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Triterpene saponins from *Chenopodium quinoa* Willd.

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ABSTRACT

Twenty triterpene saponins (**1**–**20**) have been isolated from different parts of *Chenopodium quinoa* (flowers, fruits, seed coats, and seeds) and their structures have been elucidated by analysis of chemical and spectroscopic data including 1D- and 2D-NMR. Four compounds (**1**–**4**) were identified: 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-23-oxo-olean-12-en-28-oic acid β-D-glucopyranoside (**1**), 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-27-oxo-olean-12-en-28-oic acid β-D-glucopyranoside (**2**), 3-*O*-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester (**3**), and 3-*O*-β-D-glucuronopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester (**4**). The following known compounds have not previously been reported as saponin constituents from the flowers and the fruits of this plant: two bidesmosides of serjanic acid (**5**,**6**), four bidesmosides of oleanolic acid (**7**–**10**), five bidesmosides of phytolaccagenic acid (**11**–**15**), four bidesmosides of hederagenin (**16**–**19**), and one bidesmoside of 3β,23,30-trihydroxy olean-12-en-28-oic acid (**20**). The cytotoxicity of these saponins and their aglycones was tested in HeLa cells. Induction of apoptosis in Caco-2 cells by bidesmosidic saponins **1**–**4** and their aglycones **I**–**III** was determined by flow cytometric DNA analysis. The saponins with an aldehyde group were most active. The relationships between structure and cytotoxic activity of saponins and their aglycones are discussed.

1. Introduction

Chenopodium quinoa Willd. (Chenopodiaceae), commonly known as quinoa, is a well-known staple food of the Andean communities serving as an alternative food source in other regions (Brinegar and Goundan, 1993; Delatorre-Herrera, 2003; Dini et al., 2005). The seed contains high-quality protein and is particularly rich in essential amino acids and its carbohydrate content has been reported to have a higher nutritional value than that of cereals such as corn, oats, wheat, and rice (Ruales and Nair, 1992; Lindeboon, 2005). More importantly, quinoa seed has more Ca, Fe, Mn, Mg, Cu, and K than other cereals (Ruales and Nair, 1993; Konishi et al., 2004). Quinoa seeds are a potential crop for many animal feed formulations, for direct feeding, and as a human food (Takao et al., 2005). The seeds are used in the same way as rice and wheat and are ground into flour to prepare breads, cakes, and fermented drinks (Ando et al., 2002; Bhargava et al., 2006). However, the main disadvantage has been the bitterness related to the saponins present (Brady et al., 2007).

Previous investigations on saponins isolated from seeds of *C. quinoa* have shown that they exert antimicrobial activity (Woldemichael and Wink, 2001), toxicity to brine shrimps (Meyer et al., 1990), and against viral diseases, cholesterol-lowering effects, and enhance drug absorption through mucosal membranes. They act as immunological and absorption adjuvants to enhance antigen-specific antibody and mucosal responses (Estrada et al., 1998). Saponins from *Chenopodium quinoa* are a complex mixture of triterpene glycosides that are derivatives of oleanolic acid, hederagenin, phytolaccagenic acid, serjanic acid, and 3β,23,30-trihydroxy olean-12-en-28-oic acid, which bear hydroxyl and carboxylate groups at C-3 and C-28, respectively, and are based on β-amyrin. The major carbohydrates are arabinose, glucose, and galactose; while glucuronic acid and xylose have been found to be less common (Cuadrado et al., 1995; Dini et al., 2001a,b, 2002; Mizui et al., 1988, 1990; Madl et al., 2006; Mastebroek et al., 2000; Woldemichael, 2000; Zhu et al., 2002). This study was centered on the isolation, structure elucidation, and biological activities of triterpene saponins and their aglycones in this plant. Herein, we report the isolation of twenty glycosides of 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**1**), 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**2**), serjanic acid (**3**–**6**), oleanolic acid (**7**–**10**), phytolaccagenic acid (**11**–**15**), hederagenin (**16**–**19**), and 3β,23,30-trihydroxy olean-12-en-28-oic acid (**20**). Flowers, fruits, seed coats, and seeds were investigated. For the first time we report results from flowers and fruits. A derivative of 3β-hydroxy-27-oxo-olean-12-en-28-oic acid

