

Article

Three New Phytoecdysteroids Containing a Furan Ring from the Roots of *Achyranthes bidentata* Bl.

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Abstract: Three new phytoecdysteroid compounds, named niuxixinsterone A (**1**), B (**2**) and C (**3**) with acetal functions in the side-chain were isolated from *Achyranthes bidentata* Bl. The structures were established as (20*R*,22*R*,24*S*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,14 α ,25-tetrahydroxy-5 β -ergost-7-en-6-one (**1**), (20*R*,22*R*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,25-trihydroxy-14 β -methyl-18-nor-5 β -cholesta-7,12-dien-6-one (**2**) and (20*R*,22*R*,25*R*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,5 β ,14 α ,26-pentahydroxycholest-7-en-6-one (**3**) by means of spectroscopic evidence.

Keywords: *Achyranthes bidentata* Bl.; phytoecdysteroid; niuxixinsterone

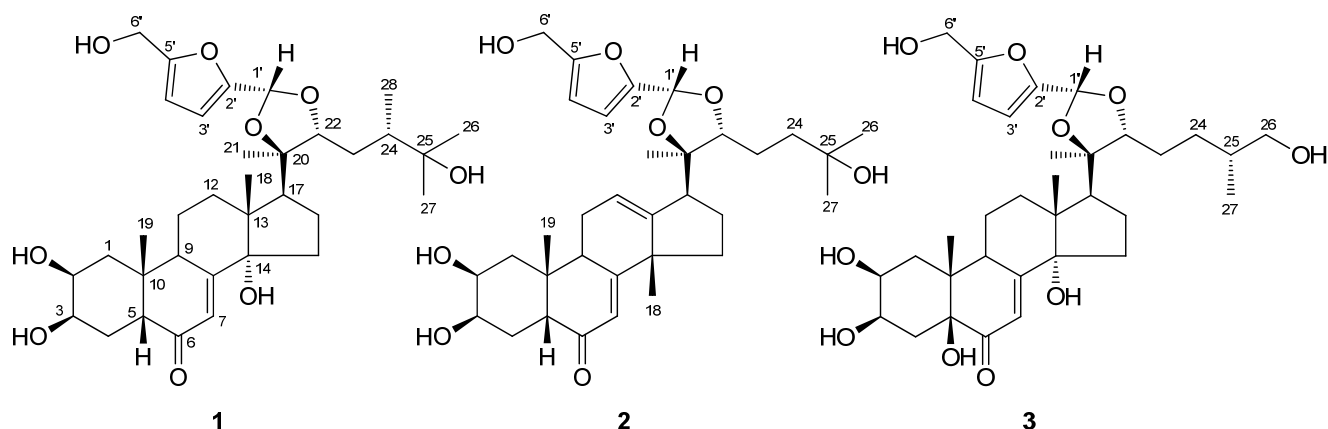
1. Introduction

Achyranthes bidentata Bl., a member of *Amaranthaceae*, is an erect perennial herbaceous plant widely distributed and grown in hilly districts of India, China, Japan and Java. The roots of *A. bidentata* named “Niuxi” in Chinese, is an important medicinal herbal and documented in the Chinese Pharmacopeia. It is usually prescribed by practitioners of Traditional Chinese Medicine (TCM) as a tonic, emmenagogue, antiarthritic, diuretic, and antifertility agent to nourish the liver and kidneys, strengthen bones and muscles, and invigorate circulation [1]. Modern pharmacological studies have

shown that the *A. bidentata* possesses immunostimulant [2,3], uteri-excitant and antifertility [4,5], antitumor [6], analgesic, antibacterial, anti-inflammatory [7], cognition-enhancing [8], antisenile [9], and anti-osteoporosis [10] activities. Its main constituents include polysaccharides [2,3], saponins [11,12] and ecdysteroids [13,14].

