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New antimicrobial pregnane glycosides from the stem of *Ecdysanthera rosea*

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ABSTRACT

Phytochemical investigation on the stem of *Ecdysanthera rosea* led to the isolation of eight new C-21 pregnane glycoside ecdysosides A–H (**1**–**8**), together with one known pregnane glycoside ecdysantheroside A (**9**). Their structures were elucidated based on extensive spectroscopic data (MS, IR, 1D and 2D NMR) analysis, as well as comparison with the reported literature data. Antimicrobial activities of all the compounds were evaluated against bacteria and yeasts. Compounds **1**, **9**, **3** and **5** exhibited moderate antibacterial activities against respective *Enterococcus faecalis* and *Providensia smartii*, with MIC value of 12.5 μg/mL. Compound **8** showed significant anti-yeast activity against *Cryptococcus neoformans* with MIC value of 12.5 μg/mL.

1. Introduction

Ecdysanthera rosea Hook. et Arn. (Apocynaceae) is mainly distributed in the southern region of China. As a Chinese folk medicine, its whole plant has been used for the treatment of traumatic injury, sore throat, and chronic nephritis [1]. Previous phytochemical investigation reported the isolation of terpenoids, flavonoids, phenolic glycosides, steroid and their glycosides from this natural medicine [2–12].

The *E. rosea* has been used as a medicinal plant with definite therapeutic effects. However, only a few steroid glycosides from this plant have been previously reported with cytotoxic activity, which is not related to its medicinal use [12], and this activated our interest to search for more compounds like them from this medicinal plant and conduct a further bioactive research. In our current phytochemical investigation, nine pregnane glycosides (**1**–**9**) were isolated from this species, of these compounds **1–5** possessed a different aglycone from the previously reported compound **9** [12]. Considering the fact that many diseases like traumatic injury and sore throat could be caused by bacteria and fungi, we evaluated their antibacterial activities against three bacterial strains and antifungal activities against three yeast species by using broth micro-dilution method. The results demonstrated that compounds **1**, **3**, **5** and **9** showed moderate activities against the corresponding bacterial strain, while compound **8** showed significant anti-yeast activity against *Cryptococcus neoformans*, comparable to nystatin, the positive control. In this paper, we report the isolation, structure elucidation and antimicrobial activities of these pregnane glycosides.

2. Experimental

2.1. General methods

Optical rotations were measured with a JASCO P-1020 digital polarimeter. UV spectra were obtained using a Shimadzu

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UV-2401 PC spectrophotometer. IR spectra were recorded on a Bruker Tensor 27 infrared spectrophotometer using KBr pellets. 1D and 2D NMR spectra were recorded on a Bruker AM-400, a DRX-500, and an Avance III 600 or AV 600 MHz spectrometer with TMS as internal standard. ESI-MS spectra were recorded on Bruker HTC/Esquire and Agilent G6230 spectrometer. HREI-MS was recorded on a Waters Auto Spec Premier P776 spectrometer. HRESI-MS was recorded on an Agilent G6230 spectrometer. Column chromatography was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Rp-18 (40-63 μm, Merk), and Sephadex LH-20 (GE Healthcare, Sweden). Fractions were monitored by TLC (GF254, Qingdao Marine Chemical Ltd., Qingdao, China), and by heating silica gel plates sprayed with 10% H_2SO_4 in ethanol. GC analysis was performed on Agilent 7890A gas chromatograph equipped with a H_2 flame ionization detector.

2.2. Plant material

The stem of *E. rosea* was collected from Xishuangbanna Autonomous Prefecture, Yunnan Province, People's Republic of China, and identified by Jingyun Cui of Xishuangbanna Botanic Garden. A voucher specimen (Cui 200811-03) has been deposited at the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air dried and crushed stems of *E. rosea* (10 kg) were extracted with MeOH (70 L, 24 h) three times at room temperature. After filtration, all the solvent was evaporated under reduced pressure at 50 °C to yield the extract (500 g). This crude extract was exhaustively partitioned between water and EtOAc to obtain the EtOAc fraction (120 g). The EtOAc fraction was then subjected to column chromatography over silica gel and eluted with chloroform–acetone (100:1→1:1) to give 6 fractions [Fr.A (38.5 g), Fr.B (13 g), Fr.C (14 g), Fr.D (9 g), Fr.E (18 g), Fr.F (26 g)] based on the TLC plate analysis. Fraction C (14 g) was isolated by Sephadex LH-20 (MeOH–H_2O 50:50) and repeated silica gel column with chloroform–methanol to yield compounds **1** (12 mg), **2** (4 mg) and **9** (22 mg). Fraction D (9 g) was chromatographed over Sephadex LH-20 (MeOH–H_2O 50:50), RP-C_{18} gel (MeOH–H_2O 10:90→80:20) and further purified by HPLC (MeCN–H_2O 40:60) to provide compounds **3** (3 mg), **4** (7 mg), **7** (11 mg) and **8** (5 mg). Fraction E (18 g) was separated over Sephadex LH-20, eluted with MeOH–H_2O (1:1) and chromatographed over RP-C_{18} gel (MeOH–H_2O 10:90→70:30), followed by HPLC (MeCN–H_2O 35:65) to give compounds **5** (6 mg), and **6** (5 mg).

2.3.1. Ecdysoside A (**1**)

White amorphous powder; $[\alpha]_D^{21}$ +36.6 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 201 (3.9), 228 (3.9), and 245 (4.0) nm; IR (KBr) ν_{max} 3442, 2971, 2876, 1757, 1709, 1631, 1455, 1383, 1195, and 1001 cm^{-1}; positive-ion ESI-MS *m/z* 509 $[M + Na]^+$; HREI-MS *m/z* 486.2617 $[M]^+$ (calcd. for $C_{28}H_{38}O_7^+$, 486.2618). ^1H and ^{13}C NMR: see Tables 1 and 2.

2.3.2. Ecdysoside B (**2**)

White amorphous powder; $[\alpha]_D^{21}$ +32.8 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε): 201 (3.9), 228 (3.9), and 245 (4.0) nm; IR (KBr) ν_{max} 3441, 2971, 1760, 1630, 1451, 1381, 1368, 1332, 1196, and 1002 cm^{-1}; positive-ion ESI-MS *m/z* 797 $[M + Na]^+$; positive-ion HRESI-MS *m/z* 797.4082 $[M + Na]^+$ (calcd. for $C_{42}H_{62}O_{13}Na^+$, 797.4088). ^1H and ^{13}C NMR: see Tables 1 and 2.

