures were established by comparison of their NMR spectra and MS data with literature data. In addition, all the isolated compounds were evaluated *in vitro* for anti-inflammatory activities against LPS-stimulated nitric oxide (NO) production in RAW 264.7 macrophages. Compounds **1–4** exhibited anti-inflammatory activities with IC_{50} values of 37.5 μM, 31.4 μM, 34.6 μM, and 56.1 μM, respectively. Furthermore, compounds **5–10** showed anti-inflammatory activities with IC_{50} values ranging from 20.3 to 42.9 μM.



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Reineckia carnea; structure identification; anti-inflammatory activity

1. Introduction

Reineckia carnea (Andr.) Kunth (referred to herein as *R. carnea*), an evergreen medicinal herb known in Chinese as "Jixiangcao," is widely used for medical treatment in China and Japan [1, 2]. In China, it has been commonly used by the folk people of

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the Miao minority as an antitussive, antidote, anti-arthritic, antipyretic, and hemostatic medicine [3–5]. Extensive phytochemical investigation of *R. carnea* revealed the presence of saponins, flavonoids, volatile oils, alkaloids, and others [6, 7]. Among these compounds, saponins have received the greatest attention because of their pharmacological properties, including anti-inflammatory and antioxidant activities. *R. carnea* is also used as a major ingredient in many herbal composition formulae for the treatment of cough and bronchitis, such as the Chinese medicine "Kesuting syrup" and "compound *R. carnea* buccal tablets." In this paper, we describe the isolation from *R. carnea* and structural elucidation of compounds **1–10** and their inhibitory activity on LPS-stimulated nitric oxide (NO) production in RAW 264.7 macrophages (Figure 1).

2. Results and discussion

Compound **1** was obtained as a white amorphous powder. The HR-ESI-MS showed a negative molecular ion peak at *m/z* 769.4155 $[M\text{-}H]^-$, corresponding to a molecular formula of $C_{39}H_{62}O_{15}$ (calcd 769.4089 $[M\text{-}H]^-$), and neutral loss (NL) fragments of D-fucose and L-rhamnose (146 Da). The ^{1}H NMR spectrum of **1** (Table 1) showed the presence of an olefinic proton at δ_H 6.61 (1H, dd, $J = 1.8$, 3.1 Hz, H-16), six methyl group signals at δ_H 0.96 (3H, s, H-18), 1.29 (3H, s, H-19), 2.24 (3H, s, H-21), 1.47 (3H, d, $J = 6.4$ Hz, H-Fuc-6′), 1.67 (3H, d, $J = 6.2$ Hz, H-Rha-6‴), and 1.75 (3H, d, $J = 6.2$ Hz, H-Rha-6″), and three anomeric protons at δ_H 5.01 (1H, d, $J = 7.7$ Hz,

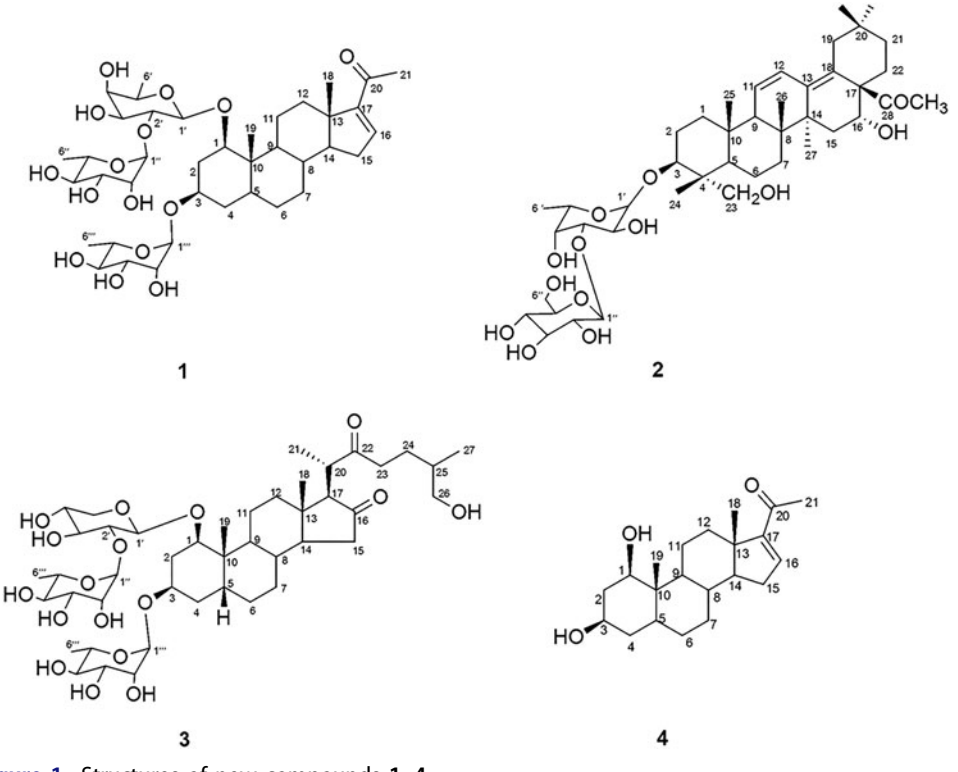

Figure 1. Structures of new compounds **1–4.**

Table 1. ^{1}H NMR and ^{13}C NMR spectral data[a] for compound **1**.