In this paper, we describe the isolation and structure elucidation of three new phytoecdysteroids with acetal functions [15] (Figure 1) isolated from the EtOH extracts of *A. bidentata*. In previous experiments, we discovered ecdysterone, inokosterone and serfurosterone A [16]. The three new phytoecdysteroids had a similar structure to serfurosterone A, and we presumed they might have similar pharmacological activity. The structure determination of the phytoecdysteroids from *A. bidentata* could establish a basis for further pharmacological experiments.

Figure 1. Structures of compounds 1-3.



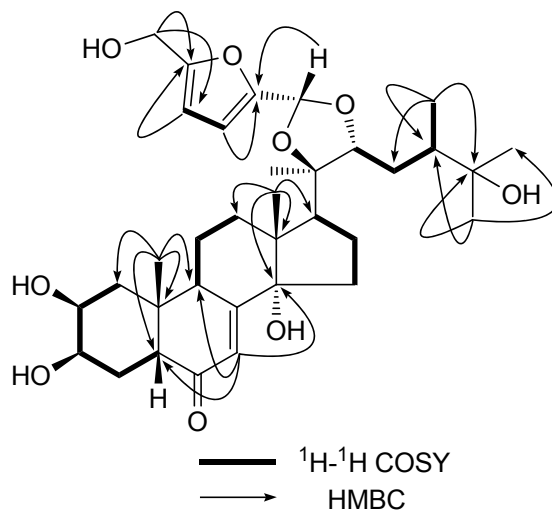
Here, we describe the isolation and structure elucidation of three new phytoecdysteroid compounds on the basis of the spectroscopic analysis, including 1D, 2D-NMR techniques and HRESIMS.

2. Results and Discussion

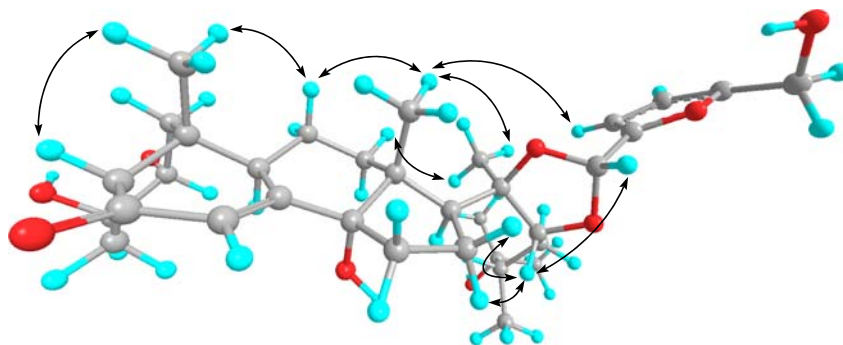
Compound **1** was obtained as a white amorphous powder and had a $[M+Na]^+$ ion peak at m/z 625.3359 in the HRESIMS, corresponding to a molecular formula of $C_{34}H_{50}O_9$. The UV spectrum was consistent with presence of a 7-en-6-one chromophore in an ecdysteroid, with a maximum value at 248 nm. By analyzing the 1H - and ^{13}C -NMR spectroscopic data, it was determined that **1** was an ecdysone analog with a furan ring-containing substituent.

In the 1H -NMR spectrum of **1**, five methyl singlets at δ_H 1.00 ($\times 2$), 1.53, 1.28 and 1.36 were attributed to CH_3 -18, CH_3 -19, CH_3 -21, CH_3 -26 and CH_3 -27, respectively. The CH_3 -28 signal appeared as a doublet at δ_H 1.19 ($J = 6.8$ Hz). In addition, signals for three olefin protons (H-7, 3' and 4'), two hydroxymethyl protons, and an acetal proton were observed in the spectrum.