2.3.3. Ecdysoside C (**3**)

White amorphous powder; $[\alpha]_D^{21}$ +7.5 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε): 201 (3.8), 228 (3.8), and 245 (3.8) nm; IR (KBr) ν_{max} 3439, 2970, 2853, 1758, 1709, 1631, 1452, 1368, 1196, and 1004 cm^{-1}; positive-ion ESI-MS *m/z* 959 $[M + Na]^+$; positive-ion HRESI-MS *m/z* 959.4617 $[M + Na]^+$ (calcd. for $C_{48}H_{72}O_{18}Na^+$, 959.4611). ^1H and ^{13}C NMR: see Tables 1 and 2.

2.3.4. Ecdysoside D (**4**)

White amorphous powder; $[\alpha]_D^{21}$ +30.7 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε): 200 (4.0), 222 (4.2), and 245 (4.0) nm; IR (KBr) ν_{max} 3442, 2972, 1760, 1604, 1450, 1381, 1266, 1196, and 1001 cm^{-1}; positive-ion ESI-MS *m/z* 941 $[M + Na]^+$; HREI-MS *m/z* 918.4970 $[M]^+$ (calcd. for $C_{49}H_{74}O_{16}^+$, 918.4977). ^1H and ^{13}C NMR: see Tables 1 and 2.

2.3.5. Ecdysoside E (**5**)

White amorphous powder; $[\alpha]_D^{21}$ +8.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 200 (4.2), 222 (4.2), and 247 (4.1) nm; IR (KBr) ν_{max} 3442, 2972, 2906, 1756, 1708, 1631, 1451, 1404, 1382, and 1002 cm^{-1}; positive-ion ESI-MS *m/z* 1121 $[M+Na]^+$; positive-ion HRESI-MS *m/z* 1121.5150 $[M+Na]^+$ (calcd. For $C_{54}H_{82}O_{23}Na^+$, 1121.5145). ^1H and ^{13}C NMR: see Tables 1 and 2.

2.3.6. Ecdysoside F (**6**)

White amorphous powder; $[\alpha]_D^{21}$ −6.4 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3442, 2969, 2853, 1755, 1631, 1452, 1381, 1367, 1267, and 1003 cm^{-1}; positive-ion ESI-MS *m/z* 801 $[M + Na]^+$; positive-ion HRESI-MS *m/z* 801.4406 $[M+Na]^+$ (calcd. for $C_{42}H_{66}O_{13}Na^+$, 801.4401). ^1H and ^{13}C NMR: see Tables 3 and 4.

2.3.7. Ecdysoside G (**7**)

White amorphous powder; $[\alpha]_D^{21}$ −4.4 (*c* 0.05, MeOH); IR (KBr) ν_{max} 3439, 3432, 2970, 2872, 1756, 1631, 1454, 1381, 1190, and 1002 cm^{-1}; positive-ion ESI-MS *m/z* 963 $[M + Na]^+$; positive-ion HRESI-MS *m/z* 963.4935 $[M + Na]^+$ (calcd. for $C_{48}H_{76}O_{18}Na^+$, 963.4929). ^1H and ^{13}C NMR: see Tables 3 and 4.

2.3.8. Ecdysoside H (**8**)

White amorphous powder; $[\alpha]_D^{21}$ −29.5 (*c* 0.04, MeOH); IR (KBr) ν_{max} 3443, 2969, 2855, 1759, 1634, 1451, 1382, 1192, 1097, and 1005 cm^{-1}; positive-ion ESI-MS *m/z* 1251 $[M+Na]^+$; negative HRESI-MS *m/z* 1227.6518 $[M - H]^-$ (calcd. for $C_{62}H_{99}O_{24}^-$, 1227.6526). ^1H and ^{13}C NMR: see Tables 3 and 4.

2.3.9. Acid hydrolysis of **1**–**9** and GC analysis

Compounds **1**–**9** (2 mg) were dissolved with 2 M HCl (1, 4dioxane/H_2O 1:1, 2 mL) and hydrolyzed on water bath at 90 °C for 2 h. After cooling, the reaction products was partitioned with $CHCl_3$ (3 × 5 mL). The aqueous layer was evaporated to dryness with MeOH until neutral. The dried residue was dissolved in 1 mL anhydrous pyridine and treated with L-cysteine methyl ester hydrochloride (1.5 mg) and stirred at 60 °C for 1 h. Trimethylsilylimidazole (1.5 ml) was added to the

Table 1
^{1}H NMR data of compounds **1**–**5** (δ in ppm, J in Hz, C_5D_5N).