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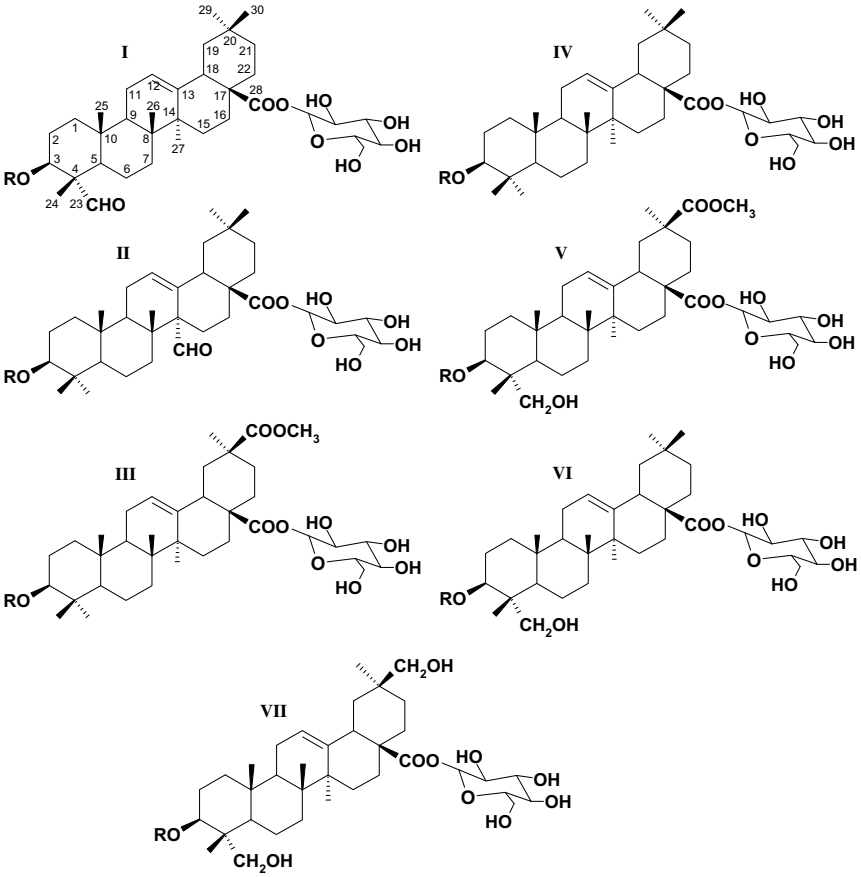
is described for the first time in the Chenopodiaceae. The occurrence of 3β-hydroxy-27-oxo-olean-12-en-28-oic acid might represent an interesting intermediate in the biosynthesis of these substances. Several reports showed that sugar moiety and type of linkage are also directly related to the structure–activity of saponins (Takechi et al., 1996; Woldemichael and Wink, 2001; Voutquenne et al., 2002). However, data in the literature for comparative structure–activity relationship studies in apoptosis induction and cytotoxicity are limited (Chwalek et al., 2006). The present paper reports on the cytotoxicity and apoptosis-inducing activity of saponins **1**–**4** and their aglycones **I**–**III**.

2. Results and discussion

Preliminary TLC comparison of saponin fractions of the four different plant parts of *C. quinoa* revealed similar results in each case. The crude saponins obtained from seed coats (10.7% yield), seeds (8% yield), flowers (4% yield), and fruits (4% yield) were fractionated by a combination of silica gel open column chromatography to afford 20 compounds (**1**–**20**) in a pure form as confirmed by HPLC analysis. Sixteen of these were identified as 3-*O*-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester (**5**) (Dini et al., 2001b), 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester (**6**) (Dini et al., 2002), quinoside D (**7**) (Mizui et al., 1990), quinoa saponin 7 (**8**) (Mizui et al., 1990), 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl oleanolic acid 28-*O*-β-D-glucopyranosyl ester (**9**) (Dini et al., 2001a), chikusetsusaponin **IVa** (**10**) (Mizui et al., 1990), quinoa saponin 4 (**11**) (Woldemichael, 2000), quinoa saponin 3 (**12**) (Woldemichael, 2000), quinoa saponin 5 (**13**) (Mizui et al., 1988), quinoa saponin 8 (**14**) (Mizui et al., 1990), 3-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl phytolaccagenic acid 28-*O*-β-D-glucopyranosyl ester (**15**) (Dini et al., 2001b), quinoa saponin 2 (**16**) (Mizui et al., 1988), *Hedera nepalensis* saponin F (**17**) (Mizui et al., 1988), quinoa saponin 9 (**18**) (Mizui et al., 1990), quinoa saponin 1 (**19**) (Mizui et al., 1988), and 3-*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl 3β,23,30-trihydroxy olean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl ester (**20**) (Madl et al., 2006), by comparison of physical data with literature values and from spectroscopic evidence. The chemical structures of the identified saponins **1**–**20** are depicted in Fig. 1.