	Aglycone of compound **1**			Sugar moieties of compound **1**	
No.	δc	δ_H	No.	δc	δ_H
1	78.2	4.03 (1H)	Fuc-1′	99.7	5.01 (1H, d, J = 7.7 Hz)
2	30.0	2.26 (1H)	2′	77.2	4.21 (1H)
		2.39 (1H)	3′	75.1	4.81 (1H)
3	70.4	4.23 (1H)	4′	74.4	4.51 (1H)
4	31.1	1.54 (1H)	5′	71.4	3.84 (1H)
		1.88 (1H)	6′	17.1	1.47 (3H, d, J = 6.4 Hz)
5	34.8	2.18 (1H)	Rha-1″	101.6	6.44 (1H, br.s)
6	26.8	1.29 (1H)	2″	72.5	4.89 (1H)
		1.70 (1H)	3″	72.5	4.21 (1H)
7	26.8	1.03 (1H)	4″	74.2	4.27 (1H)
		1.35 (1H)	5″	70.0	4.22 (1H)
8	33.6	1.50 (1H)	6″	19.0	1.75 (3H, d, J = 6.2 Hz)
9	47.0	1.34 (1H)	Rha-1‴	99.6	5.45 (1H, br.s)
10	39.8	–	2‴	72.4	4.69 (1H)
11	22.2	1.48 (1H)	3‴	72.8	4.26 (1H)
		2.16 (1H)	4‴	73.5	4.70 (1H)
12	35.6	1.60 (1H)	5‴	69.2	4.81 (1H)
		2.48 (1H)	6‴	18.8	1.67 (3H, d, J = 6.2 Hz)
13	46.5	–			
14	56.9	1.38 (1H)			
15	32.4	1.90 (1H)			
		2.15 (1H)			
16	144.6	6.61 (1H, dd, J = 1.8, 3.1 Hz)			
17	155.7	–			
18	16.3	0.96 (3H, s)			
19	16.8	1.29 (3H, s)			
20	196.3	–			
21	27.1	2.24 (3H, s)			

[a]^{1}H NMR and ^{13}C NMR were measured at 600 MHz and 150 MHz in C_5D_5N, and the experimental temperature was 298 K, and only the multiplicities of not overlapping resonances are given.

H-Fuc-1′), 5.45 (1H, br. s, H-Rha-1‴), and 6.44 (1H, br. s, H-Rha-1″). The ^{13}C NMR spectrum (Table 1) showed 39 carbon signals, including one carbonyl carbon at δ_C 196.3 (C-20) and two olefinic carbons at δ_C 144.6 (C-16) and 155.7 (C-17) that indicated the presence of an α,β-unsaturated carbonyl group. In addition, six methyls at δ_C 16.3 (C-18), 16.8 (C-19), 27.1 (C-21), 17.1 (C-Fuc-6′), 18.8 (C-Rha-6″), and 19.0 (C-Rha-6‴), and three anomeric carbons at δ_C 99.6 (C-Rha-1‴), 99.7 (C-Fuc-1′), and 101.6 (C-Rha-1″) also appeared in the ^{13}C NMR spectrum. When comparing to literature NMR data [8], the resonances of **1** were almost identical to those of (17,20-*S-trans*)-5β-pregn-16-en-1β,3β-diol-20-one 1-*O*-β-D-xylopyranosyl-(1→2)-[α-L-rhamnopyranosyl]-3-O-α-L-rhamnopyranoside, with the exception of the identity of the sugar: according to the second-level HR-ESI-MS, compound **1** has three deoxyhexose sugars. Moreover, acid hydrolysis (0.1 M HCl) of **1** resulted in the production of D-fucose and L-rhamnose, as identified by HPLC analysis using an optical rotation detector [9]. HMBC correlations (Figure 2) of H-18/C-12, C-13, C-14 and C-17, H-19/C-1, C-5, C-9 and C-10, H-1/C-3, C-5, C-10 and C-19, revealed the planar structure of the aglycone moiety to be pregn-16-en-1,3-diol-20-one. Moreover, HMBC correlations of H-Fuc-1′/C-1, H-Rha-1″/C-Fuc-2′, and H-Rha-1‴/C-3 showed that the terminal rhamnose unit was linked to C-2 of the inner fucose unit, that the fucose unit was linked at C-1 of the aglycone, and that another rhamnose unit was linked at C-3 of the aglycone. Additionally, the NOESY spectrum (Figure 2) showed correlations of

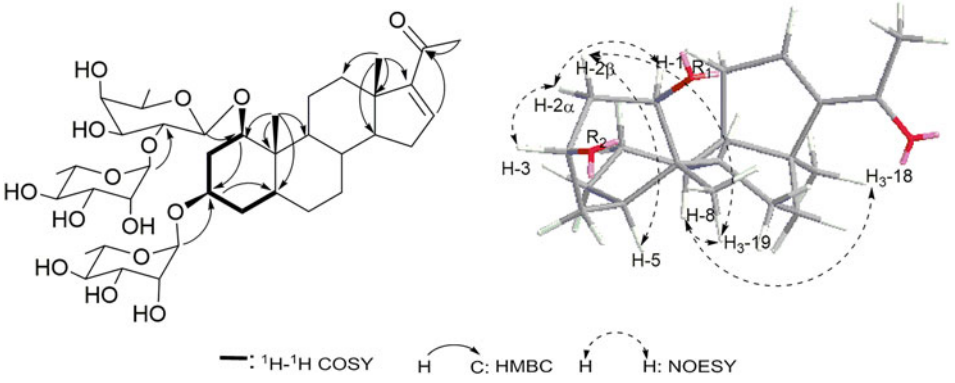

Figure 2. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of compound **1**.