Position	1	2	3	4[a]	5
1	1.02 m, 1.73 m	1.03 m, 1.73 m	1.05 m, 1.75 m	1.11 m, 1.90 m	1.04 m, 1.74 m
2	1.74 m, 2.14 m	1.70 m, 2.09 m	1.72 m, 2.11 m	1.61 m, 1.97 m	1.70 m, 2.10 m
3	3.85 m	3.78 m	3.82 m	3.54 m	3.79 m
4	2.37 m, 2.58 m	2.32 m, 2.52 m	2.34 m, 2.55 m	2.24 m, 2.39 m	2.33 m, 2.53 m
6	5.36 m	5.32 m	5,36 m	5.41 m	5.33 m
7	2.00 m, 2.13 m	1.97 m, 2.10 m	2.00 m, 2.12 m	2.09 m, 2.27 m	1.98 m, 2.10 m
8	2.93 m	2.90 m	2.92 m	2.92 m	2.90 m
9	1.20 m	1.17 m	1.20 m	1.28 m	1.17 m
11	1.60 m, 2.40 m	1.58 m, 2.38 m	1.60 m, 2.39 m	1.72 m, 2.38 m	1.58 m, 2.38 m
12	1.59 m, 2.40 m	1.55 m, 2.35 m	1.59 m, 2.40 m	1.45 m, 2.32 m	1.56 m, 2.36 m
15	5.94 d (1.5)	5.90 d (1.7)	5.94 d (1.7)	5.86 d (1.8)	5.90 d (1.5)
17	2.76 d (2.5)	2.71 d (2.4)	2.77 d (2.4)	2.51 d (2.7)	2.72 d (2.5)
19	1.11 s	1.08 s	1.10 s	1.14 s	1.07 s
20	4.85 m	4.81 m	4.85 m	4.68 m	4.81 m
21	1.38 d (6.4)	1.35 d (6.0)	1.39 d (6.4)	1.51 d (6.5)	1.36 d (6.4)
1′	5.28 dd (9.6, 1.8)	5.27 dd (9.6, 1.6)	5.30 dd (9.6, 1.5)	4.84 dd (9.6, 1.6)	5.27 br d (9.6)
2′	1.89 m, 2.42 m	1.91 m, 2.32 m	1.94 m, 2.37 m	1.58 m, 2.09 m	1.91 m, 2.33 m
3′	3.81 m	4.10 m	4.12 m	3.79 m	4.09 m
4′	3.61 m	3.51 m	3.53 m	3.20 m	3.51 m
5′	4.19 m	4.20 m	4.24 m	3.83 m	4.21 m
6′	1.57 d (6.2)	1.36 d (6.0)	1.39 d (6.2)	1.20 d (6.1)	1.35 d (6.2)
OMe	3.51 s	3.64 s	3.65 s	3.43 s	3.62 s
1″		5.12 dd (9.6, 1.6)	5.13 dd (9.7, 1.4)	4.74 dd (9.7, 1.8)	5.14 br d (9.6)
2″		1.83 m, 2.32 m	1.81 m, 2.35 m	1.63 m, 2.10 m	1.80 m, 2.32 m
3″		4.06 m	4.02 m	3.80 m	3.99 m
4″		3.49 m	3.51 m	3.21 m	3.48 m
5″		4.19 m	4.20 m	3.85 m	4.19 m
6″		1.41 d (6.2)	1.40 d (6.2)	1.21 d (6.1)	1.36 d (6.2)
OMe		3.56 s	3.42 s	3.43 s	3.40 s
1‴		4.76 dd (9.8, 1.7)	4.68 br d (9.7)	4.44 dd (9.6, 1.7)	4.61 br d (9.7)
2‴		1.72 m, 2.54 m	2.21 m, 2.35 m	1.53 m, 2.30 m	2.15 m, 2.31 m
3‴		3.48 m	3.48 m	3.70 m	3.41 m
4‴		3.47 m	4.22 m	3.15 m	4.32 m
5‴		3.59 m	3.58 m	3.45 m	3.54 m
6‴		1.56 d (6.0)	1.57 d (6.4)	1.28 d (6.0)	1.69 d (6.2)
OMe		3.45 s	3.41 s	3.40 s	3.36 s
1⁗			5.18 d (7.8)	4.64 dd (9.7, 2.1)	5.09 d (7.8)
2⁗			4.00 m	1.64 m, 1.99 m	3.94 t (8.4)
3⁗			4.26 m	3.29 m	4.14 m
4⁗			4.18 m	3.68 d (2.8)	4.00 m
5⁗			3.99 m	3.30 m	4.09 m
6⁗			4.62 d (11.7)	1.35 d (6.2)	4.30 m, 4.84 m
			4.38 dd (11.7, 5.8)		
OMe				3.39 s	
1′′′′′					5.25 d (7.7)
2′′′′′					4.06 m
3′′′′′					4.24 m
4′′′′′					4.23 m
5′′′′′					4.25 m
6′′′′′					4.54 d (11.4)
					4.39 dd (11.4, 4.8)

[a] Measured in $CDCl_3$ at 600 MHz.

reaction mixtures, and they were kept at 60 °C for 30 min. The supernatants (4 μL) were analyzed by GC, respectively, under the following conditions: Agilent 7890A gas chromatograph equipped with HP-5 (5% diphenyl polysiloxane) quartz capillary column (30 m × 0.32 mm, film thickness 0.25 μm). The oven temperature was programmed to rise from 100 to 230 at the rate of 10 °C/min, and the carrier gas was N_2 (2 mL/min); FID temperature: 300; injector temperature: 250; and split ratio: 1/5 [13,14]. The result showed that the standard samples of D-glucose and D-cymarose derivatives gives a peak at 10.86 and 11.43 min, respectively. Thus, the D-glucose in compounds **3**, **5**, **7** and **8** and D-cymarose in compounds **1**–**9** were detected by comparing the retention times of the corresponding derivatives with signals of the authentic D-glucose and D-cymarose.

2.4. Antimicrobial assays

The microorganisms used in antimicrobial assays were obtained from the American Type Culture Collection (ATCC), "centre Pasteur" of Yaounde Cameroon and "Institut Pasteur de Paris" (IP). They included three bacterial strains: *Enterococcus faecalis* ATCC 10541, *Staphylococcus aureus* ATCC 25922 and *Providensia smartii* ATCC29916; three yeasts: *Candida albicans* ATCC 2091, *C. neoformans* IP 90526 and *Candida guilimondis*. The MIC values of the isolated compounds were determined by

Table 2
^{13}C NMR data of compounds **1**–**5** in C_5D_5N (δ in ppm, *J* in Hz, C_5D_5N).

Position	1	2	3	4[a]	5
1	37.7	37.0	37.7	37.1	37.7
2	30.6	29.9	30.6	29.4	30.6
3	77.4	76.9	77.5	77.3	77.5
4	39.5	38.8	39.5	38.6	39.5
5	141.0	140.3	140.9	140.5	141.0
6	120.7	120.0	120.7	119.6	120.8
7	29.8	29.1	29.8	29.0	29.8
8	34.3	33.7	34.3	33.8	34.3
9	50.1	49.5	50.1	49.6	50.1
10	38.4	37.8	38.4	37.8	38.4
11	20.6	20.0	20.6	19.9	20.6
12	35.5	34.9	35.5	35.4	35.5
13	55.1	54.4	55.1	54.5	55.1
14	183.2	182.5	183.2	183.7	183.2
15	127.5	126.7	127.5	126.4	127.5
16	205.9	205.1	205.9	205.4	205.9
17	61.3	60.7	61.3	60.8	61.4
18	175.4	174.6	175.4	174.3	175.4
19	19.7	19.0	19.7	19.2	19.7
20	76.6	75.8	76.6	75.7	76.6
21	24.1	23.4	24.1	23.9	24.1
1′	96.7	96.1	96.7	95.9	96.7
2′	36.6	37.0	37.6	35.4	37.6
3′	79.4	77.8	78. 4	76.9	78.4
4′	74.7	83.1	83.8	82.4	83.8
5′	71.4	68.8	69.5	68.4	69.5
6′	19.6	18.3	19.0	18.2	19.0
OMe	58.5	58.6	59.2	57.9	59.2
1″		100.2	100.9	99.6	100.9
2″'		36.7	36.9	35.4	36.8
3″'		77.6	77.9	77.2	77.9
4″'		82.9	83.5	82.5	83.6
5″'		68.7	69.4	68.2	69.4
6″'		18.3	19.0	18.2	19.0
OMe		58.6	58.7	58.1	58.7
1‴		101.9	103.1	101.2	103.2
2‴		37.0	33.3	36.3	33.3
3‴		81.1	80.3	79.2	80.3
4‴		75.9	73.9	82.3	73.5
5‴		72.7	71.4	70.5	71.5
6‴		18.4	18.4	18.3	18.6
OMe		56.8	56.6	56.8	56.7
1⁗			105.3	100.6	105.2
2⁗			76.3	31.9	76.0
3⁗			78.9	70.8	78.8
4⁗			72.3	66.7	72.3
5⁗			79.1	77.8	78.2
6⁗			63.6	16.8	70.9
OMe				55.7	
1⁗′					106.1
2⁗′					75.6
3⁗′					79.0
4⁗′					72.1
5⁗′					79.1
6⁗′					63.1

[a] Measured in $CDCl_3$ at 125 MHz.

