Chemical Constituents of Ajuga parviflora
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Two new antifungal withanolides, (1) and (2) have been isolated from the defatted methanolic extract of Ajuga parviflora. Their structures were established as 3β,17β,20-trihydroxy-1-oxo-(20R, 22R)-witha-5,14,24-trienolide (1), 28-hydroxy-14,20-epoxy-1-oxo-(22R)-witha-2,5,24-trienolide (2) on the basis of spectroscopic data including two dimensional NMR techniques. The pyrrolizidine alkaloids seneconine (3) and integerrimine (4) are also reported for the first time from A. parviflora.

Introduction

Ajuga parviflora Benth. (Labiatae) is an annual or short lived perennial herb growing in the hilly regions of northern Pakistan. Species belonging to genus Ajuga have been used as folk medicinal plants as anthelmintic, antifungal, hypoglycaemic, antitumor and antimicrobial agents [1,2]. During a search for new bioactive compounds from medicinal plants, we found that the defatted methanolic extract of A. parviflora, showed strong brine shrimp bioactivity. This prompted us to study the chemical constituents of this plant. Herein we report the isolation and structural elucidation of two new withanolides, (1) and (2), which showed moderate antifungal activity against T. schoenemii and R. Solani respectively. Two pyrrolizidine alkaloids have also been isolated and characterized as seneconine (3) and integerrimine (4) by comparison of their spectral data with those in the literature [3,4].

Results and Discussion

Withanolide (1) showed in the UV spectrum an absorption at λ max 222 nm characteristic of the dimethyl substituted α,β-unsaturated δ-lactone [5]. The IR spectrum showed the presence of hydroxyl groups (3460 cm⁻¹), a six membered cyclic ketone (1724 cm⁻¹), and an α,β unsaturated δ-lactone (1712 cm⁻¹). The assignment of 1H NMR signals was made through comparison with reported spectra of 3β-hydroxy-2,3-dihydrowithanolide-H [6] and withaperuvin F [7]. The methyl singlets at δ 1.19, 1.29 and 1.30 could be assigned to Me-18, 19 and 21, while downfield singlets at δ 1.89 and 1.99 were due to Me-27 and 28, respectively. The presence of two trisubstituted double bonds was inferred from downfield singlets at δ 5.54 and δ 5.26. The broad doublet at δ 5.54 (J = 5.4 Hz) was due to H-6. The α-carbinyl hydrogen at C-3 was observed as multiplet at δ 3.63. Comparison of the 13C NMR data of 1 with those of 3β-hydroxy-2,3-dihydrowithanolide-H [6] confirmed the presence of carbonyl group at C-1 (δ 212.4). Acetylation of 1 provided the monoacetate 1a which still showed the presence of hydroxyl absorption in the IR spectrum confirming the presence of tertiary hydroxyl groups. One of these could be assigned to C-20, as signal of H-22 was observed as a double doublet at δ 4.80 (1H, dd, J = 12, 4.5 Hz), and that of Me-21 as a singlet. The 20-hydroxywithanolide skeleton was also revealed from mass spectral peaks at m/z 125.0603 (C₇H₁₀O₂), 152.0837 (C₈H₁₂O₂) and 169.0863 (C₉H₁₄O₃). The remaining hydroxyl group was located at C-17 by the HMBC experiment. It showed 3J correlations at δ 87.2 with protons at δ 1.19 (Me-18) and 1.30 (Me-21). The signals of rings C,D and δ lactone in the 1H and 13C NMR spectra were very similar to those of withaperuvin F [7] and physaloside C [8], allowing us to assign the remaining double bond at C-14. The remaining problem was to assign the configuration at C-17 and C-22. The β-configuration could be assigned for the hydroxyl group at C-17 on the basis of characteristic pyri-
dine induced downfield shift of Me-18 (0.32 ppm) and Me-21 (0.35) in the \(^1\)H NMR as reported earlier for 17β hydroxy withanolides [9,10]. It has been found that when C-22 has an S-configuration, H-22 appears as a broad singlet with a half-line width of ~ 5 Hz while in the R configuration it appears in the \(^1\)H NMR spectrum as a double doublet with two coupling constants, characteristic for axial – axial and axial – equatorial interactions with H2-23 [11]. In the case of 1, H-22 resonated as a double doublet revealing R configuration at C-22. The compound 1 was, therefore, assigned the structure 3β,17β,20-trihydroxy-1-oxo-
\((20R, 22R)\)

