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Steroidal constituents isolated from the seeds of *Withania somnifera*

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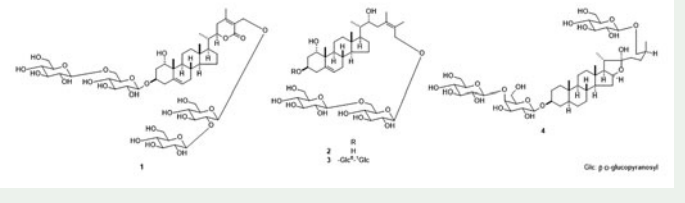
ABSTRACT

A new withanolide glycoside (**1**), two new ergostanol glycosides (**2** and **3**), and a new furostanol glycoside (**4**), along with nine known steroidal derivatives (**5**–**12**) were isolated from the seeds of *Withania somnifera*. The structures of the new compounds were determined using spectroscopic analysis and hydrolysis. The cytotoxic activities of the isolated compounds were evaluated against Ca9-22 human gingival carcinoma cells, HSC-2 human mouth carcinoma cells, and HL-60 human promyelocytic leukemia cells. Only **12** exhibited cytotoxic activity against these cell lines with IC_{50} values of 0.38, 0.54, and 1.5 μM, respectively.



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1. Introduction

Withania somnifera is an evergreen shrub belonging to the Solanaceae family, which is found in diverse areas of Africa, the countries of the Mediterranean littoral, and India. *W. somnifera* has been frequently used in Ayurvedic medicine for over 3,000 years and is often referred to as "Ashwagandha" or "Indian ginseng" (Jana and Charan 2018). The extracts of *W. somnifera* exhibit antibacterial, antifungal, and anticancer properties (John 2014; Dar et al. 2015). The chemical constituents in the leaves and fruits of *W. somnifera* have been well studied, and a variety of withanolide derivatives, such as withaferin A, and alkaloids have been isolated and identified (John 2014). Currently, the withaferin A content of Sardinian plant of *W. somnifera* has been shown to be

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higher than that of the Indian and Sicilian ones (Scartezzini et al. 2007). However, the chemical composition of the seeds of *W. somnifera* have not been fully surveyed, which prompted our group to carry out a phytochemical study on the seeds of this plant. As a result, a new withanolide glycoside (**1**), two new ergostanol glycosides (**2** and **3**), a new furostanol glycoside (**4**), and nine known steroidal derivatives (**5**–**12**) were isolated from *W. somnifera* seeds. The new compounds (**1**–**4**) were identified based on the analysis of their spectroscopic data and hydrolysis. The cytotoxic activities of the isolated compounds against Ca9-22 human gingival carcinoma cells, HSC-2 human mouth carcinoma cells, and HL-60 human promyelocytic leukemia cells were evaluated.

2. Results and discussion

The seeds of *W. somnifera* (1.0 kg) were extracted with hot MeOH. The concentrated MeOH extract (90 g) was passed through a Diaion HP-20 porous polymer polystyrene resin column and successively eluted with MeOH/H_2O (1:4), EtOH, and EtOAc. The EtOH eluted fraction (45 g) was repeatedly separated using silica gel and octadecylsilanized (ODS) silica gel column chromatography (CC), and preparative ODS HPLC to obtain 12 compounds (**1**–**12**). The structure of the known compounds were elucidated as (20*S*,22*R*)-1α,3β,27-trihydroxywitha-5,24(25)-dienolide (**5**) (Sahai 1985), (20*S*,22*R*)-3β-[(β-D-glucopyranosyl)oxy]-1α-hydroxywitha-5,24(25)-dienolide (**6**) (Nakano et al. 2013), (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α-hydroxywitha-5,24(25)-dienolide (**7**) (Matsuda et al. 2001), (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α,20-dihydroxywitha-5,24(25)-dienolide (**8**) (Matsuda et al. 2001), (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α,27-dihydroxywitha-5,24(25)-dienolide (**9**) (Matsuda et al. 2001), (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α,27-dihydroxywitha-5,24(25)-dienolide (**10**) (Zhao et al. 2002), (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-27-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α-hydroxy-5,24(25)-dienolide (**11**) (Zhao et al. 2002), and (20*S*,22*R*)-5β,6β-epoxy-4β,27-dihydroxy-1-oxowitha-2,24(25)-dienolide (**12**) (Fuska et al. 1982) (Figure 1).