Table 3
^{1}H NMR data of compounds **6**–**9** (δ in ppm, *J* in Hz, C_5D_5N).

Position	6	7	8
1	1.14 m, 1.80 m	1.10 m, 1.80 m	1.13 m, 1.76 m
2	1.27 m, 2.11 m	1.69 m, 2.09 m	1.72 m, 2.11 m
3	3.81 m	3.81 m	3.85 m
4	2.29 m, 2.50 m	2.49 m, 2.89 m	2.32 m, 2.51 m
6	5.38 m	5.38 m	5.40 m
7	2.00 m, 2.61 m	2.00 m, 2.60 m	2.03 m, 2.63 m
8	2.51 m	2.47 m	2.52 m
9	1.22 m	1.20 m	1.23 m
11	1.50 m, 2.42 m	1.47 m, 2.40 m	1.50 m, 2.43 m
12	1.28 m, 1.96 m	1.25 m, 1.93 m	1.27 m, 1.96 m
15	1.86 m, 1.99 m	1.88 m, 2.59 m	1.89 m, 1,97 m
16	2.03 m	2.01 m	2.04 m
17	2.15 m	2.13 m	2.16 m
19	1.36 s	1.02 s	1.05 s
20	4.65 m	4.66 m	4.69 m
21	1.24 d (6.2)	1.23 d (6.2)	1.26 d (6.1)
1′	5.25 br d (9.2)	5.25 br d (9.2)	5.29 br d (9.5)
2′	1.88 m, 2.29 m	1.87 m, 2.29 m	1.92 m, 2.33 m
3′	3.41 m	4.06 m	4.10 m
4′	3.49 m	3.49 m	3.49 m
5′	4.18 m	4.20 m	4.23 m
6′	1.1.36 d (6.1)	1.37 d (6.5)	1.40 d (5.7)
OMe	3.38 s	3.59 s	3.63 s
1″	5.10 br d (9.3)	5.09 br d (9.7)	5.14 br d (9.2)
2″	1.79 m, 2.30 m	1.76 m, 2.29 m	1.86 m, 2.35 m
3″	4.07 m	3.98 m	3.98 m
4″	3.51 m	3.46 m	3.48 m
5″	4.19 m	4.15 m	4.19 m
6″	1.39 d (6.2)	1.35 d (6.6)	1.40 d (6.6)
OMe	3.61 s	3.38 s	3.59 s
1‴	4.68 br d (9.2)	4.63 br d (9.7)	4.72 br d (9.6)
2‴	2.15 m, 2.27 m	2.17 m, 2.30 m	1.76 m, 2.51 m
3‴	4.06 m	3.43 m	3.56 m
4‴	3.88 m	4.14 m	3.49 m
5‴	3.56 m	3.52 m	3.52 m
6‴	1.53 d (6.3)	1.53 d (6.3)	1.41 d (5.3)
OMe	3.50 s	3.36 s	3.41 s
1⁗		5.12 d (7.7)	4.92 d (9.6)
2⁗		3.94 m	1.75 m, 2.52 m
3⁗		4.21 m	3.62 m
4⁗		4.12 m	3.52 m
5⁗		3.93 m	3.54 m
6⁗		4.56 d (11.2)	1.46 m
		4.34 dd (11.4, 5.7)	
OMe			3.51 s
1⁗′			4.90 d (9.7)
2⁗′			2.01 m, 2.31 m
3⁗′			3.46 m
4⁗′			4.20 m
5⁗′			3.55 m
6⁗′			1.56 d (6.2)
OMe			3.48 s
1⁗′′			5.19 d (7.6)
2⁗′′			4.01 m
3⁗′′			4.04 m
4⁗′′			4.21 m
5⁗′′			3.99 m
6⁗′′			4.61 d (11.2)
			4.39 dd (11.2, 5.4)

the broth microdilution method in 96-well microtitre. The 96-well plates were prepared by dispensing into each well 100 μL of Mueller Hinton broth for bacteria and Sabouraud dextrose broth for fungi. The compounds were initially prepared in 10% DMSO in broth medium at 400 μg/mL for compounds or 50 μg/mL for the reference antibiotics. A volume of 100 μL of each test sample was added into the first wells of the microtitre plate (whose wells were previously loaded with 100 μL of broth medium). Serial two-fold dilutions of the test samples were made and 100 μL of inoculum standardized at 10^6 CFU/mL for bacteria, and 2.5×10^5 CFU/mL for yeasts (at 600 nm, Jenway 6105 UV/Vis spectrophotometer 50 Hz/60 Hz). This gave final concentration ranges from 100 to 0.781 μg/mL for the compounds and 12.5 to 0.097 μg/mL for reference substances. The plates were sealed with parafilm, then agitated with a plate

Table 4
^{13}C NMR data of compounds **6**–**9** (δ in ppm, *J* in Hz, C_5D_5N).

Position	6	7	8
1	38.8	37.3	37.8
2	31.5	30.0	30.5
3	78.5	77.0	77.5
4	40.4	38.9	39.5
5	141.1	139.7	140.1
6	123.0	121.6	122.1
7	28.7	27.2	27.8
8	40.1	38.6	39.2
9	47.4	45.9	46.4
10	38.0	37.3	37.9
11	22.7	21.2	21.7
12	34.8	33.3	33.8
13	61.1	59.6	60.1
14	86.3	84.8	85.3
15	27.0	25.6	26.1
16	36.6	36.9	35.7
17	58.3	56.8	57.3
18	180.0	178.5	179.2
19	21.0	21.0	20.1
20	84.6	82.8	83.4
21	22.6	21.0	21.6
1′	97.5	96.0	96.5
2′	38.4	36.9	37.4
3′	80.1	77.7	78.3
4′	84.3	83.1	83.4
5′	70.2	68.8	69.3
6′	19.9	18.3	18.9
OMe	56.6	58.5	59.1
1″	101.7	100.2	100.8
2″	38.0	36.2	37.2
3″	79.2	77.2	78.0
4″	84.4	82.9	83.4
5″	70.3	68.7	69.2
6″	19.8	18.3	18.8
OMe	60.0	58.1	59.2
1‴	104.0	102.4	102.2
2‴	34.0	32.6	37.8
3‴	79.0	79.5	79.2
4‴	67.9	73.4	83.0
5‴	72.9	70.7	71.2
6‴	18.8	17.6	18.9
OMe	59.9	55.9	56.5
1⁗		104.7	100.4
2⁗		75.6	37.7
3⁗		78.2	79.7
4⁗		71.6	83.3
5⁗		78.3	71.9
6⁗		62.9	19.0
OMe			57.6
1⁗′			101.5
2⁗′			33.4
3⁗′			80.3
4⁗′			73.7
5⁗′			71.9
6⁗′			18.2
			57.2
1⁗″			105.1
2⁗″			76.3
3⁗″			78.7
4⁗″			72.1
5⁗″			78.9
6⁗″			63.4