**Withanolide (2)** \(C_{38}H_{36}O_5\) was obtained as a white amorphous solid. The UV spectrum displayed a characteristic absorption at 220 nm for an α,β unsaturated δ-lactone [5]. In the IR spectrum the absorption bands for hydroxyl, δ-lactone and α,β unsaturated ketone appeared at 3420, 1710 and 1696 cm\(^{-1}\), respectively. The downfield signals in the \(^1\)H NMR spectrum of 2 were similar to steroidal 2,5 dien-1-one skeleton on the basis of their chemical shifts and well known splitting patterns at δ 6.76 (1H, ddd, \(J = 10, 4.9, 2.4\) Hz, H-3), δ 5.85 (1H, dd, \(J = 10, 2.4\) Hz, H-2), δ 5.58 (1H, br.d, \(J = 5.7\) Hz, H-6). The \(^1\)H and \(^{13}\)C NMR data were in agreement with the published data of related withanolides [12,13] with similar ring A. \(^1\)H NMR spectrum included three quaternary methyls at δ 0.88 (3H, s, Me-18), 1.23 (3H, s, Me-19), 1.25 (3H, s, Me-21) and one vinylmethyl at δ 1.92 (3H, s). The absence of one vinylmethyl and the appearance of two AB doublets at δ 4.1 and 4.17 (2H, ABd, \(J = 11.8\) Hz) which moved downfield to δ 4.76 and 4.81 (2H, ABd, \(J = 12.1\) Hz) in the corresponding monoacetate 2a suggested the presence of hydroxymethylene at either C-24 or C-25. Its oxy-l-oxo-(22β)-witha-2,5,24-trienolide. The \(^{13}\)C NMR spectrum was in complete agreement to the assigned structure and in each case the assignment was confirmed by the HMBC and HMQC experiments.

**Experimental**

Optical rotations were measured on a JASCO DIP-360 polarimeter and UV (MeOH) on Hitachi U-3200, IR on Jasco-A-302 spectrometers, FAB-MS and HR-EIMS on Finnigan MAX 112 and JEOL JMS HX-110 spectrometers, respectively. The \(^1\)H and \(^{13}\)C NMR spectra were recorded in Bruker AM-300 instrument using TMS as an internal reference. The 2D NMR experiments (HMBC and HMQC) were performed on the same instrument using the same solvent. The chemical shifts are in (δ) and coupling constants (\(J\)) are in Hz.

**Collection, extraction and isolation: Ajuga parviflora** (Labiateae), whole plant, was collected in July 1997 from Swat in NWFP Province, Pakistan and identified by Dr. J. Shah. A voucher specimen (No. PUH14918) has been kept in the herbarium in Peshawar University. Whole plant material (20 kg) of A. parviflora was shade dried, ground and extracted thrice with MeOH at room temperature. The combined extracts were evaporated under reduced pressure to obtain a crude syrup which was defatted through repeated extraction with hexane. The defatted extract was subjected to vacuum liquid chromatography (VLC) [silica gel 60 \(\delta\) PF\(_{254}\) (1.5 kg) hexane-EtOAc and then EtOAc-MeOH in increasing order of polarity]. The fractions obtained from EtOAc-MeOH (9:1) showed four spots on TLC. These were combined and subjected to low pressure liquid chromatography [Lobar 9 Lichroprep (Si 60 Merck Column)]; eluted first with EtOAc – MeOH (9:5:0.5) then with
EtOAc-MeOH (9:1) The fractions obtained from EtOAc-MeOH (9:5:0.5) was a binary mixture which could be separated through preparative TLC (silica gel 60 F254; CH2Cl2-MeOH-NH3 85:14:1) to obtain the pyrrolizidine alkaloids 3 (19.4 mg) and 4 (20.2 mg). The fractions obtained from EtOAc-MeOH (9:1) was subjected to preparative TLC (silica gel 60 F254; CHCl3-C6H6-MeOH-H2O 4:4:4:0.3) to afford withanolides 1 (22.3 mg) and 2 (20.7 mg) respectively.

Withanolide (1): Amorphous powder, [α]D = 75 (c=0.46, MeOH). IR (KBr): ν = 3460, 1724, 1712 cm⁻¹. UV (MeOH): λmax (log ε) = 222 nm (3.89). FABHRMS: m/z = 471.2667 [M+H]+ (caked for C29H40O6 471.2686). EIMS: m/z (rel.int.%) = 452 (11), 309 (20), 283 (27), 240 (25), 169 (45), 152 (100), 125 (77), 109 (64), 97 (33). 1H NMR (300 MHz, CDCl3 + few drops CD2OD): δ = 1.19 (3H, s, 18-H), 1.29 (3H, s, 19-H), 1.30 (3H, s, 21-H), 1.89 (3H, s, 27-H), 1.99 (3H, s, 28-H), 2.76 (1H, dd, J = 14, 6Hz, 2-Hβ), 2.63 (1H, dd, J = 14, 2.8 Hz, 2-Hα), 3.63 (1H, m, 3-H), 5.54 (1H, d, J = 5.4 Hz, 6-H) 5.26 (1H, br, s, 5-H), 4.80 (1H, dd, J22α,23α = 12, Hz, J22α,23β = 4.5 Hz, 22-Hβ). 13C NMR (75 MHz, CDCl3 + few drops CD2OD): δ = 11.5 (C-27), 17.8 (C-18), 18.4 (C-19), 20.1 (C-21), 20.19 (C-28), 21.8 (C-11), 29.8 (C-7), 31.9 (C-23), 34.9 (C-8), 35.2 (C-9), 36.3 (C-16), 39.6 (C-12), 40.2 (C-4), 47.2 (C-2), 51.7 (C-10), 55.2 (C-13), 67.9 (C-3), 77.3 (C-20), 80.9 (C-22), 87.2 (C-17), 116.8 (C-15), 121.0 (C-25), 124.8 (C-6), 134.8 (C-5), 151.5 (C-14), 153 (C-24), 166.3 (C-26), 212.4 (C-1).