H-8*β*/H-5 and H-19, and H-9α/H-1 and H-3, indicating α-axial configurations of H-1 and H-3, and *β*-orientations of H-5, H-19, 1-OH, and 3-OH, confirming an A/B *cis* ring junction pattern. Furthermore, NOE correlations of H-7α/H-9 and H-7*β*/H-8, and H-8/H-18 and H-19, signified a B/C *trans* ring junction pattern; NOE correlations of H-15*β*/H-8, and H-14/H-15α and H-16, indicated a C/D *trans* ring junction pattern; and NOESY correlations of H-21/H-16 signified that the 16-en-20-one was in an *S-trans* configuration. Therefore, the structure of **1** was characterized as (17,20-*S-trans*)-5*β*-pregn-16-en-1*β*,3*β*-diol-20-one 1-*O*-α-L-rhamno pyranosyl-(1→2)-*β*-D-fucopyranosyl-3-*O*-α-L-rhamnopyranoside.

Compound **2** was obtained as a white amorphous powder. The HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 791.4679 $[M\text{-}H]^{-}$, consistent with the molecular formula $C_{43}H_{68}O_{13}$ (calcd 791.4660 $[M\text{-}H]^{-}$), and NL fragments of D-fucose (146 Da) and D-glucose (162 Da). The NMR data (Table 2) of **2** revealed eight proton signals arising from methyl at δ_H 0.74 (3H, s, H-24), 0.76 (3H, s, H-26), 0.88 (3H, s, H-29), 0.98 (3H, s, H-30), 1.00 (3H, s, H-25), 1.28 (3H, s, H-27), 1.31 (3H, d, $J = 6.4$ Hz, H-Fuc-6′), and 1.95 (3H, s, 28-CH_3), with corresponding carbon signals at δ_C 13.1 (C-24), 17.9 (C-26), 25.6 (C-29), 33.2 (C-30), 19.4 (C-25), 22.4 (C-27), 17.2 (C-Fuc-6′), and 24.8 (28-CH_3), respectively (HSQC spectrum). Two vinyl protons at δ_H 6.49 (1H, dd, $J = 10.8$, 2.9 Hz, H-11) and 5.63 (1H, d, $J = 10.8$ Hz, H-12), together with the two olefinic groups producing signals at δ_C 127.0 (C-11), 127.3 (C-12), 137.5 (C-13), and 133.0 (C-18), showed that the two double bonds could only be located at the $\Delta^{11(12)}$ and $\Delta^{13(18)}$ positions. This was further confirmed by the ^{1}H-^{1}H COSY cross-peaks between δ_H 2.05 (H-9) and δ_H 6.49 (H-11)/5.63 (H-12) and the HMBC correlations (Figure 3) between the proton signals at δ_H 1.74, 2.52 (H_2-19), and δ_H 1.28 (H-27), and the olefinic quaternary carbon at δ_C 137.5 (C-13). In addition, two anomeric protons at δ_H 4.44 (1H, d, $J = 7.7$ Hz, H-Fuc-1′) and 4.58 (1H, d, $J = 7.7$ Hz, H-Glc-1″) that connected to carbons at δ_C 105.9 (C-Fuc-1′), 106.0 (C-Glc-1″) were also present in the NMR spectra. These resonances of **2** were consistent with those of saikosaponin b2 [10] with the only key exceptions being the absence of a carbon signal at δ_C 65.3 (C-28), and the presence of two new carbon signals, at δ_C 182.1 and 24.2, and a methyl proton signal at δ_H 1.95. Moreover, the HMBC correlation (Figure 3) between δ_H 1.95 (CH_3) and δ_C 182.1 (C-28) revealed the existence of a -$COCH_3$