shaker to mix their contents and incubated at 35 °C for 24 h for bacteria, 48 h for yeast.

For bacteria, MICs were determined upon addition of 50 μL (0.2 mg/mL) p-iodonitrotetrazolium chloride (INT, Sigma-Aldrich, South Africa). Viable bacteria reduced the yellow dye to a pink color. For yeasts, MICs were determined by visualizing the turbidity of the wells. The MIC corresponded to the lowest well concentration where no color turbidity change was observed, indicating no growth of microorganism. All tests were performed in triplicates. Gentamycin for bacteria, and nystatin for yeast were used as positive controls, and the MIC value was defined as the lowest concentration that inhibited visible growth.

3. Results and discussion

The MeOH extracts of *E. rosea* was partitioned between EtOAc and water. The EtOAc fraction was subjected to silica gel, Sephadex LH-20 and ODS column chromatography and finally by semi-preparative HPLC to yield eight new pregnane glycosides **1**–**8** and a known compound **9** [12]. The aglycone of compounds **1**–**5** was identified with the same skeleton of pregna-5,14-dien-18-oic acid,3,20-dihydroxy-16-oxo-,γ-lactone, (3β,20R), and compounds **6**–**9** with the same aglycone of pregn-5-en-18-oic acid,3,14,20-trihydroxy-,γ-lactone,(3β,14β,20R) by detailed analysis of their NMR spectra data in association with literature information. The D-configuration of glucose in the compounds was identified by GC analysis of acid hydrolysis products. For the deoxysugars, since only the D-glucose and D-cymarose could be obtained, the L-configuration of cymarose in the compounds was determined mainly by comparison of their ^{13}C NMR chemical shift values with reported data. By analyzing the reported literature data, it was confirmed that the chemical shift of C-2 is at 35.0–38.0 ppm in β-D-cymaropyranosyl and α-D-cymaropyranosyl. On the other hand, this values are between 31.0 and 34.0 ppm in β-L-cymaropyranosyl and α-L-cymaropyranosyl [12–20]. Therefore, compounds **1**–**8** were identified as ecdysosides A–H and the known compound **9** was determined to be ecdysantheroside A.

The HREI-MS spectra gave a single peak at *m/z* 486.2617 $[M]^+$ (calcd. for 486.2618) which indicated that the molecular formula of compound **1** was $C_{28}H_{38}O_7$ with 10° of unsaturation. The IR spectrum gave characteristic signals at 3442, 1709 cm^{-1} for hydroxyl and carbonyl groups, respectively. The ^{13}C NMR and DEPT spectrum of **1** (Table 2) exhibited twenty eight carbon signals for four methyls (one oxygenated), seven methylenes, eleven methines, and six quaternary carbons, of which 7 carbon signals were assigned to the sugar part, and 21 carbon signals were assigned to a C21-steroidal aglycone. In the ^{1}H NMR spectra, a characteristic anomeric proton at δ_H 5.28 (dd, $J = 9.6$, 1.8 Hz, H-1′) indicated the presence of a sugar unit with β configuration based on its large coupling constant value (9.6 Hz) and supported by the ROESY experiment. The terminal methyl group at δ_H 1.57 (d, $J = 6.2$ Hz, Me-6′), methoxy methyl group at δ_H 3.51 (s, OMe-3′) and methylene at δ_H 2.42 (m, H-2′a) could be assigned to the sugar moiety by the correlations from δ_H 5.28 (H-1′) to δ_C 36.6 (C-2′) and δ_C 79.4 (C-3′); δ_H 1.57(Me-6′) to δ_C 71.4 (C-5′) and δ_C 74.7 (C-4′); and δ_H 3.51 (OMe-3′) to δ_C 79.4 (C-3′) in the HMBC spectrum. Furthermore, the detailed COSY correlations between δ_H 2.42 (m, H-2′a), δ_H 5.28 (H-1′) and δ_H 3.81 (H-3′); δ_H 3.81 (m, H-3′) and δ_H 3.61 (m, H-4′); and δ_H 4.19 (m, H-5′), δ_H 3.61 (H-4′) and δ_H 1.57 (H-6′) revealed the presence of a 2, 6-dideoxysugar. The sugar unit was further determined to be β-D-cymaropyranose based on its chemical shift of δ_C 36.6 (C-2′) which was very close to the reported data

of 36.7 ppm in the same solvent (pyridine), and >35.0 ppm [13,14,16], this was also supported by the GC analysis of the sugar derivative from compound **1** after acid hydrolysis. Besides the sugar unit, the aglycone should include two carbonyl groups and two double bonds which accounted for 4° of unsaturation, the remaining 5° of unsaturation suggested that the aglycone had a pentacyclic skeleton. The literature data suggested that the aglycone in compound **1** was a known C-21 pregnane [2]. As the methyl groups at C-18 and C-19 in pregnane were typical methyl groups with β-configuration [21] in the ROESY spectral data, the correlation between δ_H 1.11 (H-19) and δ_H 2.93 (H-8) suggested the β-configuration of H-8; the correlation between δ_H 1.11 (H-19) and δ_H 2.37 (H-4a) suggested the α-configuration of H-4b, therefore, the correlation from δ_H 2.58 (H-4b) to δ_H 3.85 (H-3) indicated the β-configuration of the hydroxyl group at C-3. Likewise, the correlation between δ_H 2.93 (H-8) and δ_H 2.40 (H-12a) suggested the β-configuration of H-12a which revealed the α-configuration of H-12b, thus, the observation of the correlation between δ_H 1.59 (H-12b) and δ_H 1.20 (H-9) suggested the α-configuration of H-9. In addition, as the correlation between δ_H 1.38 (H-21) and δ_H 2.76 (H-17) with δ_H 1.59 (H-12b) could be observed, it suggested the α-configuration of C-21 and H-17. The above observation associated with literature data showed that the aglycone had a skeleton of ecdysantherin [2]. Moreover, the linkage position of the sugar moiety was at the C-3 hydroxyl group of the aglycone by the unambiguous correlation from δ_H 5.28 (H-1′) to δ_C 77.4 (C-3) in the HMBC spectra. Thus, the structure of compound **1** was elucidated as shown in Fig. 1, and named ecdysoside A.