The ^{13}C -NMR spectrum of **1** had one carbonyl at δ_C 203.4 (C-6), three oxymethine at δ_C 68.2, 68.1 and 85.4 (C-2, 3 and 22), three oxyquaternary carbons at δ_C 80.4, 85.5 and 72.1 (C-14, 20 and 25), six olefinic carbons at δ_C 121.7, 165.4, 152.2, 110.1, 107.8 and 157.4 (C-7, 8, 2', 3', 4' and 5'), together with an acetal carbon at δ_C 97.8. The full connectivity of **1** was deduced from the HSQC, 1H - 1H -COSY and HMBC correlations (Figure 2).

Figure 2. Selected 2D NMR correlations of Niuxixinsterone A (**1**).

The stereochemistry of **1** was elucidated from NOESY data and ^1H - ^1H coupling constants. The $\text{H}_3\text{-19}/\text{H}_{\beta}\text{-11}$, $\text{H}_3\text{-19}/\text{H}_{\beta}\text{-1}$, and $\text{H}_3\text{-19}/\text{H}_{\beta}\text{-5}$ correlations in the NOESY spectrum were indicative of a *cis*-A/B ring junction, whereas the $\text{H}_{\beta}\text{-11}/\text{H}_3\text{-18}$, $\text{H}_{\beta}\text{-15}/\text{H}_3\text{-18}$, $\text{H}_{\beta}\text{-12}/\text{H}_3\text{-18}$, and $\text{H-9}/\text{H}_{\alpha}\text{-12}$ cross-peaks indicated a *trans*-C/D ring junction. The relative configurations of the hydroxyl groups at C-2 and C-3 were elucidated from the coupling constant (11.8 Hz, brd) between $\text{H}_{\beta}\text{-1}$ and H-2, a broad singlet of H-3, HMBC correlations between $\text{H}_3\text{-19}$ and C-1, and NOESY correlation between H-2 and $\text{H}_{\alpha}\text{-4}$. A NOESY correlation between $\text{H}_{\alpha}\text{-12}$ and H-17 indicated that C-17 side-chain was β -oriented [17]. All of the above data were in good agreement with the structural features of 2 β ,3 β ,14 α -trihydroxy-5 β -cholest-7-en-6-one.

Figure 3. Selected NOESY correlations of niuxixinsterone A (**1**).

In compound **1** the high chemical shifts of C-20 (δ_{C} 85.5) and C-22 (δ_{C} 85.4) proved the oxygen substitution. The NOESY correlation between H-22 and H-1' in **1** and the chemical shift of C-1' (97.8 ppm) verified the existence of an acetal-type five-membered ring. Moreover, the H-1'/C-2', H-3'/C-2', H-4'/C-5', H-6'/C-4' and H-6'/C-5' HMBC cross-peaks revealed a 5-hydroxymethyl-furfurylidene substituent on C-1'. The characteristic ^{13}C -NMR chemical shifts and the low coupling constant value $^3J_{\text{H-30}, \text{31}} = 3.2$ Hz furnished further support for the structure [16]. Furthermore, the $\text{H}_{\beta}\text{-12}/\text{H}_3\text{-21}$, $\text{H}_3\text{-18}/\text{H}_3\text{-21}$, $\text{H}_3\text{-18}/\text{H-3'}$, H-22/H₂-16 and H-22/H-1' NOESY correlations revealed the absolute configurations of 20*R* and 22*R* as shown in Figure 3. In addition, the absolute configurations of C-20

and C-22 were consistent with previously reported similar phytoecdysteroids with acetal functions [16]. Comparison of ^{13}C -NMR chemical shifts of C-23~28 in **1** with 24-*epi* makisterone A and makisterone A [18,19] indicated a 24*S* configuration. Accordingly, compound **1** was identified as (20*R*,22*R*,24*S*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,14 α ,25-tetrahydroxy-5 β -ergost-7-en-6-one.