Compound **1** was obtained as an amorphous powder and its molecular formula was identified as $C_{52}H_{82}O_{25}$ based on high-resolution electrospray ionization time-of-flight mass spectroscopy (HRESITOFMS; 1129.5044 $[M+Na]^+$, calculated for $C_{52}H_{82}O_{25}Na$: 1129.5043) and ^{13}C-NMR spectroscopy. The ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of **11**, showing the following ^{1}H- and ^{13}C-NMR features: Four methyl groups [δ_H 2.26 (3H, s, Me-28), 0.96 (3H, s, Me-19), 0.92 (3H, d, $J = 6.4$ Hz, Me-21), and 0.55 (3H, s, Me-18); δ_C 20.9 (C-28), 19.4 (C-19), 13.4 (C-21), and 11.6 (C-18)], an α,β-unsaturated δ-lactone group [δ_H 4.46 (1H, m, H-22), 2.43 (1H, dd, $J = 17.1$, 14.0 Hz, H-23a), and 2.07 (1H, dd, $J = 17.1$, 8.1 Hz, H-23b); δ_C 166.1 (C-26), 158.1 (C-24), 123.2 (C-25), 78.3 (C-22), and 30.0 (C-23)], an olefinic group [δ_H 5.55 (br s, H-6); δ_C 139.2 (C-5) and 123.9 (C-6)], two oxygenated methine groups [δ_H 4.75 (1H, m, $W_{1/2} = 21.8$ Hz, H-3) and 4.05 (1H, br s, H-1); δ_C 74.3 (C-3) and 72.3 (C-1)], an oxygenated methylene group [δ_H 5.02 (d, $J = 11.4$ Hz, H-27a) and 4.82 (d, $J = 11.4$ Hz, H-27b); δ_C 63.3 (C-27)], two quaternary carbons [δ_C 42.7 (C-13) and 41.9 (C-10)], and four anomeric protons and

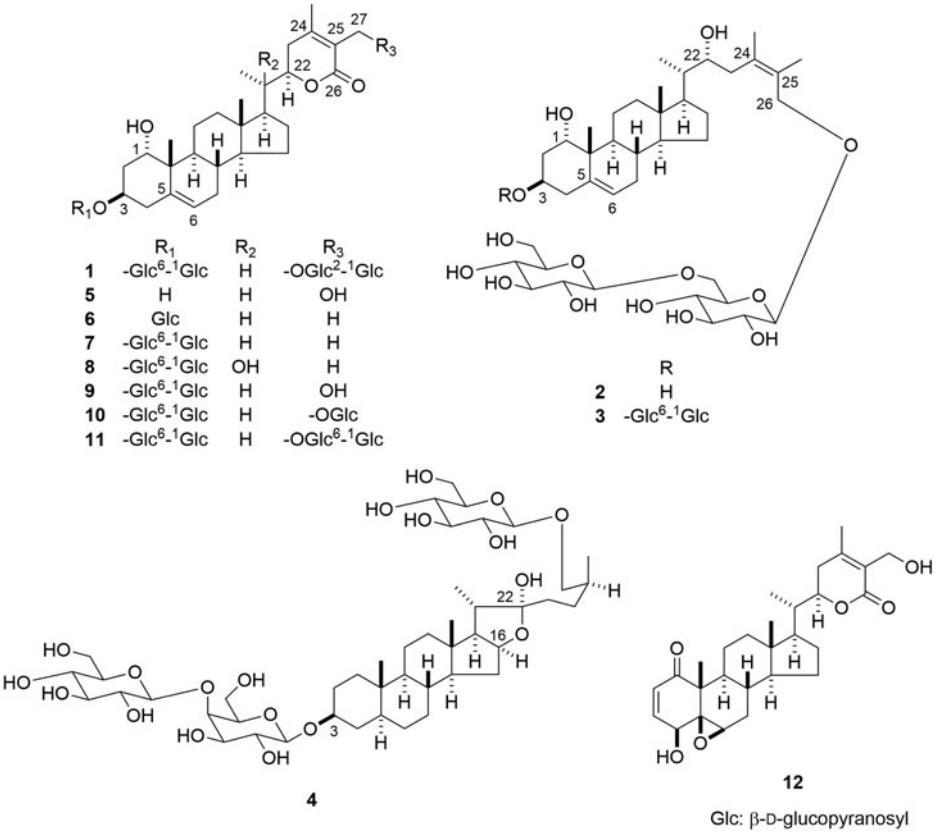

Figure 1. Structures of **1**–**12**.