Table 2. ^{1}H NMR and ^{13}C NMR spectral data[a] for compound **2.**

	Aglycone of compound **2**			Sugar moieties of compound **2**	
No.	δc	δ_H	No.	δc	δ_H
1	39.4	1.02 (1H)	Fuc-1′	105.9	4.44 (1H, d, J = 7.7 Hz)
		1.90 (1H)	2′	71.7	3.67 (1H)
2	26.5	1.80 (1H)	3′	85.5	3.62 (1H)
		1.97 (1H)	4′	72.2	3.65 (1H)
3	83.7	3.66 (1H)	5′	71.5	3.35 (1H)
4	44.3	–	6′	17.2	1.31 (3H, d, J = 6.4 Hz)
5	48.5	1.27 (1H)	Glc-1″	106.0	4.58 (1H, d, J = 7.7 Hz)
6	19.2	1.02 (1H)	2″	75.7	3.30 (1H)
		1.49 (1H)	3″	78.2	3.38 (1H)
7	33.2	1.33 (1H)	4″	72.7	3.87 (1H)
		1.48 (1H)	5″	78.0	3.29 (1H)
8	42.2	–	6″	62.7	3.69 (1H)
9	55.1	2.05 (1H, d , J = 4.2 Hz)			3.85 (1H)
10	37.6	–			
11	127.0	6.49 (1H, dd, J = 10.8, 2.9Hz)			
12	127.3	5.63 (1H, d, J = 10.8 Hz)			
13	137.5	–			
14	42.7	–			
15	32.3	1.44 (1H)			
		1.95 (1H)			
16	69.3	4.07 (1H)			
17	45.7	–			
18	133.0	–			
19	39.7	1.74 (1H, d, J = 13.5 Hz)			
		2.52 (1H, d, J = 13.5 Hz)			
20	33.5	–			
21	36.1	1.32 (1H)			
		1.56 (1H)			
22	24.2	1.59 (1H)			
		2.04 (1H)			
23	65.3	3.30 (1H)			
		3.68 (1H)			
24	13.1	0.74 (3H, s)			
25	19.4	1.00 (3H, s)			
26	17.9	0.76 (3H, s)			
27	22.4	1.28 (3H, s)			
28	182.1	–			
29	25.6	0.88 (3H, s)			
30	33.2	0.98 (3H, s)			
28-CH_3	24.8	1.95 (3H, s)			

[a]^{1}H NMR and ^{13}C NMR were measured at 600 MHz and 150 MHz in CD_3OD, the experimental temperature was 298 K, and the only the multiplicities of not overlapping resonances are given.

group, which connected to C-17. Meanwhile, NOESY correlations (Figure 3) of H_3-24/H_3-25β, H-5α/H-3, and H-15β/H-16 indicated that 16-OH was in an α-axial configuration and that H-24 and 3-OH were in β-orientations. Acid hydrolysis (0.1 M HCl) of **2** afforded D-glucose and D-fucose, which were identified by HPLC equipped with an optical rotation detector [9]. Thus, compound **2** was characterized as 3β,16α,23-trihydroxy-11,13(18)-dien-28-methylketone-oleane 3-*O*-β-D-glucopyranosyl-(1→3)-β-D-fucopyranoside.

Compound **3** was obtained as a white amorphous solid. The HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 871.4838 $[M\text{-}H]^-$, consistent with the molecular formula $C_{44}H_{72}O_{17}$ (calcd 871.4770 $[M\text{-}H]^-$), and NL fragments of L-rhamnose (146 Da) and D-xylose (132 Da). The ^{1}H NMR spectrum (Table 3) of **3** showed six methyl groups, of which two were singlet signals (δ_H 0.71 and 1.35) and four were

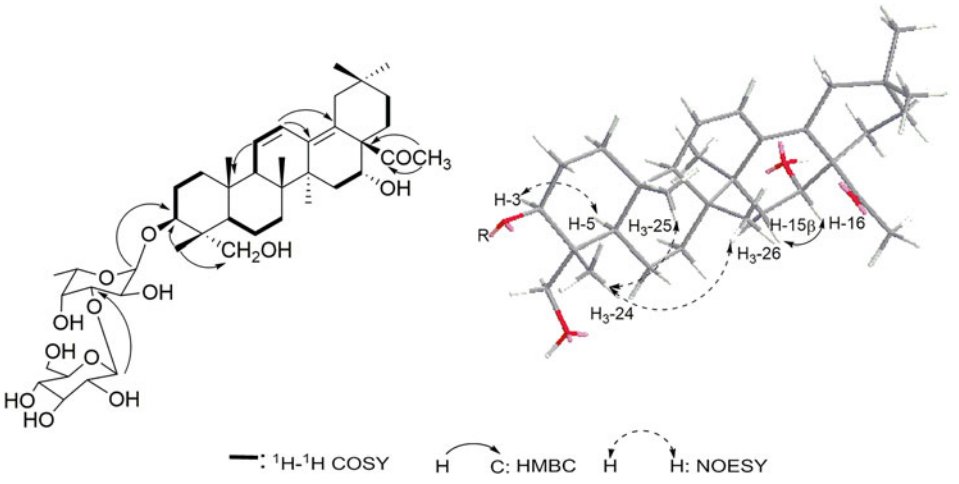

Figure 3. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of compound **2.**

doublet signals (δ_H 1.02, 1.15, 1.72, and 1.80), and three anomeric protons at δ_H 6.53 (1H, br. s, H-Rha-1″), 5.47 (1H, br. s, H-Rha-1‴), and 5.09 (1H, d, $J = 6.3$ Hz, H-Xyl-1′). The ^{13}C NMR spectrum (Table 3) showed 44 carbon signals, including two carbonyl carbons at δ_C 213.3 and 217.9, and three anomeric carbons at δ_C 99.9 (C-Rha-1‴), 100.2 (C-Xyl-1′), and 101.7 (C-Rha-1″). HMBC correlations (Figure 4) were observed from δ_H 0.71 (H-18) to δ_C 39.3 (C-12)/41.8 (C-13) and 66.6 (C-17), from δ_H 1.02 (H-21) to δ_C 43.8 (C-20), 66.6 (C-17) and 213.3 (C-22), and from δ_H 2.76 (H-17) to δ_C 13.1 (C-18)/213.3 and 217.9. Moreover, the DEPT-135 spectrum showed that the peak at δ_C 67.5 was downfield, implying that the carbonyl signal at δ_C 213.3 arose from C-22 and the one at δ_C 217.9 from C-16. In addition, correlations in the HMBC spectrum (Figure 4) of H-Xyl-1′/C-1 and H-Rha-1″/C-Xyl-2′ showed that the terminal rhamnose unit was linked at C-2′ of the inner xylose unit, which was linked at C-1 of the aglycone. Furthermore, the correlation between H-Rha-1‴ and C-3 indicated that another rhamnose unit was linked at C-3 of the aglycone. Acid hydrolysis (0.1 M HCl) of **3** released L-rhamnose and D-xylose, which were identified by HPLC analysis using an optical rotation detector [9]. NOESY correlations of H-8β/H-5 and H-19, and H-9α/H-1 and H-3, indicated α-configurations for H-1 and H-3, and β-orientations for H-5, H-19, 1-OH and 3-OH, confirming an A/B *cis* ring junction pattern. Moreover, correlations of H-17/H-21 and H-14α suggested α-configurations for H-17 and H-21. Therefore, the structure of compound **3** was characterized as 1β,3β,26-trihydroxy-16,22-dioxo-cholestane 1-O-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-3-O-α-L-rhamnopyranoside.