Compound **1** was isolated as a white powder. It gave a positive Liebermann–Burchard test. The molecular formula of **1** was established as $C_{47}H_{74}O_{18}Na$ by positive-mode ESI-MS (*m*/*z* 949.5 $[M+Na]^+$). Its HR-ESI-MS gave a peak at 949.4698 $[M+Na]^+$ (calculated for $C_{47}H_{74}O_{18}Na$, 949.4772). The ^1H NMR spectrum of **1** (Table 1) shows signals for six tertiary methyl groups (δ 0.80, 0.91, 0.92, 0.98, 1.08, and 1.15), an olefinic broad singlet (δ 5.40, H-12), and an oxymethine proton at δ 3.99, *dd*, *J* = 11.8 and 4.0 Hz, H-3. These signals indicated a pentacyclic skeleton in **1**. The ^1H NMR spectrum further showed double doublets at δ 3.20 (*J* = 13.0 and 2.6 Hz) attributed to H-18, a characteristic signal of the oleanane-type skeleton (Seebacher et al., 2003). A singlet for an aldehyde proton was resonated at δ 9.39 in the ^1H NMR spectrum. The ^{13}C NMR spectrum (Table 1) of compound **1** showed signals corresponding to 47 carbons, resolved into 6 methyl, 13 methylene, 20 methine, and 8 quaternary carbons. Among them, 17 signals were assigned to the oligosaccharide moiety. The ^{13}C NMR spectrum of **1** showed olefinic carbon signals at δ_C 123.40 (C-12) and 143.81 (C-13). The chemical shifts of C-3 (δ 81.98) and C-28 (δ 176.13) indicated a bisdesmosidic glycoside (Woldemichael and Wink, 2001; Sahu and Achari, 2001). The ^1H and ^{13}C NMR spectra of **1** exhibited three anomeric protons resonated as doublets at δ 4.95 (*J* = 7.3 Hz), 5.30 (*J* = 7.8 Hz), and 6.29 (*J* = 8.0 Hz), which corresponded to the carbon signals at δ 106.54, 106.24, and 95.80, respectively. The sugars were identified as two units of glucose, and one unit of arabinose by authentic samples and a detailed study of COSY, HSQC, and HMBC spectra. The β-anomeric configurations of the glucose and arabinose units were deduced from their $^3J_{H1,H2}$ coupling constants (7.4–8.0 Hz) (Woldemichael and Wink, 2001). The sequence of the sugar connected to the C-3 of the aglycone was deduced from the HMBC correlations of anomeric H-1 (δ 4.95) of the arabinose moiety with carbon signal at δ 81.98 (C-3) and 11.72 (C-24), indicating the attachment of arabinose at C-3. The anomeric H-1 (δ 5.30) of the glucose moiety exhibited HMBC correlation with δ 84.01 (C-3) of the arabinose moiety, indicating connectivity between C-1 and C-3. Subsequently, the glucose sugar substituted at C-3 of α-L-arabinoside was also inferred from the analysis of chemical shifts data in the literature (Jiang et al., 2006; Yoshimitsu et al., 2007). The presence of a second monodesmosidic moiety at C-28 was deduced by the HMBC interactions of anomeric H-1 of glucose (δ 6.29) with C-28 carbonyl (δ_C 176.13). The C-23 aldehyde proton (δ 9.36) showed HMBC interactions with carbon signals at δ 11.72 (C-24), 53.01 (C-4), and 81.98 (C-3). The ^1H NMR subspectra of individual monosaccharide units were obtained by using selective irradiation of the easily identifiable anomeric proton signals, as well as irradiations of other non-overlapping proton signals in a series of COSY and HSQCAD experiments (Table 2). Basic hydrolysis of **1** gave the prosapogenin; acid hydrolysis of the resulting prosapogenin then yielded arabinose. Acid hydrolysis of **1** gave the aglycone 3β-hydroxy-23-oxo-olean-12-en-28-oic acid along with arabinose and glucose. On the basis of the above evidence, compound **1** was a new compound, determined as 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-23-oxo-olean-12-en-28-oic acid β-D-glucopyranoside.

Compound **2** was isolated as a white powder. It gave a positive Liebermann–Burchard test. The molecular formula of **2** was established as $C_{47}H_{74}O_{18}Na$ by positive-mode ESI-MS (*m*/*z* 949.5). Its HR-ESI-MS gave a peak at 949.4671 $[M+Na]^+$ (calculated for $C_{47}H_{74}O_{18}Na$, 949.4772). The ^1H NMR spectrum of compound **2** showed signals for six methyl groups at δ 1.08, 0.97, 0.85, 1.09, 0.74, and 0.84 assigned to Me-23, Me-24, Me-25, Me-26, Me-29, and Me-30 of the triterpene moiety of the oleanane-type (Seebacher et al., 2003), respectively. These signals were correlated with their corresponding carbon signals at δ 32.01, 28.05, 16.22, 16.73, 32.92, and 23.92 in the HSQC spectrum. The ^1H and ^{13}C NMR spectra of **2** exhibited three anomeric protons resonated as doublets at δ 4.95 (*J* = 7.3 Hz), 5.30 (*J* = 7.8 Hz), and 6.29 (*J* = 8.0 Hz), which corresponded to the carbon signals at 106.61, 106.01, and 95.74, respectively. The sugars were identified as two units of glucose and one unit of arabinose by comparison with authentic samples. The β-anomeric configurations of the glucose and arabinose units were deduced from their $^3J_{H1,H2}$ coupling constants (7.4–8.0 Hz) (Table 2) (Bouguet-Bonnet et al., 2002; Woldemichael and Wink, 2001). Compound **2** exhibited a triplet for the olefinic proton H-12 which appeared at low field position δ 5.45 in the ^1H NMR spectrum, and signals for double bonds at δ 123.40 and 143.81 in the ^{13}C NMR spectrum (Table 1). Two carbonyl signals, resonating at δ 176.12 and 209.01 were also shown in the ^{13}C NMR spectrum and assigned to a 28-glycosyl ester carbonyl and aldehyde group, respectively. The NMR spectra of **2** contained one singlet at δ 2.14, suggesting that one of the methyl groups of oleanolic acid was replaced by an aldehyde group. With respect to this type of triterpene, the signals from C-13 are shifted upfield by 7.3 ppm; the C-12 appeared at 5.1 ppm because of higher deshielding in compound **2**. This observation indicates that the aldehyde function is in close proximity of the double bond and located at C-14 instead of Me-27. The glycosyl ester function was proposed to be at C-28 in view of the ^{13}C NMR spectrum, which showed δ values of 23.43, 46.72, and 33.62 for C-16, C-17, and C-22, respectively. In the HMBC spectrum the pro-