carbons [δ_H 5.34 (d, $J = 7.7$ Hz), 5.13 (d, $J = 7.7$ Hz), 5.03 (d, $J = 7.9$ Hz), and 4.92 (d, $J = 7.7$ Hz); δ_C 105.6, 105.3, 103.1, and 102.4]. Enzymatic hydrolysis of **1** with naringinase gave **5** and D-glucose. A comparison of the ^{1}H- and ^{13}C-NMR spectra of the sugar moieties in **1** with those of **11** suggested that **1** was a bisdesmoside of **5**, which differed from **11** in the sugar chain sequence of the diglycoside attached to C-26 in the aglycone. The ^{1}H-^{1}H correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC), and HSQC-TOCSY data obtained for **1** allowed the sequential assignment of the ^{1}H- and ^{13}C-NMR signals observed for each sugar unit, indicating that the sugar moieties in **1** consist of two terminal β-D-glucopyranosyl units [Glc″: δ_H 5.13 (d, $J = 7.7$ Hz, H-1″); δ_C 105.3, 75.2, 78.3, 71.4, 78.2, and 62.4 (C-1″–6″); Glc⁗: δ_H 5.34 (d, $J = 7.7$ Hz, H-1⁗); δ_C 105.6, 76.3, 78.1, 71.3, 78.2, and 62.5 (C-1⁗–6⁗)], a 2-monosubstituted inner β-D-glucopyranosyl unit [Glc‴: δ_H 5.03 (d, $J = 7.9$ Hz, H-1‴); δ_C 102.4, 83.3, 78.4, 71.0, 78.1, and 62.3 (C-1‴–C-6‴)], and a 6-monosubstituted inner β-D-glucopyranosyl unit [Glc′: δ_H 4.92 (d, $J = 7.7$ Hz, H-1′); δ_C 103.1, 75.0, 78.4, 71.3, 76.9, and 69.6 (C-1′–C-6′)]. The heteronuclear multiple bond correlation (HMBC) spectrum of **1** exhibited long-range correlations between H-1″ of Glc″ (δ_H 5.13) and C-6′ of Glc′ (δ_C 69.6), H-1′ of Glc′ (δ_H 4.92) and C-3 of the aglycone (δ_C 74.3), H-1⁗ of Glc⁗ (δ_H 5.34) and C-2‴ of Glc‴ (δ_C 83.3), and between H-1‴ of Glc‴ (δ_H 5.03) and C-27 of the aglycone (δ_C 63.3). Thus, **1** was

established to be (20*S*,22*R*)-3β-[(*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-27-[(*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl)oxy]-1α-hydroxywitha-5,24(25)-dienolide.