Compound **2**, a white amorphous powder, was assigned a molecular formula of $\text{C}_{33}\text{H}_{46}\text{O}_8$ by HRESIMS, which exhibited a $[\text{M}+\text{Na}]^+$ ion peak at m/z 593.3091. The UV spectrum was consistent with presence of the ecdysteroid chromophore as above. Extensive NMR analysis of the parent nucleus indicated that compounds **2** and **1** were structurally different in terms of the presence of additional one olefin signal and the loss of one oxyquaternary carbon signal.

Comparison of ^1H - and ^{13}C -NMR data from the spectra of **2** and (20*R*,22*R*)-2 β ,3 β ,20,22,26-pentahydroxy-14 β -methyl-18-nor-5 β -cholesta-7,12-dien-6-one [13] revealed the close similarity between them, with practically identical values for C-1 to C-19, which suggests the structure of the tetracyclic ring system of these two ecdysteroids to be the same. The attachment of the parent nucleus was also determined by ^1H - ^1H COSY and HMBC spectrum. The H_3 -18/C-8, 13 and 14, H_α -17/C-12, 13 and 14, and H_α -12/C-9, 11, 13, 14 HMBC cross-peaks and the H_α -11/ H_α -12 ^1H - ^1H COSY correlations revealed that the linkage position of double bond into position C-12 and C-13 and CH_3 -18 substituent on C-14. The 14 β - CH_3 configuration was confirmed in ^1H - ^1H -ROESY spectrum by observation of NOE contacts of H-7/ H_β -15 and H-9/ H_α -15.

Detailed comparison of chemical shifts of C-17 side-chain of compound **2** with **1** indicated that both compounds had the same side-chain, but compound **2** had one less methyl signal at C-24. Thus, the structure of **2** was formulated as (20*R*,22*R*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,25-trihydroxy-14 β -methyl-18-nor-5 β -cholesta-7,12-dien-6-one.

Compound **3**, a white amorphous powder, was established by HRESIMS (m/z 627.3150 $[\text{M}+\text{Na}]^+$) as $\text{C}_{33}\text{H}_{48}\text{O}_{10}$ with a maximal UV absorption at 247 nm. Comparison of the ^{13}C -NMR spectroscopic data of the tetracyclic ring system between **3** and **1** indicated that the major difference was a methine at δ_{C} 51.4 (C-5) in **1** being replaced by an oxyquaternary at δ_{C} 80.0 in **3**. The HMBC correlations between H-7 and H_3 -19 with the signal at δ_{C} 80.0, respectively, which established the presence of an OH substituent at C-5. The 5 β -OH configuration was indicated by the upfield resonance for the CH_3 -19 at δ_{C} 17.1 [20].

In compound **3**, three methyl singlets at δ_{H} 1.12, 0.99 and 1.38 were reasonably attributed to the methyl groups at C-18, C-19 and C-21 by a HMBC experiment. Additionally, the methyl doublet δ_{H} 1.04 is correlated in HMBC to C-23, C-24 and C-26, which is only in agreement with the presence of this methyl group at C-25. Comparison of ^{13}C -NMR chemical shifts of C-23 ~ 27 in **3** with palythoalone B [17] and 25*R*-inokosterone [21] indicated a 25*R* configuration.

Furthermore, the ^1H - ^1H COSY and HMBC correlations implied that **3** also displayed a furan ring-containing substituent at C-20 and C-22. The absolute configurations of substituents at positions 20 and 22 were derived from the observed NOE contacts and comparison with the known compound [16]. Consequently, **3** was elucidated as (20*R*,22*R*,25*R*)-20-*O*,22-*O*-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,5 β ,14 α ,26-pentahydroxycholest-7-en-6-one.

Many plants have been found to be rich in ecdysteroids [22]. A multiplicity of ecdysteroids have been isolated from members of the *Amaranthaceae* [23-27]. The common structural features are characteristic of the ecdysteroids: Δ^7 -6-keto grouping in ring B; *cis*-junction of A/B rings; hydroxyl

groups in positions 1, 2, 3, 5, 11, and 14 of the steroidal core; and the side chain usually containing an (*R*)-C22-group. In most cases, phytoecdysteroids are isolated in a free state, although many their derivatives (ethers, esters, and glycosides) have also been found.