Compound	Aglycone	R	Flower	Fruit	Seed	Seed coat
1	**I**	β-D-Glc(1→3)-α-L-Ara	+	+	+	+
2	**II**	β-D-Glc(1→3)-α-L-Ara	+	+	+	+
3	**III**	α-L-Ara	+	+	++	++
4	**III**	β-D-GlcA	+	+	++	++
5	**III**	β-D-Glc(1→2)-β-D-Glc(1→3)-α-L-Ara	+	+	++	++
6	**III**	β-D-Glc(1→3)-α-L-Ara	+	+	+	++
7	**IV**	β-D-Xyl(1→3)-β-D-GlcA	+	+	+	+
8	**IV**	β-D-Glc(1→2)-β-D-Glc(1→3)-α-L-Ara	+	+	+	+
9	**IV**	β-D-Glc(1→3)-α-L-Ara	+	+	+	+
10	**IV**	β-D-GlcA	+	+	++	++
11	**V**	β-D-Glc(1→3)-α-L-Ara	++++	++++	++++	++++
12	**V**	α-L-Ara	+++	+++	++++	++++
13	**V**	β-D-Glc(1→3)-β-D-Gal	++	++	++	++
14	**V**	β-D-Glc(1→2)-β-D-Glc(1→3)-α-L-Ara	++	++	+	++
15	**V**	β-D-Glc(1→4)-β-D-Glc(1→4)-β-D-Glc	++	++	+	++
16	**VI**	β-D-Glc(1→3)-β-D-Gal	+	+	+	+
17	**VI**	α-L-Ara	+	+	+	+
18	**VI**	β-D-GlcA	++	++	++	++
19	**VI**	β-D-Glc(1→3)-α-L-Ara	+++	+++	++	+++
20	**VII**	β-D-Glc(1→3)-α-L-Ara	+	+	++	++++

(+) trace quantities, (++) low quantities, (+++) moderate quantities, (++++) high quantities

Fig. 1. Structures of saponins **1–20** (aglycones **I–VII**, 3β-hydroxy-23-oxo-olean-12-en-28-oic acid, **I**; 3β-hydroxy-27-oxo-olean-12-en-28-oic acid, **II**; serjanic acid, **III**; oleanolic acid, **IV**; phytolaccagenic acid, **V**; hederagenin, **VI**; 3β,23,30-trihydroxy olean-12-en-28-oic acid, **VII**).

ton signal at δ 2.14 showed long-range correlation with the carbonyl aldehyde at δ 209.01 indicating that Me-27 was oxidized to CHO. The anomeric H-1 (δ 5.30) of the glucose moiety exhibited HMBC correlation with δ 84.32 (C-3) of the arabinose moiety, pointing to a connectivity between C-1 and C-3. Subsequently, the glucose sugar substituted at C-3 of α-L-arabinoside was also inferred from the analysis of chemical shifts data in the literature (Jiang et al., 2006; Yoshimitsu et al., 2007). The shift values of C-3 at δ 88.38 and C-28 at δ 176.12 indicated that **2** is 3,28-bisdesmoside (Woldemichael and Wink, 2001). Basic hydrolysis of **2** gave the prosapogenin; acid hydrolysis of the resulting prosapogenin then yielded arabinose. Acid hydrolysis of **2** gave the aglycone 3β-hydroxy-27-oxo-olean-12-en-28-oic acid and the arabinose and glucose. Thus the structure of **2** proved to be the new compound 3β-[(*O*-β-D-glucopyranosyl-(1 → 3)-α-L-arabinopyranosyl)oxy]-27-oxo-olean-12-en-28-oic acid β-D-glucopyranoside.