Compound **2** ($C_{40}H_{66}O_{14}$) was obtained as an amorphous powder. The ^{1}H-NMR spectrum of **2** exhibited signals for five typical steroidal methyl protons [δ_H 2.09 (3H, s, Me-27), 2.00 (3H, s, Me-28), 1.20 (3H, d, $J = 6.8$ Hz, Me-21), 1.14 (3H, s, Me-19), and 0.71 (3H, s, Me-18)], an olefinic proton [δ_H 5.65 (1H, br d, $J = 4.4$ Hz, H-6)], and two anomeric protons [δ_H 5.13 (1H, d, $J = 7.8$ Hz) and 4.86 (1H, d, $J = 7.7$ Hz)]. The ^{13}C-NMR spectrum showed signals for five steroid methyl carbons [δ_C 19.9 (C-19), 19.0 (C-28), 17.5 (C-27), 13.0 (C-21), and 12.0 (C-18)], two pairs of olefinic carbons [δ_C 140.2 (C-5), and 123.8 (C-6); 133.2 (C-25) and 127.3 (C-24)], and two anomeric carbons [δ_C 105.5 and 103.1]. The spectral features of **2** are closely related to those observed for (22*R*,24*Z*)-1α,3β,22,26-tetrahydroxyergosta-5,24(25)-diene 26-*O*-β-glucopyranoside (cilistol v), which was isolated from *Solanum cilistum* (Zhu et al. 2001). However, the molecular formula of **2** was larger than that of cilistol v by $C_6H_{10}O_5$, which corresponds to a hexosyl unit. Enzymatic hydrolysis of **2** with naringinase yielded an aglycone (**2a**) whose ^{1}H- and ^{13}C-NMR spectra were in agreement with those of the aglycone of cilistol v and D-glucose. The above-mentioned data implied **2** was a derivative of cilistol v bearing one more D-glucopyranosyl moiety. Analysis of the ^{1}H-^{1}H COSY and HSQC spectra of the sugar moieties in **2** revealed that it was composed of a 6-monosubstituted inner β-D-glucopyranosyl unit (Glc′) and a terminal β-D-glucopyranosyl unit (Glc″). In the HMBC spectrum of **2**, long-range correlations were observed between H-1″ of Glc″ (δ_H 4.86) and C-6′ of Glc′ (δ_C 70.1), and between H-1′ of Glc′ (δ_H 5.13) and C-26 of the aglycone (δ_C 70.2). Therefore, **2** was deduced to be (22*R*,24*Z*)-1α,3β,22-trihydroxyergosta-5,24(25)-dien-26-yl *O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Compound **3** was obtained as an amorphous powder. The HRESITOFMS and ^{13}C-NMR data allowed the molecular formula of **3** to be assigned as $C_{52}H_{86}O_{24}$, which was larger than that of **2** by $C_{12}H_{20}O_{10}$, indicating the presence of two additional hexosyl groups. The ^{1}H- and ^{13}C-NMR spectra of **3** exhibited four anomeric protons and carbons at δ_H 5.11 (d, $J = 7.8$ Hz, H-1″ of Glc″)/δ_C 105.1, 5.10 (d, $J = 7.8$ Hz, H-1‴′ of Glc‴′)/δ_C 105.2, 4.91 (d, $J = 7.7$ Hz, H-1′ of Glc′)/δ_C 103.0, and 4.85 (d, $J = 7.8$ Hz, H-1‴ of Glc‴)/δ_C 103.7. Enzymatic hydrolysis of **3** was carried out to give **2a** and D-glucose. When the ^{1}H- and ^{13}C-NMR spectra of **3** were compared with those of **2**, the ^{13}C-NMR signal assigned to C-3 was shifted downfield by 8.1 ppm, whereas those corresponding to C-2 and C-4 moved upfield by 1.4 and 5.7 ppm, respectively, which implied that both the C-3 and C-26 hydroxy groups were glycosylated. The ^{1}H-^{1}H COSY, TOCSY, HSQC, and HSQC-TOCSY spectra obtained for **3** indicate the presence of two 6-monosubstituted β-D-glucopyranosyl units (Glc′ and Glc‴) and two terminal β-D-glucopyranosyl units (Glc″ and Glc‴′). HMBC correlations were observed from H-1″ of Glc″ [δ_H 5.11 (d, $J = 7.8$ Hz)] to C-6′ of Glc′ (δ_C 69.5), H-1′ of Glc′ [δ_H 4.91 (d, $J = 7.7$ Hz)] to C-3 of the aglycone (δ_C 74.2), H-1‴′ of Glc‴′ [δ_H 5.10 (d, $J = 7.8$ Hz)] to C-6‴ of Glc‴ (δ_C 69.8), and H-1‴ of Glc‴ [δ_H 4.85 (d, $J = 7.8$ Hz)] to C-26 of the aglycone (δ_C 70.0) in **3**. Thus, **3** was assigned as (22*R*,24*Z*)-26-[(β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)oxy]-1α,22-dihydroxyergosta-5,24(25)-dien-3β-yl β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Compound **4** was obtained as an amorphous powder. The HRESITOFMS and ^{13}C-NMR data showed **4** had a molecular formula of $C_{45}H_{76}O_{19}$. In the ^{1}H- and ^{13}C-NMR spectra recorded for **4**, signals for four characteristic steroidal methyl groups [δ_H 1.27 (3H, d,