However, the distribution of ecdysteroids with a furan ring in the side-chain in natural sources is very limited. They have been found so far only in the plant *Serratula wolffii* [16]. The new isolates **1-3** constitute a series of ecdysteroids containing a furan ring in the side-chain, all of them with an acetal group at C-1', which have not been reported previously from *A. bidentata*. It is difficult to decide whether the three ecdysteroids are originally present in the plant or if it is artifacts formed during root drying, but we consider that these compounds are genuine compounds as they can be characterized by their UPLC-MS retention time and fragmentation pattern in ethanol extracts of fresh root. It is well known that 20,22-condensation reaction can take place under certain conditions [28], so compounds **1-3** are new endogenous compounds.

3. Experimental

3.1. General

The melting points (uncorrected) were measured on a Kofler micromelting point apparatus. Optical rotations were measured with a PE-241 digital polarimeter. IR spectra were recorded on an IR-47 spectrometer. The NMR spectra were recorded on Bruker DPX 400 (400 MHz for ¹H-NMR and 100 MHz for ¹³C-NMR), respectively. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as an internal standard, and coupling constants are given in Hz. The HRESIMS analyses were conducted on IonSpec Ultima 7.0T FTICR. Preparative HPLC (Waters, Delta 600-2487) was performed on Pegasil ODS II (5 μ m, 10 $\times$ 250 mm, Senshu Pak, Japan). Macroporous absorption resin (D101 Crosslinked Polystyrene, Nan Kai, Tian Jin, China) was employed for column chromatography. Silica gel (100–200 mesh) for column chromatography and silica gel H for TLC were obtained from Qingdao Marine Chemical Factory, Qingdao, Shandong Province, China. ODS-A (120 A, 50 μ m) was obtained from YMC Co.

3.2. Plant Material

The roots of *A. bidentata* were purchased from the Medicinal Materials Planting Base of Anhui University of Traditional Chinese Medicine in 2007. The original plant was identified by Zhenyue Wang of Heilongjiang University of Chinese Medicine. A voucher specimen (No. 20071062) was deposited at the Herbarium of Heilongjiang University of Chinese Medicine, China.

3.3. Extraction and Isolation

The air dried roots of *A. bidentata* (12 kg) were ground to the particle size passing through standard No.10 mesh sieve and extracted with 95% EtOH (3 $\times$ 10 L) for 2 h. The EtOH extracts (5.5 kg) were concentrated under reduced pressure and fractioned by D101 macroporous resin column (8 $\times$ 60 cm) with H₂O, 50% and 95% EtOH-H₂O to give three fractions (H₂O fraction, 50% EtOH-H₂O fraction, 95% EtOH-H₂O fraction). The 50% EtOH-H₂O fraction, which showed potent proliferating activities on osteoblast-like cell formation, was subjected to further isolation. Thus, the fraction (108 g) was

column chromatographed on silica gel with a gradient of CH₂Cl₂/MeOH (30:1 to 3:1) solvents as eluents to afford eight fractions: Fr.4 (20 g; eluted with CH₂Cl₂/MeOH 20:1 to 5:1) was further submitted to silica gel chromatography, and afforded four subfractions A1–A4. Compounds **1** (15.1 mg, *t_R* = 27.8 min) and **2** (17.5 mg, *t_R* = 38.6 min) were obtained by prep. HPLC chromatography of the sub-fraction A2 (1.3 g; eluted with MeOH/H₂O 9:20). A3 (4 g; eluted with MeOH/H₂O 1:5 to 1:0) was separated on ODS-A column, to produce five sub-fractions (B1–B5). The sub-fraction B3 (0.9 g) was purified by prep. HPLC with MeOH/H₂O (2:5) to afford **3** (25.2 mg, *t_R* = 30.9 min).