Compound **3** was isolated as a white powder. It gave a positive Liebermann–Burchard test. The molecular formula of **3** was established as $C_{42}H_{66}O_{14}Na$ by positive-mode ESI-MS (*m*/*z* 817.5). Its HR-ESI-MS gave a peak at 817.4352 $[M+Na]^+$ (calculated for

Table 1
^{13}C NMR spectroscopic assignments (δ) of the aglycone and monosaccharide moieties of saponins **1**–**4**[a]

Aglycone					Monosaccharide				
C	**1**	**2**	**3**	**4**	**C**	**1**	**2**	**3**	**4**
					C-3	Arabinose	Arabinose	Arabinose	Glucuronic acid
1	36.32	38.49	38.52	38.51	1	106.54	106.61	105.93	107.55
2	23.29	26.38	26.51	26.50	2	71.92	72.10	73.89	80.01
3	81.98	88.38	87.22	87.21	3	84.01	84.32	75.49	76.60
4	53.01	39.40	39.53	39.53	4	69.22	69.34	70.45	75.22
5	46.82	55.87	56.01	56.02	5	67.49	67.12	67.68	76.21
6	18.63	18.32	18.47	18.41	6				172.34
7	30.62	35.72	33.53	33.52		Glucose	Glucose		
8	38.45	41.91	39.72	39.74	1	106.24	106.01		
9	46.02	49.74	47.51	47.55	2	74.78	76.45		
10	34.32	37.40	37.01	37.06	3	78.41	78.21		
11	24.14	24.14	23.33	23.37	4	71.13	71.73		
12	123.40	123.40	123.12	123.12	5	78.29	78.03		
13	143.81	143.81	144.22	144.24	6	62.34	62.71		
14	40.21	59.14	42.04	42.15	C-28	Glucose	Glucose	Glucose	Glucose
15	26.20	21.33	28.25	28.12	1	95.80	95.74	95.89	95.71
16	21.50	23.43	23.91	23.84	2	74.84	74.00	75.01	74.12
17	48.11	46.72	46.23	46.15	3	78.30	78.78	80.21	79.30
18	40.02	46.71	42.62	42.52	4	72.10	71.11	71.57	71.12
19	44.53	42.22	43.34	43.25	5	78.61	78.13	79.67	78.84
20	28.72	43.63	44.22	44.12	6	62.52	69.58	62.78	62.21
21	32.01	30.62	30.74	30.65					
22	30.42	33.62	34.55	34.42					
23	210.74	32.01	28.42	28.34					
24	11.72	28.05	17.54	17.86					
25	13.17	16.22	15.45	15.42					
26	15.15	16.73	16.92	16.89					
27	23.89	209.01	26.04	26.15					
28	176.13	176.12	177.24	177.24					
29	31.12	32.92	28.52	28.39					
30	21.84	23.92	180.26	180.26					
$-COOCH_3$			51.78	51.77					

[a] Chemical shifts were referenced according to the highest field signals of pyridine-d_5 (8.71 ppm for ^1H and 149.9 ppm for ^{13}C).

Table 2
^1H MR chemical shift data (δ_H, J, Hz) of the monosaccharide moieties of saponins **1**–**4**[a]

	1	**2**	**3**	**4**
C-3	Arabinose	Arabinose	Arabinose	Glucuronic acid
1	4.95 *d*(7.3)	4.95 *d*(7.3)	4.90 *d*(7.7)	5.14 *d*(7.0)
2	4.59	4.59	4.59	4.13
3	4.08	4.08	4.08	4.35
4	4.41	4.40	4.41	4.61
5	3.62, 4.21	3.62, 4.21	3.62, 4.21	4.67
6				5.14 *d*(7.0)
	Glucose	Glucose		
1	5.30 *d*(7.8)	5.30 *d*(7.8)		
2	4.02	4.00		
3	4.24	4.22		
4	4.23	4.21		
5	4.23	4.22		
6	4.37, 4.29	4.52		
C-28	Glucose	Glucose	Glucose	Glucose
1	6.29 *d*(8.0)	6.29 *d*(8.0)	6.29 *d*(8.0)	6.29 *d*(8.0)
2	4.17	4.16	4.17	4.22
3	3.97	3.96	3.97	4.04
4	4.38	4.35	4.38	4.36
5	4.28	4.27	4.28	4.28
6	4.42, 2H	4.42, 2H	4.42, 2H	4.44, 2H

[a] Chemical shifts were referenced according to the highest field signals of pyridine-d_5 (8.71 ppm for ^1H and 149.9 ppm for ^{13}C).

$C_{42}H_{66}O_{14}Na$, 817.4351). The presence of a peak at m/z 501 $[M+H-162-132-Na]^+$ corresponds most probably to the loss of one glucose and one arabinose molecule from m/z 817. The ion at m/z 656 $[M+H-162]^+$ corresponds to a loss of glucose in the positive-mode ESI-MS. On acidic hydrolysis, the aglycone moiety of compound **3** gave signals in the ^1H and ^{13}C NMR spectra, which corresponded to serjanic acid (Table 1). The signal for C-30 in two cases was at δ 177.24–180.26, confirming the presence of a methyl ester at this position. Acid hydrolysis of **3** afforded serjanic acid, glucose, and arabinose. The sugars were identified by comparison with authentic samples. The NMR data comparison between **3** with **6**, which was reported by Dini et al. (2002), indicated that both ^1H and ^{13}C NMR signals were in good agreement with those of serjanic acid. The sequence of the sugar connected to the C-3 of the aglycone was deduced from the HMBC correlations of anomeric H-1 (δ 4.90) of the arabinose moiety with a carbon signal at δ 87.22 (C-3), indicating the attachment of arabinose at C-3. The presence of a second monodesmosidic moiety at C-28 was deduced by the HMBC interactions of anomeric H-1 of glucose (δ 6.29) with the C-28 carbonyl (δ_C 180.26). Thus, the structure of **3** was shown to be the new compound 3-*O*-α-L-arabinopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester.