$J = 7.0$ Hz, Me-21), 0.99 (3H, d, $J = 6.6$ Hz, Me-27), 0.82 (3H, s, Me-18), and 0.62 (3H, s, Me-19); δ_C 17.2 (C-27), 16.5 (C-18), 16.2 (C-21), and 12.1 (C-19)] and three anomeric protons and carbons [δ_H 5.23 (1H, d, $J = 7.9$ Hz), 4.89 (1H, d, $J = 7.4$ Hz), and 4.78 (1H, d, $J = 7.8$ Hz); δ_C 106.4, 104.7, and 102.3] were observed. In addition, the hemiacetal carbon signal observed at δ_C 110.5 and a positive color reaction in Ehrlich's test implied that **4** was a furostanol glycoside. Compound **4** was treated with β-D-glucosidase, which gave the corresponding spirostanol glycoside (**4a**) (Mahato et al. 1981) and D-glucose. On the other hand, acid hydrolysis of **4** with 1 M HCl gave (25*S*)-5α-spirostan-3β-ol (**4b**) (Sashida et al. 1992), D-galactose, and D-glucose. The NMR data, including ^{1}H-^{1}H COSY, TOCSY, HSQC, and HSQC-TOCSY spectra, indicated that the sugar moieties in **4** consist of two terminal β-D-glucopyranosyl units [Glc′: δ_H 5.23 (d, $J = 7.9$ Hz, H-1′); δ_C 106.4, 75.7, 78.2, 71.8, 78.2, and 62.6 (C-1′–6′) and Glc″: δ_H 4.78 (d, $J = 7.8$ Hz, H-1″); δ_C 104.7, 74.9, 78.1, 71.3, 78.2, and 62.3 (C-1″–6″)] and a 4-monosubstituted β-D-galactopyranosyl unit [Gal: δ_H 4.89 (d, $J = 7.4$ Hz, H-1‴); δ_C 102.3, 73.1, 74.9, 79.6, 75.2, and 60.9 (C-1‴–6‴)]. In the HMBC spectrum of **4**, long range correlations were observed from H-1′ of Glc′ (δ_H 5.23) to C-4‴ of Gal (δ_C 79.6), H-1‴ of Gal (δ_H 4.89) to C-3 of the aglycone (δ_C 77.2), and H-1″ of Glc″ (δ_H 4.78) to C-26 of the aglycone (δ_C 75.2). The configuration of the C-22 hydroxy group in **4** was determined to be 22α based on the NOE correlation observed between the signals corresponding to H-20 (δ_H 2.18) and H_2-23 (δ_H 2.06). Accordingly, **4** was determined to be (25*S*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxy-5α-furostan-3β-yl *O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside.

The cytotoxic activities of the isolated compounds were evaluated against Ca9-22, HSC-2, and HL-60 cells. As expected from the previously reported data, **12** (withaferin A) showed considerable cytotoxic activity against Ca9-22, HSC-2, and HL-60 cells when compared with the positive controls, etoposide and doxorubicin, with IC_{50} values of 0.38, 0.54, and 1.5 μM, respectively. Ca9-22 and HSC-2 oral cancer cells were found to be more sensitive to **12** than HL-60 leukemia cells. The other compounds did not show any cytotoxic activity (IC_{50} >20 μM).

3. Experimental

See supplementary material.k

4. Conclusions

A phytochemical investigation of the seeds of *W. somnifera* gave 12 steroidal derivatives (**1**–**12**), including four new steroidal glycosides (**1**–**4**). The structures of the new compounds were determined using extensive spectroscopic analysis, including two-dimensional NMR spectroscopy and hydrolysis. The cytotoxic activities of the isolated compounds against Ca9-22, HSC-2, and HL-60 cells were evaluated. Only **12** showed cytotoxic activity against Ca9-22, HSC-2, and HL-60 cells with IC_{50} values of 0.38, 0.54, and 1.5 μM, respectively.

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Disclosure statement

The authors declare no conflict of interest.

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