Compound **4** was obtained as a white amorphous powder. The molecular formula was deduced as $C_{21}H_{32}O_3$ from the HR-ESI-MS result, which showed a positive molecular ion peak at *m/z* 333.2434 $[M+H]^+$. The ^{1}H NMR spectrum (Table 4) of **4** showed three methyls at δ_H 0.95 (3H, s), 1.33 (3H, s) and δ_H 2.26 (3H, s) and the presence of an olefinic proton at δ_H 6.63 (1H, dd, $J = 1.6$, 3.0 Hz, H-16) . The DEPT and ^{13}C NMR (Table 4) spectrum displayed 21 carbon signals, including 3 methyls, 7 methylenes, 7 methines, and 4 quaternary carbons. Among them, three methyl carbon

Table 3. ^{1}H NMR and ^{13}C NMR spectral data[a] for compound **3**.

	Aglycone of compound **3**			Sugar moieties of compound **3**	
No.	δc	δ_H	No.	δc	δ_H
1	79.2	4.08 (1H)	Xyl-1′	100.2	5.09 (1H, d, J = 6.3 Hz)
2	31.5	2.33 (1H)	2′	77.0	4.29 (1H)
		2.42 (1H)	3′	79.6	4.28 (1H)
3	71.0	4.22 (1H)	4′	71.4	4.27 (1H)
4	31.8	1.63 (1H)	5′	67.1	3.67 (1H)
		1.87 (1H)			4.37 (1H)
5	35.7	1.98 (1H)	Rha-1″	101.7	6.53 (1H, br.s)
6	26.2	1.31 (1H)	2″	72.6	4.85 (1H)
		1.54 (1H)	3″	72.8	4.67 (1H)
7	27.3	1.78 (1H)	4″	74.3	4.28 (1H)
		2.23 (1H)	5″	69.5	4.85 (1H)
8	33.4	1.39 (1H)	6″	19.1	1.80 (3H, d, J = 6.1 Hz)
9	46.7	1.44 (1H)	Rha-1‴	99.9	5.47 (1H, br.s)
10	39.6	–	2‴	72.5	4.85 (1H)
11	22.6	1.49 (1H)	3‴	72.5	4.67 (1H)
		2.21 (1H)	4‴	74.1	4.35 (1H)
12	39.3	1.49 (1H)	5‴	70.1	4.42 (1H)
		1.89 (1H)	6‴	18.7	1.72 (3H, d, J = 6.1 Hz)
13	41.8	–			
14	51.6	1.38 (1H)			
15	40.3	2.90 (1H)			
		3.00 (1H)			
16	217.9	–			
17	66.6	2.76 (1H, d, J = 10.6 Hz)			
18	13.1	0.71 (3H, s)			
19	16.7	1.35 (3H, s)			
20	43.8	2.72 (1H)			
21	15.7	1.02 (3H, d, J = 6.5 Hz)			
22	213.3	–			
23	37.5	1.71 (1H)			
		2.08 (1H)			
24	27.7	1.78 (1H)			
		2.23 (1H)			
25	36.2	1.98 (1H)			
26	67.5	3.74 (1H)			
		3.81 (1H)			
27	17.3	1.15 (3H, d, J = 6.7 Hz)			

[a]^{1}H NMR and ^{13}C NMR were measured at 600 MHz and 150 MHz in C_5D_5N, the experimental temperature was 298 K, and only the multiplicities of not overlapping resonances are given.

signals at δ_C 16.1, 19.2, and 26.6 were ascribed to C-18, C-19, and C-21, and one carbonyl carbon at δ_C 196.2 (C-20) and two olefinic carbons at δ_C 144.6 (C-16) and 155.3 (C-17) indicated the presence of an α,β-unsaturated carbonyl moiety [11]. HMBC correlations (Figure 5) of H-19/C-1, C-5, C-9 and C-10, H-18/C-12, C-13, C-14 and C-17, H-1/C-3, C-5, C-10 and C-19, H-3/C-2 and C-4, H-6/C-4,C-7, C-8 and C-10, H-8/C-9, C-11 and C-14, H-16/C-13, C-14, C-15, C-17 and C-20, H-21/C-17 and C-20, revealed the planar structure of compound **4** as pregn-16-en-1,3-diol-20-one. Meanwhile, the NOESY correlations (Figure 5) of H-8β/H-5 and H-19, H-8β and H-18, H-9α/H-1 and H-3, indicated α-axial configurations of H-1 and H-3, and β-orientation of H-5, H-8, H-18, H-19, 1-OH and 3-OH, which supported the A/B *cis* ring junction pattern. Therefore, the structure of 4 was characterized as 5β-pregn-16-ene-1β,3β-diol-20-one, as a natural product [12].