(20*R*,22*R*,24*S*)-20-*O*,22-*O*-(5'-Hydroxymethyl)-furfurylidene-2β,3β,14α,25-tetrahydroxy-5β-ergost-7-en-6-one (*niuxixinsterone A*, **1**): white amorphous powder, mp: 227–228 °C. [α]_D²⁰ +55 (c 0.05, MeOH). IR (KBr) cm^{−1}: 3460, 1660, 1454, 1380, 1062. UV (MeOH) $\lambda_{\max}$ (log ϵ) nm: 226 (4.10), 248 (3.20), 323(1.15). HRESIMS (positive ion mode) *m/z*: 625.3359 [M+Na]⁺, (calc. for C₃₄H₅₀O₉Na 625.3353). ¹H-NMR and ¹³C-NMR data are shown in Table 1.

Table 1. ¹H- and ¹³C-NMR data for compounds **1–3** in C₅D₅N. (δ in ppm, *J* in Hz).

No	1		2		3	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1α	2.12 (1H, m)	37.9	2.05 (2H, m)	37.9	2.21 (1H, m)	34.8
1β	1.90 (1H, m)				2.09 (1H, m)	
2α	4.17 (1H, d, 11.8)	68.2	4.17 (1H, m)	68.0	4.23 (1H, m)	67.9
3α	4.23 (1H, br. s)	68.1	4.42 (1H, br. s)	68.3	4.18 (1H, m)	69.9
4α	1.70 (1H, m)	32.4	1.90 (1H, m)	32.3	1.98 (1H, dd, 14.4, 2.8)	36.0
4β	1.97 (1H, m)		2.25 (1H, m)		2.10 (1H, m)	
5β	2.98 (1H, dd, 13.2, 3.6)	51.4	2.97 (1H, dd, 13.2, 4.0)	50.4		80.0
6		203.4		202.4		200.9
7	6.24 (1H, d, 2.0)	121.7	6.16 (1H, d, 2.4)	123.1	6.20 (1H, d, 2.4)	120.0
8		165.4	-	146.5		166.1
9α	3.52 (1H, m)	34.7	2.90 (1H, m)	39.5	3.60 (1H, m)	38.2
10		38.6		40.3		44.7
11α	1.78 (1H, m)	21.0	2.19 (1H, m)	21.7	1.88(2H, m)	21.4
11β	1.61 (1H, m)		1.86 (1H, m)			
12α	2.39 (1H, m)	31.7	6.04 (1H, m)	122.1	1.96 (1H, m)	31.7
12β	1.86 (1H, m)				1.88 (1H, m)	
13		47.7		173.6		47.8
14		84.0		48.9		83.9
15α	2.15 (1H, m)	31.5	1.83 (1H, m)	38.8	2.55 (1H, m)	31.7
15β	1.86 (1H, m)		1.50 (1H, m)		2.00 (1H, m)	
16α	2.15 (2H, m)	22.5	1.78 (2H, m)	26.2	2.47 (1H, m)	23.0
16β					1.98 (1H, m)	
17α	2.89 (1H, t, 8.6)	50.6	3.11 (1H, t, 9.2)	49.5	2.82 (1H, t, 9.2)	49.9
18β	1.00 (3H, s)	17.3	1.09 (3H, s)	25.4	1.12 (3H, s)	17.3
19β	1.00 (3H, s)	24.4	0.96 (3H, s)	23.3	0.99 (3H, s)	17.1
20		85.5		84.9		85.8

Table 1. Cont.