Compound **4** was isolated as a white powder. It also gave a positive Liebermann–Burchard test. The molecular formula of **4** was established as $C_{43}H_{66}O_{16}Na$ by positive-mode ESI-MS (m/z 861.5). Its HR-ESI-MS gave a peak at 861.4243 $[M+Na]^+$ (calculated for $C_{43}H_{66}O_{16}Na$, 861.4249). The presence of a peak at m/z 501 $[M+H-176-162-Na]^+$ corresponds most probably to loss of one glucuronic acid and one glucose molecule from m/z 861. The ion at m/z 686 $[M+H-176]^+$ corresponds to a loss of glucuronic acid in the positive-mode ESI-MS. On acid hydrolysis, **4** afforded serjanic acid and sugars that were identified as glucuronic acid and glucose by comparing with authentic samples. In addition, the anomeric ^{13}C signals at δ 107.55 and 95.71 and their corresponding protons at δ 5.14 (^1H, δ, J = 7.0 Hz) and 6.29 (^1H, δ, J = 8.0 Hz), respectively, were strongly indicative of two hexoses. The COSY and HSQCAD spectra allowed identification of the spin systems of the two monosaccharide residues (Table 2). The linkage sites of the monosaccharide moieties were first inferred from the hydrolysis

experiment. This was indicative of C-28 esterification of the glucose moiety and the C-3 linkage of glucuronic acid. Confirmation of this came from the HMBC spectrum by which $^3J_{CH}$ correlations were observed between C-1 (δ 107.55) and H-3 (δ 3.40) of glucuronic acid and between C-28 (δ 177.24) and H-1 (δ 6.29) of glucose. The relative stereochemistry of both monosaccharides was determined as β-D-glucuronopyranose and β-D-glucopyranose on the basis of the characteristic $J_{1,2}$ coupling constants (Woldemichael and Wink, 2001). Thus the structure of **4** proved to be the new compound 3-*O*-β-D-glucuronopyranosyl serjanic acid 28-*O*-β-D-glucopyranosyl ester.

Cytotoxic activities of isolated compounds **1**–**4** and their aglycones: 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**I**), 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**II**), and serjanic acid (**III**) were evaluated in HeLa cells employing the MTT assay (Sladowski et al., 1993). Cytotoxicity of the isolated compounds, expressed as IC_{50} is summarized in Table 3.

The cytotoxic effects of saponins **1** and **2** were very similar (IC_{50} > 100 μg/ml). These compounds have same molecular weight and both bear the 1 → 3 linkage of sugars at C-3 and one glucose unit at C-28; the aglycones of saponins **1** and **2** are different however, being 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**I**) and 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**II**), both having the same IC_{50} of 25.4 μg/ml. It appears that the CHO groups at C-23 or C-27 are correlated with the obtained increased cytotoxicity. They can form Schiff's bases with amino groups of protons and DNA bases. A similar result was observed by Woldemichael and Wink (2001) in a study of the structure–activity relationships of saponins and their prosapogenins. Hederagenin, with an IC_{50} of 15–23 μg/ml has shown to be more potent than oleanolic acid with an IC_{50} of 62–99 μg/ml, suggesting that the substitution of a hydroxymethylene group at C-23 enhances the activity of the oleanolic acid. Indeed, a number of monodesmosidic saponins of plant origin have shown potent cytotoxic activity, such as 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranosyl hederagenin (IC_{50} 6.3–8.7 μg/ml), no activity has been observed when a sugar is attached at C-28, as in the case of β-D-Glu*p*-(1 → 6)-β-D-Glu*p* (Hung et al., 2005). Moreover, the presence of a free carboxylic acid at C-28 seems to be essential for cytotoxicity (Bang et al., 2005). However, cytotoxic effects of aglycones **I** and **II**, both with an IC_{50} of 25.4 μg/ml, were closely comparable to that of hederagenin (**VI**).

The cytotoxic effects of bidesmosidic saponins **3** and **4** were very similar (IC_{50} > 100 μg/ml). These compounds have the same sapogenin (**III**; serjanic acid, IC_{50} 50 μg/ml), but a different sugar unit at C-3. Compound **3** contains a sugar at C-3, arabinose, but **4** has glucuronic acid in this position. Both compounds have glucose linked to C-28 and the same aglycone. Oleanolic acid has been shown to be non-cytotoxic (IC_{50} > 50 μg/ml) in HL-60 cells (Mimaki et al., 1999). This indicates that the substitution of the 30-*O*-methyl of spergulagenic acid does not increase cytotoxicity of the aglycone, as compared with oleanolic acid (**IV**).