Additionally, two known triterpenoid saponins and four flavones were identified, by comparing their spectra (NMR and MS) and physicochemical data with those

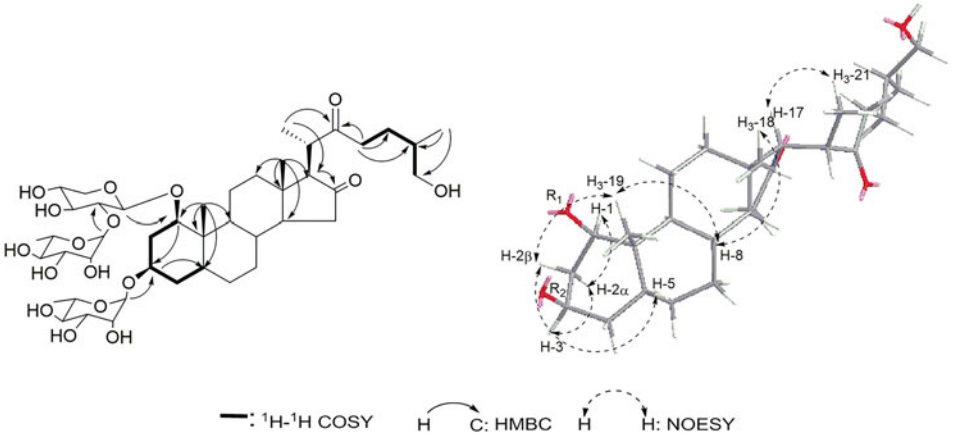

Figure 4. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of compound **3.**

Table 4. ^{1}H NMR and ^{13}C NMR spectral data[a] for compound **4.**

No.	Compound **4**	
	δc	δ_H
1	73.1	4.00 (1H)
2	34.4	2.07 (1H)
		2.10 (1H)
3	68.1	4.39 (1H)
4	34.4	1.40 (1H)
		1.61 (1H)
5	31.3	1.90 (1H)
6	26.2	1.38 (1H)
		1.53 (1H)
7	27.0	1.18 (1H)
		1.29 (1H)
8	32.8	1.76 (1H)
9	42.5	1.33 (1H)
10	40.4	–
11	20.9	1.53 (1H)
		2.24 (1H)
12	35.4	1.41 (1H)
		2.60 (1H)
13	46.3	–
14	56.2	1.39 (1H)
15	32.2	1.89 (1H)
		2.16 (1H)
16	144.6	6.63 (1H, dd, J = 1.6, 3.0 Hz)
17	155.3	–
18	16.1	0.95 (3H, s)
19	19.2	1.33 (3H, s)
20	196.2	–
21	26.6	2.26 (3H, s)

[a]^{1}H NMR and ^{13}C NMR were measured at 600 MHz and 150 MHz in C_5D_5N, the experimental temperature was 298 K, and only the multiplicities of not overlapping resonances are given.

reported in the literature, as prosaikogenin D (**5**) [13], saikosaponin b2 (**6**) [10], isorhamnetin-3-*O*-rutinoside (**7**) [14], farrerol (**8**) [15], luteolin (**9**) [16], and 1,2,8-trihydroxy-5,6-dimethoxyxanthone (**10**) [17] (Figure 1).

All isolated compounds were evaluated for their inhibitory activity on LPS-induced NO production in RAW 264.7 macrophages. Compounds **1–4** showed IC_{50} values of

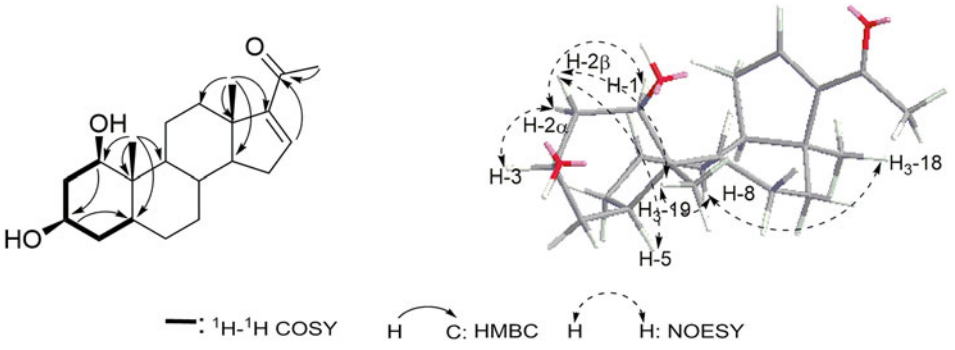

Figure 5. Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of compound **4.**

37.5 μM, 31.4 μM, 34.6 μM, and 56.1 μM, respectively. Compounds **5–10** also showed inhibitory activities with IC_{50} values ranging from 20.3 to 42.9 μM (Table 5, Figure 6). These results showed that the steroids, saponins, and flavones isolated from *R. carnea* all had anti-inflammatory activities consistent with the literature data [18–21].