Acetylation of 1: A solution of the sample (10 mg) in pyridine (2 ml) and Ac2O (2 ml) was stirred overnight at room temperature and usual workup provided the corresponding acetyl derivative 1a as an amorphous solid, [α]D = -64 (c=0.40, CHCl3). IR (CHCl3): ν = 3435, 1734, 1722, 1710 cm⁻¹. UV (MeOH): λmax (log ε) = 223 nm (3.83). FABHRMS: m/z = 513.2829 (caked for C39H41O7 513.2852). EIMS: m/z (rel.int.%): = 512 (2), 452 (15), 283 (30), 169 (40), 152 (100), 125 (81). 1H NMR (300 MHz, CDCl3): δ = 1.20 (3H, s, 18-H), 1.28 (3H, s, 19-H), 1.32 (3H, s, 21-H), 1.91 (3H, s, 27-H), 2.0 (3H, s, 28-H), 2.04 (3H, s, COCH3), 2.77 (1H, dd, J = 13.8, 5.8Hz, 2-H), 2.64 (1H, dd, J = 13.8, 4.1Hz, 2-H), 4.64 (1H, m, 3-H), 5.56 (1H, d, J = 5.1 Hz, 6-H), 4.82 (1H, dd, J22α,23α = 12.2 Hz, J22α,23β = 3.5Hz, 22-H).

Withanolide (2): Amorphous powder, [α]D = 53 (c=0.52, MeOH) IR (KBr): ν = 3420, 1710, 1696 cm⁻¹. UV (MeOH): λmax (log ε) = 218 nm (3.92). FABHRMS: m/z = 453.2546 [M+H]+ (caked for C28H37O5 453.2550). EIMS: m/z (rel.int.%): = 283 (5), 267 (24), 169 (15), 141 (85), 124 (100). 1H NMR (300 MHz, CDCl3 + few drops CD2OD): δ = 0.88 (3H, s, 18-H), 1.23 (3H, s, 19-H), 1.25 (3H, s, 21-H), 1.92 (3H, s, 27-H), 4.10, 4.17 (2H, Abd, J2α,2β = 11.8 Hz each 28-H), 4.24 (1H, dd, J2α,2β = 12.6, J2α,2β = 5.5Hz, 22-H), 5.58 (1H, br,d, J = 5.7 Hz, 6-H), 5.85 (1H, d, J = 10 Hz, 2-H), 6.76 (1H, ddd, J = 10, 4.9, 2.4 Hz, 3-H). 13C NMR (75 MHz, CDCl3 + few drops CD2OD): δ = 14.1 (C-27), 14.3 (C-18), 16.4 (C-21), 16.8 (C-19), 20.7 (C-15), 21.5 (C-11), 29.8 (C-23), 31.5 (C-16), 32.5 (C-8), 33.0 (C-4), 34.0 (C-7), 39.6 (C-9), 40.0 (C-12), 47.2 (C-13), 48.6 (C-17), 50.4 (C-10), 59.4 (C-28), 74.8 (C-20), 81.4 (C-22), 83.7 (C-14), 120.6 (C-25), 125.2 (C-6), 127.1 (C-2), 134.0 (C-5), 146.0 (C-3), 152.0 (C-24), 166.0 (C-26), 205 (C-1).

Withanolide (3): Acetylation was carried out (as described for 1) to obtain monoacetate 2a. [α]D = 55 (c=0.51, CHCl3). IR (CHCl3): ν = 1736, 1712, 1696 cm⁻¹. UV (MeOH): λmax (log ε) = 218 nm (3.79). FABHRMS: m/z = 495.2720 (caked for C39H41O7 495.2746). EIMS: m/z (rel.int.%): = 494 (2), 434 (4), 283 (5) 141 (42) 124 (100). 1H NMR (300 MHz CDCl3): δ = 0.88 (3H, s, 18-H), 1.24 (3H, s, 19-H), 1.15 (3H, s, 21-H), 1.93 (3H, s, 27-H), 2.05 (3H, s, COCH3), 4.25 (1H, dd, J2α,2β = 12.5 Hz, J2α,2β = 4.5 Hz, 22-H), 4.76, 4.81 (2H, Abd, J2α,2β = 12 Hz each, 28-H), 5.58 (1H, brd, J = 5.5 Hz, 6-H), 5.85 (1H, d, J = 10 Hz, 2-H), 6.76 (1H, dd, J = 10, 4.9, 2.4Hz, 3-H).