Compound **2** had the molecular formula of $C_{42}H_{62}O_{13}$, which was identified by its positive HRESI-MS at *m/z* 797.4082 $[M+Na]^+$ (calcd. for 797.4088). The similar signals to those of compound **1** in NMR spectra suggested that compound **2** contained the same aglycone skeleton as **1** except for the sugar moiety. In ^{1}H NMR spectrum of compound **2**, the characteristic anomeric protons at δ_H 5.27 (dd, J = 9.6, 1.6 Hz, H-1′), δ_H 5.12 (dd, 9.6 1.6 Hz, H-1″), 4.76 (dd, J = 9.8 1.7 Hz, H-1‴) assumed the presence of three sugar units and this could be confirmed by its molecular formula and acid hydrolysates of compound **2**. All the three sugar units were identified with a β-configuration on the basis of their large coupling constant (>9.0 Hz). The ^{13}C and DEPT NMR spectrum of **2** showed three methylene groups at δ_C 37.0 (C-2′), 36.7 (C-2″), and 37.0 (C-2‴), three methyl groups at δ_C 18.3 (C-6′), 18.3 (C-6″), and 18.4 (C-6‴), and three methoxyl groups at δ_C 58.6 (−OMe), 58.6 (−OMe), and 56.8 (−OMe) which were assigned to the sugar moiety. These observations further suggested the presence of three 2, 6-dideoxysugar units. According to the detailed 2D NMR spectral data together with acid hydrolysis experiment, all the sugar signals were assigned to three cymaropyranoses. The sugar units were determined to be with D-configuration by comparing their corresponding ^{13}C NMR chemical shifts at δ_C 37.0 (C-2′) [14], δ_C 36.7 (C-2″) [13,14,16], and δ_C 37.0 (C-2‴) [14] which were >35.0 ppm with literature data. Furthermore, the straight sugar chain and its sequence were revealed by the correlations from δ_H 5.27 (H-1′) to δ_C 76.9 (C-3), from δ_H 5.12 (H-1″) to δ_C 83.1 (C-4′) and from δ_H 4.76 (H-1‴) to δ_C 82.9 (C-4″) in HMBC experiment. Thus, the structure of compound **2** was elucidated as shown in Fig. 1, and named ecdysoside B.

The molecular formula of compound **3** was deduced to be $C_{48}H_{72}O_{18}$ on the basis of its HREI-MS *m/z* 959.4617 $[M+Na]^+$ (calcd. for 959.4611). Detailed analysis of the ^{1}H and ^{13}C NMR data of compound **3** with those of **2** indicated that **3** had the same aglycone as **2** but with a different sugar moiety. The anomeric proton signals at δ_H 5.30 (dd, J = 9.6, 1.5 Hz, H-1′), δ_H 4.68 (dd, J = 9.7, 1.5 Hz, H-1″), δ_H 5.18 (d, J = 7.8 Hz, H-1‴), δ_H 5.13 (dd, J = 9.7, 1.4 Hz, H-1⁗) in ^{1}H NMR spectrum implied the presence of four sugar units with a β-configuration. The 1D and 2D NMR spectral data supported the presence of one β-D-glucose, and two β-D-cymarose residues, together with another β-L-cymarose unit, which were further confirmed by their characteristic chemical shifts of δ_C 37.6 (C-2′) [13,14], δ_C 36.9 (C-2″) [16], δ_C 33.3 (C-2‴) [17]. In the HMBC spectrum, the key correlations from δ_H 5.30 (H-1′) to δ_C 77.5 (C-3), from δ_H 4.68 (H-1″) to δ_C 83.8 (C-4′), from δ_H 5.18 (H-1‴) to δ_C 83.5 (C-4″) and from δ_H 5.13 (H-1⁗) to δ_C 80.3 (C-4‴) suggested that the sugar moiety was also a straight chain and its linkage position was at the C-3 hydroxyl group of the aglycone. As a result, the above data allowed the structural assignment of **3** as shown in Fig. 1, and named ecdysoside C.

The molecular formula $C_{49}H_{74}O_{16}$ of compound **4** could be determined according to its HREI-MS *m/z* 918.4970 (calcd. for 918.4977). The ^{13}C and DEPT NMR spectra revealed the same aglycone unit as in compound **3** except for the sugar moiety. In the ^{1}H NMR spectra, it gave out four anomeric protons at δ_H 4.84 (dd, J = 9.6, 1.6 Hz, H-1′), δ_H 4.64 (dd, 9.7 2.1 Hz, H-1″), δ_H 4.74 (dd, 9.7 1.8 Hz, H-1‴) and δ_H 4.44 (dd, 9.6 1.7 Hz, H-1⁗). By further analyzing these anomeric proton signals with their large coupling constant they were assigned to four sugar units with a β-linkage. In the HSQC, HMBC and COSY spectra, the β-D-configuration of three cymarose residues and the β-L-configuration of the remaining cymarose residues were determined and further supported by comparison of the characteristic chemical shift values of δ_C 35.4 (C-2′) [14], δ_C 35.4 (C-2″) [13], δ_C 36.3 (C-2‴) [13], and δ_C 31.9 (C-2⁗) [14,17] with reported data. In addition, the sequence of the sugar chain could be determined by the HMBC correlations from δ_H 4.84 (H-1′) to δ_C 77.3 (C-3), from δ_H 4.74 (H-1″) to δ_C 82.4 (C-4′), from δ_H 4.44 (H-1‴) to δ_C 82.5 (C-4″), and from δ_H 4.64 (H-1⁗) to δ_C 82.3 (C-4‴). Therefore, the structure of **4** was elucidated as shown in Fig. 1, and named ecdysoside D.