3. Experimental

3.1. General experimental procedures

The IR spectra were recorded on a TENSOR-27 instrument (Bruker, Karlsruhe, Germany). The HR-ESI-MS was measured on a Triple TOF^{TM} 5600^{+} system with a Duo Spray source (AB SCIEX, Foster City, CA, USA). 1D and 2D NMR spectra were recorded on a AVANCE600 instrument (Bruker, Karlsruhe, Germany) with TMS as an internal standard. Optical rotations were recorded on a Model 343 polarimeter (PerkinElmer, Massachusetts, USA). The analytical HPLC was performed on a HPLC system (Shimadzu, Kyoto, Japan), and separation was performed on a Cosmosil C_{18} column (4.6 × 250 mm, 5.0μm). Semipreparative HPLC (Waters, Massachusetts, USA) was performed on a system comprising a Waters 550 pump equipped with a Waters 2487 detector and a Cosmosil C_{18} column (10 mm × 250 mm, 5.0 μm, Kyoto, Japan). Sephadex LH-20 gel was purchased from GE Healthcare Bio-Sciences AB (GE, Uppsala, Sweden). HP20 macroporous resin ODS gel (MeOH as mobile phases) was purchased from Mitsubishi Chemical Corporation (Mitsubishi, Tokyo, Japan). Silica gel was purchased from Qingdao Haiyang Chemical Group Corporation (Qingdao, China). D-fucose (140651-201504), L-rhamnose (111683-201502), D-glucose (111833-201506), and D-xylose (111508-201605) were purchased from National Institute for Food and Drug Control (Beijing, China).

3.2. Plant material

Reineckia carnea was collected on March in 2014 from Anshun city of Guizhou Province, and identified by Vice Director of Pharmacists Bei Wu, Nanchang Institute for Food and Drug Control. A voucher specimen (No. Z-140310-01) has been deposited at Jiangxi University of Traditional Chinese Medicine, Nanchang, China.

Table 5. IC_{50} values of compounds **1–10** on LPS-stimulated NO production in RAW 264.7 macrophages.

Compounds	IC_{50} (μM)	Compounds	IC_{50} (μM)
1	37.5 ± 3.9	**6**	30.7 ± 3.2
2	31.4 ± 3.1	**7**	20.3 ± 2.7
3	34.6 ± 4.3	**8**	27.7 ± 4.6
4	56.1 ± 7.6	**9**	25.4 ± 4.2
5	42.9 ± 6.3	**10**	22.9 ± 3.4
Quercetina	20.7 ± 2.8		

[a]Quercetin was used as the positive control.

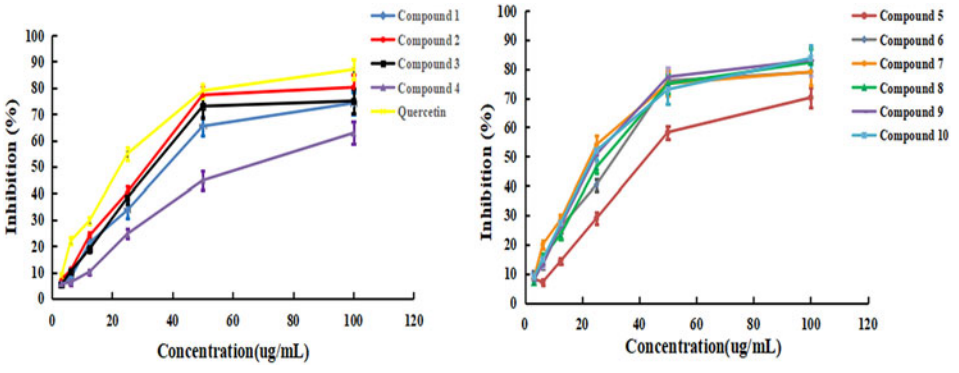

Figure 6. The dose-response curve of NO production of compounds **1–10**.

3.3. Extraction and isolation

The air-dried whole plant of *R. carnea* (20 kg) was powdered and extracted with 80% EtOH under reflux for three times at 80 °C. After removing the solvent, the concentrated residue was successively partitioned with petroleum ether, EtOAc and *n*-BuOH. The EtOAc extract (305 g) was subjected to column chromatography (CC) on silica gel, eluting with gradient solvent system (CH_2Cl_2-MeOH, 1:0–0:1) to give seven fractions (E-1∼E-7). E-6 was subjected to CC on MCI gel, and further separated by semipreparative HPLC (MeCN/H_2O = 23%/77%; 2.0 ml/min; 203 nm) to obtain compound **1** (10.0 mg; t_R = 22 min), compound **2** (5.3 mg; t_R = 25 min), compound **5** (2.7 mg; t_R = 36 min), compound **6** (6.4 mg; t_R = 20 min). The *n*-BuOH extract (812 g) was subjected to HP-20 macroporous resin column, and the 60% EtOH eluate (241 g) was separated by silica gel CC, eluting with gradient solvent system (CH_2Cl_2-MeOH, 20:1–0:1) to obtain five fractions (S-1∼S-5), and fraction S4 was further separated by semipreparative HPLC (MeOH/H_2O = 32%/68%; 2.0 ml/min, 254 nm) to yield compound **7** (4.8 mg; t_R = 32 min), compound **3** (5.3 mg; t_R = 27 min), compound **4** (4.6 mg; t_R = 34 min). The 95% EtOH eluate (79 g) was separated by ODS gel CC to yield four fractions (O-1∼O-4), and fraction O3 was further separated by semipreparative HPLC (2.0 ml/min, 254 nm) with MeCN-H_2O (13:87) as mobile phase to give compound **9** (13.4 mg; t_R = 26 min), compound **8** (2.8 mg; t_R = 28 min), compound **10** (3.7 mg; t_R = 32 min).