Table 3
Cytotoxicity of saponins **1**–**4** and their sapogenins **I**–**III** in HeLa cells as compared to apoptosis induce in Caco-2 cells

Compound	IC_{50} (μg/ml)	Sub-G_1 population (%)
1	>100	13.18
2	>100	13.18
3	>100	25.50
4	>100	26.40
I	25.4	51.40
II	25.4	51.40
III	50.5	50.23

Serial compound dilutions (0.195–100 μg/ml) were incubated with HeLa cells for 24 h and cell viability was evaluated by MTT reduction to the blue-colored formazan in living cells. The compounds activity was compared with the untreated samples (control). All values were means of triplicates.

To elucidate whether cell growth inhibitory effects of bidesmosidic saponins and their aglycones were associated with apoptosis, we performed DNA analysis for hypoploid sub-G_1 peaks in cells treated with 100 μg/ml bidesmosidic saponins **1**–**4** and their aglycones **I**–**III** for 24 h. Apoptosis levels induced by saponins **1**–**4** were 13.18%, 13.18%, 25.50%, and 26.40%, respectively, and those induced by their aglycones **I**–**III** were 51.40%, 51.40%, and 50.23%, respectively. Observed apoptotic effects on Caco-2 cells were in agreement with the cytotoxicity assay (Table 3). The bidesmosidic saponins **1**–**4** have an $IC_{50} \sim 100$ μg/ml or 100 μM and showed weak apoptosis at ~13–26%; while the aglycones **I**–**III** with a lower $IC_{50} \sim 25$–50 μg/ml showed higher apoptosis at ~50%. Our compounds exhibited relative high $IC_{50} \sim 100$ μM, which were comparable to that reported for silybin (Varghese et al., 2005). However, silybin even with relatively high *in vitro* IC_{50} has been shown to be effective in inhibiting tumor formation *in vivo* (Varghese et al., 2005). On the other hand, some natural products such as an alkaloid emetine can be highly cytotoxic exhibited IC_{50}'s of 0.17 and 0.52 μM in jurkat T-cells and healthy human PBMC, respectively (Möller and Wink, 2007). Bidesmosidic saponins can not interfere with biomembrane and are therefore usually nontoxic, except if they have further functional groups. Monodesmosidic saponins can dive into biomembrane with their lipophilics side chain and complex cholesterol, whereas their hydrophilic sugar chain binds to extracellular glycoproteins and glycolipids. Aglycones are not active on biomembrane, but can be active towards other targets, e.g. proteins if they have reactive functional groups such as aldehyde function. Our results indicate significant differences in structures–activity relationship of the isolated bidesmosidic saponins depending on nature and position of functional group within structure of the aglycones.

3. Conclusion

An interesting feature of the saponins isolated from this plant is the presence of the aldehyde group at the C-23 and C-27 of the corresponding aglycones of saponins **1** and **2**, respectively. These sapogenins were reported for the first time in aerial parts of *Climacoptera obtusifolia* (Chenopodiaceae) (Yeskaliyeva et al., 2006) and of *Fagonia cretica* (Zygophyllaceae) (Khalik et al., 2000). It is interesting to note that the reported serjanic acid also occurs in Phytolaccaceae (Caryophyllales) (Balsevich et al., 2006) and Sapindaceae (Sapindales) (Larhsini et al., 2003). Moreover, two new saponins with aglycones, 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**I**) and 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**II**), both having an aldehyde function in the β-amyrin skeleton, have been found in the families Caryophyllaceae (Caryophyllales) and Zygophyllaceae (Sapindales), (Yeskaliyeva et al., 2006; Khalik et al., 2000). This metabolite might represent an intermediate in *Chenopodium* saponin oxidative biosynthetic steps leading from a methyl group to the corresponding aldehyde function. The following aglycone moieties identified in *C. quinoa* are representative of oxidative products at C-23 from alcohol to aldehyde and C-27 from methyl to aldehyde: 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**I**), and 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**II**, Fig. 1). Phytolaccagenic acid (**V**) may originate from serjanic acid (**III**) by subsequent oxidative enzymatic steps involving the formation of the corresponding alcohol substituted at C-23. Additionally, hederagenin (**VI**) might originate from oleanolic acid (**IV**) by a selective oxidative demethylation at C-23 and, in turn, hederagenin can be a substrate for the production of 3β,23,30-trihydroxy olean-12-en-28-oic acid (**VII**), following a mechanism involving a stereochemically specific enzyme able to insert one hydroxyl group into the 30β position of the triterpene skeleton. Although these mechanisms must be demonstrated, the suggested hypothesis can give

an idea of the biosynthesis of these substances. Most of the identified saponins from *C. quinoa* have arabinose, glucose, galactose, and glucuronic acid as the first sugar at C-3 and, when present, glucose at C-28. This suggests a high enzymatic selectivity for the sugar position independent of the sapogenins involved.