The positive HRESI-MS peak at *m/z* 1121.5150 (calcd. For 1121.5145) suggested the molecular formula of compound **5** to be $C_{54}H_{82}O_{23}$ which was further supported by its ^{13}C and DEPT NMR spectra. The evidences from NMR spectra indicated that the aglycone of **5** was the same as that of compound **4**. Their major differences appeared on the sugar moiety as the ^{1}H NMR spectra showed five anomeric proton signals at δ_H 5.27 (br d, J = 9.6 Hz, H-1′), δ_H 5.14 (br d, J = 9.6 Hz, H-1″), δ_H 4.61 (br d, J = 9.7 Hz, H-1‴), δ_H 5.09 (d, J = 7.8 Hz, H-1⁗) and δ_H 5.25 (d, J = 7.7 Hz, H-1⁗), supporting the presence of five sugar units with β-configuration. Based on the detailed correlations of the 2D spectra data, the sugar moiety was deduced to be a straight chain consisting of two D-glucose, together with two D-cymarose and one L-cymarose which had similar chemical shift values of 37.6 (C-2′) [13,14], δ_C 36.8 (C-2″) [14,16], and δ_C 33.3 (C-2‴) [17] as those in literature. The unambiguous HMBC correlations from δ_H 5.27 (H-1′) to δ_C 77.5 (C-3), from δ_H 5.14 (H-1″) to δ_C 83.8 (C-4′), from δ_H 4.61 (H-1‴) to δ_C 83.6 (C-4″), from δ_H 5.09 (H-1⁗) to δ_C 73.5 (C-4‴), and from δ_H 5.25 (H-1⁗) to δ_C 70.9 (C-6⁗), revealed the sequence of the sugar chain and it connected to the C-3 of aglycone in compound **5**. Therefore,

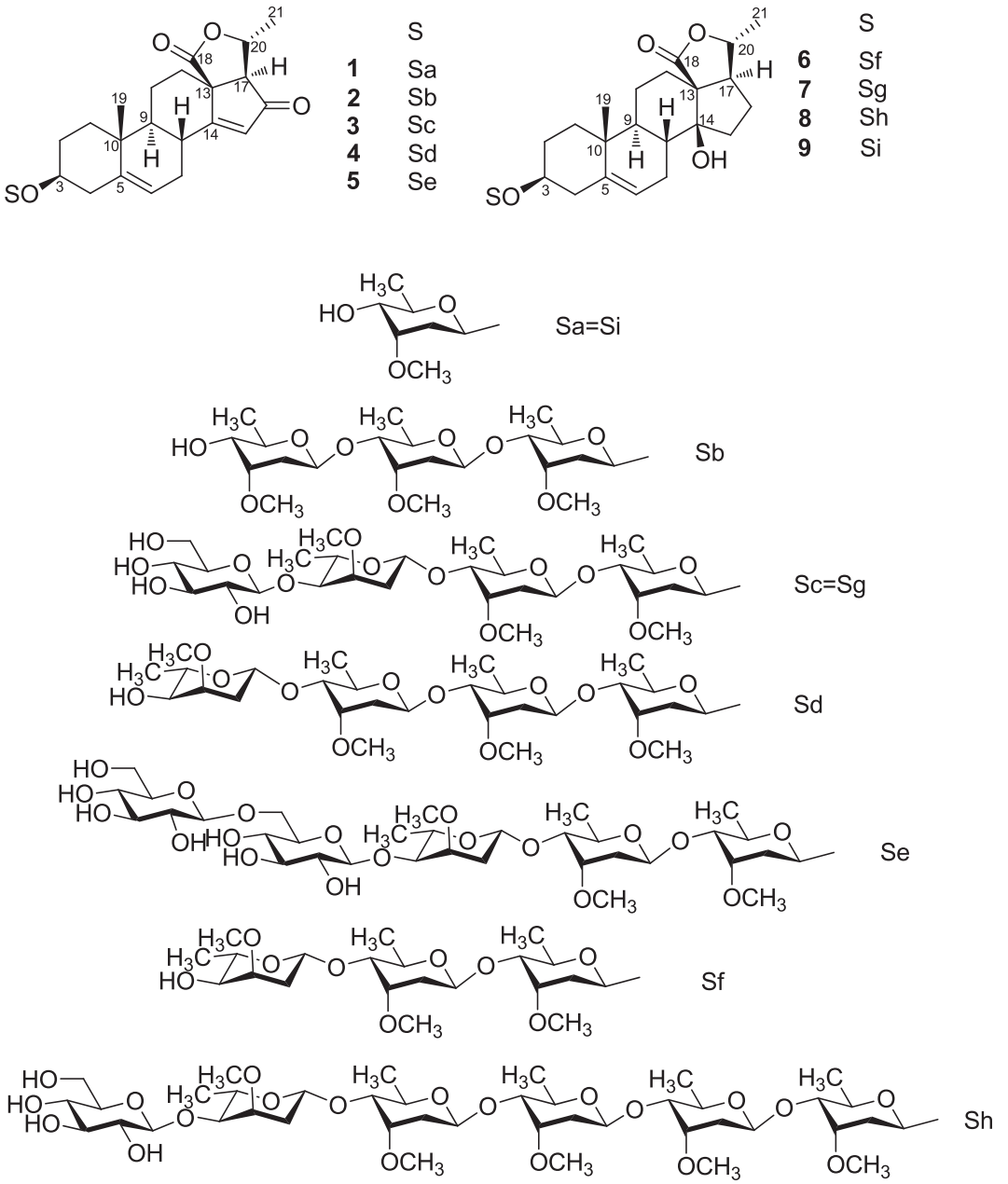

Fig. 1. Structures of pregnane glycosides **1–9** from *Ecdysanthera rosea.*

compound **5** was elucidated as shown in Fig. 1, and named ecdysoside E.

The molecular formula of compound **6** was determined to be $C_{42}H_{66}O_{13}$ on the basis of the positive HRESI-MS *m/z* 801.4406 $[M+Na]^+$ (calcd. for 801.4401). The similar IR and NMR spectra data indicated that compound **6** possessed the same aglycone but different sugar moiety to ecdysantheroside A [12]. The anomeric protons at δ_H 5.25 (br d, $J = 9.2$ Hz, H-1′), δ_H 5.10 (br d, $J = 9.3$ Hz, H-1″) and δ_H 4.68 (br d, $J = 9.2$ Hz, H-1‴) suggested that there were three sugar units, and the assumption was further confirmed by analyzing the derivatives of its acid hydrolyzate. The 1D and 2D NMR spectral data indicated that the sugar moiety consisted of three 2, 6-dideoxy sugar units. Furthermore, the presence of two β-D-cymarose and one β-L-cymarose was determined on the basis of their coupling constant in ^{1}H NMR spectrum and characteristic chemical shift values of 38.4 (C-2′) [16], δ_C 38.0 (C-2″) [13,14], δ_C 34.0 (C-2‴) [19] which were in good agreement with previously reported data. Moreover, the HMBC correlations observed from δ_H 5.25 (H-1′) to δ_C 78.5 (C-3), from δ_H 5.10 (H-1″) to δ_C 84.3 (C-4′), and from δ_H 4.68 (H-1‴) to δ_C 84.4 (C-4″) indicated the structure of a straight sugar chain, which was placed at C-3 of the aglycone. Thus, compound **6** was elucidated as shown in Fig. 1, and named ecdysoside F.