3.3.1. (17,20-S-trans)-5β-Pregn-16-en-1β,3β-diol-20-one 1-O-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranosyl-3-O-α-L-rhamnopyranoside (1)

A white amorphous powder; $[\alpha]_D^{20}$-46.3 (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3413, 2918, 1665, 1459, 1074 cm^{-1}; ^1H NMR (600 MHz, in C_5D_5N) and ^{13}C NMR (150 MHz, in C_5D_5N) spectral data, see Table 1; HRESIMS: *m/z* 769.4155 $[M\text{-}H]^-$ (calcd for $C_{39}H_{61}O_{15}$, 769.4089).

3.3.2. 3β,16α,23-trihydroxy-11,13(18)-dien-28-methylketone-oleane 3-O-β-D-gluco-pyranosyl-(1→3)-β-D-fucopyranoside (2)

A white amorphous powder; $[\alpha]_D^{20}$-29.5 (*c* 0.12, MeOH); IR (KBr) ν_{max}: 3389, 2921, 2883, 1728, 1072 cm^{-1}; ^1H NMR (600 MHz, in CD_3OD) and ^{13}C NMR (150 MHz, in CD_3OD) spectral data, see Table 2; HRESIMS: *m/z* 791.4679 $[M\text{-}H]^-$ (calcd for $C_{43}H_{67}O_{13}$, 791.4660).

3.3.3. 1β,3β,26-Trihydroxy-16,22-dioxo-cholestane 1-O-α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-3-O-α-L-rhamnopyranoside (3)

A colorless amorphous solid; $[\alpha]_D^{20}$-36.6 (*c* 0.12, MeOH); IR (KBr) ν_{max}: 3410, 2918, 1667, 1652, 1070 cm^{-1}; ^1H NMR (600 MHz, in C_5D_5N) and ^{13}C NMR (150 MHz, in C_5D_5N) spectral data, see Table 3; HRESIMS: *m/z* 871.4838 $[M\text{-}H]^-$ (calcd for $C_{44}H_{71}O_{17}$, 871.4770).

3.3.4. 5β-Pregn-16-ene-1β,3β-diol-20-one (4)

A white amorphous powder; $[\alpha]_D^{20}$ +27.4 (*c* 0.13, MeOH); IR (KBr) ν_{max}: 3413, 2922, 1664, 1456, 1114 cm^{-1}; ^1H NMR (600 MHz, in C_5D_5N) and ^{13}C NMR (150 MHz, in C_5D_5N) spectral data, see Table 4; HRESIMS: *m/z* 333.2434 $[M+H]^+$ (calcd for $C_{21}H_{33}O_3$, 333.2385).

3.4. Acid hydrolysis of compounds (1–3)

A solution of each of **1–3** (each 3.0 mg) in 2 ml HCl (0.1 M HCl) was refluxed at 80 °C for 3 h. After cooling, the reaction mixture was extracted with EtOAc (2 ml × 3) to remove the aglycone. The aqueous layer was purified by CC over silica gel eluted with EtOAc–MeOH–H_2O (6:1:1) to yield sugar, which was then compared with an authentic sugar sample by co-TLC (EtOAc–MeOH–H_2O, 6:1:1, R_f 0.12 for xylose, R_f 0.16 for rhamnose, R_f 0.28 for glucose, R_f 0.42 for fucose). On the basis of the acid hydrolysate of **1–3**, D-xylose, L-rhamnose, D-glucose, and D-fucose were confirmed by comparison of optical rotation of the liberated sugar with that of an authentic sample [D-xylose, [α]20 D +28.8 (*c* 0.02, H_2O); L-rhamnose, [α]20 D +12.4(*c* 0.15, H_2O); D-glucose, [α]20 D +55.2 (*c* 0.05, H_2O); D-fucose, [α]20 D +75.6 (*c* 0.08, H_2O)].

3.5. Analysis of NO production

RAW 264.7 macrophages were seeded into 96-well plates with 2×10^5 cells/well for 24 h and then pre-incubated with different concentrations of compounds for 1 h

before stimulation with or without LPS (1 μg/ml) for 24 h. The NO concentration in culture medium was determined by Griess reagent kit. The absorbance at 545 nm was measured using a multifunction microplate reader (Molecular Devices, Flex Station 3) [22]. Sodium nitrite was used as a standard to calculate the nitrite concentration. Inhibition (%) = $(1-(A_{LPS\ +\ sample} - A_{untreated})/(A_{LPS}-A_{untreated})) \times 100$. The experiments were performed in triplicates, and the data were expressed as mean ± SD. Quercetin was used as a positive control [23].

Disclosure statement

No potential conflict of interest was reported by the authors.

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