No	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
21	1.53 (3H, s)	22.6	1.45 (3H, s)	21.5	1.38 (3H, s)	21.3
22	4.20 (1H, dd, 10.4, 3.6)	85.4	4.23 (1H, dd, 10.0, 1.6)	83.4	4.14 (1H, dd, 9.6, 3.2)	82.9
23	2.39 (1H, m)	31.3	2.03 (1H, m)	26.2	1.85 (1H, m)	27.4
	1.86 (1H, m)		2.16 (1H, m)		1.52 (1H, m)	
24	1.94 (1H, m)	44.5	2.17 (1H, m)	42.2	1.90 (1H, m)	31.6
			1.82 (1H, m)		1.55 (1H, m)	
25		72.1		69.2	1.82 (1H, m)	36.7
26	1.28 (3H, s)	25.4	1.41 (3H, s)	29.6	3.63 (2H, m)	66.8
27	1.36 (3H, s)	28.9	1.43 (3H, s)	30.5	1.04 (3H, d, 6.4)	17.1
28	1.19 (3H, d, 6.8)	16.6				
1'	6.11 (1H, s)	97.8	6.21 (1H, s)	96.8	6.28 (1H, s)	96.4
2'		152.2		151.8		153.4
3'	6.66 (1H, d, 3.2)	110.1	6.73 (1H, d, 3.2)	110.3	6.61 (1H, d, 3.2)	109.1
4'	6.44 (1H, d, 3.2)	107.8	6.45 (1H, d, 3.2)	107.8	6.43 (1H, d, 3.2)	107.7
5'		157.4		157.6		157.3
6'	4.83 (2H, s)	57.2	4.85 (2H, s)	57.2	4.86 (2H, s)	57.2

(20R,22R)-20-O,22-O-(5'-Hydroxymethyl)-furfurylidene-2 β ,3 β ,25-trihydroxy-14 β -methyl-18-nor-5 β -cholesta-7,12-dien-6-one (niuxixinsterone B, **2**): white amorphous powder, mp: 230–231 °C. $[\alpha]_D^{20} +46$ (c 0.025, MeOH). IR (KBr) cm^{-1} : 3472, 1655, 1460, 1380, 1059. UV (MeOH) λ_{max} (log ϵ) nm: 224 (4.05), 248 (3.23), 321 (1.09). HRESIMS (positive ion mode) m/z : 593.3091 $[\text{M}+\text{Na}]^+$, (calc. for $\text{C}_{33}\text{H}_{46}\text{O}_8\text{Na}$ 593.3090). ^1H -NMR and ^{13}C -NMR data are shown in Table 1.

(20R,22R,25R)-20-O,22-O-(5'-Hydroxymethyl)-furfurylidene-2 β ,3 β ,5 β ,14 α ,26-pentahydroxycholest-7-en-6-one (niuxixinsterone C, **3**): white amorphous powder, mp: 235–236 °C. $[\alpha]_D^{20} +52$ (c 0.06, MeOH). IR (KBr) cm^{-1} : 3480, 1656, 1456, 1380, 1062. UV (MeOH) λ_{max} (log ϵ) nm: 225 (4.09), 247 (3.51), 320 (1.07). HRESIMS (positive ion mode) m/z : 627.3150 $[\text{M}+\text{Na}]^+$, (calc. for $\text{C}_{33}\text{H}_{48}\text{O}_{10}\text{Na}$ 627.3145). ^1H -NMR and ^{13}C -NMR data are shown in Table 1.

4. Conclusions

In conclusion, three new phytoecdysteroids, (20R,22R,24S)-20-O,22-O-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,14 α ,25-tetrahydroxy-5 β -ergost-7-en-6-one (**1**), (20R,22R)-20-O,22-O-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,25-trihydroxy-14 β -methyl-18-nor-5 β -cholesta-7,12-dien-6-one (**2**) and (20R,22R,25R)-20-O,22-O-(5'-hydroxymethyl)-furfurylidene-2 β ,3 β ,5 β ,14 α ,26-pentahydroxy-cholest-7-en-6-one (**3**), were isolated from the EtOH extract of *Achyranthes bidentata* Bl. The discovery of compounds **1–3** is a further addition to the diverse phytoecdysteroids compounds.

Acknowledgments

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Sample Availability: Samples of niuxixinsterone A, B and C are available from the authors.