Compound **7** with the molecular formula $C_{48}H_{76}O_{18}$ was determined on the basis of its positive HRESI-MS *m/z* 963.4935 $[M+Na]^+$ (calcd. for 963.4929). The same aglycone unit as **6** was confirmed by the ^1H and ^{13}C NMR spectra. For the sugar moiety, the characteristic anomeric protons in ^1H NMR spectrum at δ_H 5.25 (br d, $J = 9.2$ Hz, H-1′), δ_H 5.09 (br d, $J = 9.7$ Hz, H-1″), δ_H 4.63 (br d, $J = 9.7$ Hz, H-1‴) and δ_H 5.12 (d, $J = 7.7$ Hz, H-1⁗) indicated the presence of four sugar units with β configuration. Detailed analysis of its NMR spectroscopic data suggested the presence of a straight sugar chain composed of one β-D-glucose as well as two β-D-cymaropyranose and one β-L-cymaropyranose which had characteristic chemical shift values of 36.9 (C-2′) [16], δ_C 36.2 (C-2″) [15,16], δ_C 32.6 (C-2‴) [14,16] in compound **7**. The correlations from δ_H 5.25 (H-1′) to δ_C 77.0 (C-3), from δ_H 5.09 (H-1″) to δ_C 83.1 (C-4′), from δ_H 4.63 (H-1‴) to δ_C 82.9 (C-4″), from δ_H 5.12 (H-1⁗) to δ_C 73.4 (C-4‴) in HMBC experiment suggested the sequence of the sugar moiety in compound **7** and its location was similar to compound **6**. Therefore, the structure of **7** was elucidated as shown in Fig. 1, and named ecdysoside G.

Compound **8** was found to possess a molecular formula of $C_{62}H_{99}O_{24}$ based on its positive HRESI-MS *m/z* 1227.6518 $[M-H]^-$ (calcd. for 1227.6526). The ^1H and ^{13}C NMR spectra showed the same aglycone part as **7**. The ^1H NMR data showed six anomeric protons at δ_H 5.29 (br d, $J = 9.5$ Hz, H-1′), δ_H 5.14 (br d, $J = 9.2$ Hz, H-1″), δ_H 4.72 (br d, $J = 9.6$ Hz, H-1‴), δ_H 4.92 (d, $J = 9.6$ Hz, H-1⁗), δ_H 4.90 (d, $J = 9.7$ Hz, H-1⁗′) and δ_H 5.19 (d, $J = 7.6$ Hz, H-1⁗″), which indicated the presence of six sugar units with β-configuration in compound **8**. On the basis of 1D and 2D NMR spectral data, it was suggested that, besides one β-D-glucose unit, the sugar moiety included four β-D-cymaropyranose and one β-L-cymaropyranose which had characteristic chemical shift values of 37.4 (C-2′), δ_C 37.2 (C-2″), δ_C 37.8 (C-2‴), δ_C 37.7 (C-2⁗) [13,14] and δ_C 33.4 (C-2⁗′) [17], corroborating reported literatures. The sequence of the straight sugar chain could be further determined by the HMBC correlations from δ_H 5.29 (H-1′) to δ_C 77.5 (C-3), δ_H 5.14 (H-1″) to δ_C 83.4 (C-4′), δ_H 4.72 (H-1‴) to δ_C 83.4 (C-4″), δ_H 4.92 (H-1⁗) to δ_C 83.0 (C-4‴), δ_H 4.90 (H-1⁗′) to δ_C 84.4 (C-4⁗), *and* δ_H 5.19 (H-1⁗″) to δ_C 73.7 (C-4⁗′), and it was deduced to be at C-3 of the aglycone. Thus, the structure of **8** was elucidated as shown in Fig. 1, and named ecdysoside H.

Compounds **1**–**9** were evaluated for their antibacterial activities against *E. faecalis* ATCC 10541 (EF), *S. aureus* ATCC 25922 (SA), and *P. smartii* ATCC29916 (29916), and for their antifungal activities against *C. albicans* ATCC 2091 (CA), *C. neoformans* IP 90526 (CN), and *C. guilimondis* (CG) using the broth micro-dilution method. The result revealed that compounds **1** and **9** showed moderate antibacterial activities against *E. faecalis* (MIC value of 12.5 μg/mL), while compounds **3** and **5** showed moderate antibacterial activities against *P. smartii* ATCC29916 (MIC value of 12.5 μg/mL). The compound **8** showed significant antiyeast activity, compared to nystatin against *C. neoformans* (MIC value of 12.5 μg/mL). However, the antimicrobial activities of compounds **2**, **4**, **6** and **7** were relatively low (Table 5).

The phytochemical investigation shows that most of the pregnane glycosides isolated from the stem of *E. rosea* have a straight sugar chain which consisted of glucose and cymarose units. In addition, all the aglycones of these compounds have the same skeleton of C-21 pregnane. In our present bioactive research of these pregnane glycosides, some of them displayed *in vitro* antimicrobial activities against specify microbes, which might suggest that the pregnane glycosides are a kind of possible active constituents in this plant.

Table 5
Minimum inhibitory concentration (MIC, μg/mL) of compounds **1**–**9** against bacteria and yeasts.

	EF	SA	29916		CA	CN	CG
1	12.5	50	25	**1**	NA	50	NA
2	50	NA	NA	**2**	NA	100	NA
3	50	25	12.5	**3**	NA	25	100
4	NA	100	50	**4**	NA	100	100
5	50	50	12.5	**5**	NA	25	NA
6	NA	NA	NA	**6**	100	50	50
7	NA	NA	NA	**7**	100	25	25
8	25	50	25	**8**	50	12.5	50
9	12.5	50	25	**9**	NA	25	NA
Gentamycin	1.562	0.195	0.195	Nystatin	12.5	12.5	3.125

NA: not active (MIC > 100 μg/mL).
Bacteria strains: EF: *Enterococcus faecalis* ATCC 10541, SA: *Staphylococcus aureus* ATCC 25922, 29916: *Providensia smartii* ATCC29916.
Yeasts strains: CA: *Candida albicans* ATCC 2091, CN: *Cryptococcus neoformans* IP 90526, CG: *Candida guilimondis*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.10.008.

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