4. Experimental

4.1. General experimental procedures

^{1}H and ^{13}C NMR were measured on a Varian 500 MHz AC NMR spectrometer at the operating frequencies of 500.13 and 125.67 MHz, respectively. The saponins were examined as solutions in pyridine-d_5 in 5-mm tubes at 25 °C. Two-dimensional (2D) NMR experiments (H,H DQF-COSY; H,H TOSY; H,C HSQC; H,C HSQCAD; H,C HMBC) were carried out on all compounds using the pulse sequences from the Varian user library. On the basis of 2D-NMR analyses, assignments of ^{1}H and ^{13}C signals were obtained. High performance liquid chromatography was carried out on a Knauer Eurochrom 2000 HPLC system with a Lichrospher 100, RP-18 (Merck), column of 250 × 4 mm or 250 × 8 mm i.d. Peaks were detected at 206 nm with a linear gradient of 20–64% CH_3CN in 0.1% trifluoroacetic acid in water at flow rate 1 ml/min during 30 min (Woldemichael and Wink, 2001). The LC-ESI-MS system consisted of an HPLC from Latek Laborgeräte GmbH (Heidelberg, Germany) coupled with a Micromass VG Quattro II mass spectrometer from Waters (Manchester, United Kingdom). ESI-MS was operated under Waters MassLynx 4.0 Software. Mass spectrometric detection of sodium adducts of triterpene glycosides was performed in the positive ion mode over the range of 400–1400 m/z and chromatograms were processed using Waters MassLynx 4.0 software. HR-ESI was measured on a Bruker ApexQe FT-ICR-Instrument (Bruker Daltonik GmbH, Bremen). Column chromatography was performed on silica gel (Kieselgel 70–230 mesh and 230–400 mesh, Merck), and thin layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} (0.25 mm, Merck). Spray reagents were for the saponins: Liebermann–Burchard reagent and 10% H_2SO_4 in methanol reagent and heated at 120 °C in an oven for 3 min; for the sugars: aniline phthalate reagent and heated at 100 °C in an oven for 5 min. For cytotoxicity using a Microplate Reader (Tecan Ultra, Tecan Deutschland GmbH, Germany). All reagents used were of analytical grades.

4.2. Plant material

Powdered seed coats, powdered seeds, and powdered flowers of *C. quinoa* were obtained from Avelup (Temuco, Chile). Entire fruits of *C. quinoa* were obtained from three-month-old plants grown in greenhouses at the Botanical Garden, University of Heidelberg, Germany. Plants were harvested in mid-June 2005 after the beginning of the fruiting season and transported to the laboratory. The fruits were ground and air-dried, finely powdered and then used for extraction. All samples were stored at −20 °C until analysis.

4.3. Extraction and isolation

A total of 300 g of powdered seeds were defatted at room temperature with petroleum ether and extracted with MeOH by exhaustive maceration (3 × 2.5 l) to yield 32 g of residue which was successively dissolved in water and partitioned with EtOAc and *n*-BuOH to yield 2.5 g and 14 g of concentrate, respectively. Also, 300 g of powdered seed coat, and 200 g of powdered flowers were extracted with MeOH by exhaustive maceration (3 × 2.5 l) to yield 36 g, and 12 g of each residue, respectively. The methanolic extracts of seed coats (36 g) and flowers (12 g) were dissolved in H_2O, respectively. The H_2O extracts of each different plant part were partitioned between EtOAc (to yield 3 g and 2 g) and *n*-BuOH (to yield 16 g and 4 g), respectively.

A batch of 140 g of powdered fruits was extracted with MeOH by exhaustive maceration (3 × 2.5 l) to yield 9 g of each residue. The methanolic extracts of fruits were dissolved in H_2O. The H_2O extract was partitioned between EtOAc and *n*-BuOH to yield EtOAc 0.5 g and *n*-BuOH 4 g. Ten grams of *n*-BuOH of seed coats, ten grams of *n*-BuOH of seeds, four grams of *n*-BuOH of flowers, and four grams of *n*-BuOH of fruits were dissolved in *n*-BuOH saturated with water and submitted to a column chromatography (column 400 × 35 mm) with 40–60 μm of silica gel, Merck. Ten milliliter fractions were collected and checked by TLC on silica gel (Merck), developed with $CHCl_3$–EtOH–H_2O (4:2:0.4 v/v/v), respectively. Chromatograms were sprayed with Liebermann–Burchard reagent and heated to 120 °C in an oven for 3 min. Approximately, 400 fractions at 10 ml each, were collected for each plant part by fraction collector eluted with $CHCl_3$–MeOH–H_2O (7.5:2.3:0.2 → 4:5:1 v/v/v) used as solvent system. Fractions showing similar profiles were combined. Column chromatography gave 12 fractions for each plant part, each fraction containing two to four major saponins. Single saponins were separated from each fraction by silica gel Lichrospher RP-18 eluted with acetonitrile–water (20–64%). In this way 20 (**1**–**20**) pure compounds were isolated. Individual saponins were separated from the fractions by means of reversed-phase chromatography on a 400 × 25 mm, 40–63 μm, Lichrospher 100, RP-18 column (Merck), eluted with aqueous CH_3CN. Fraction I eluted with 20% CH_3CN afforded compound **1** (8 mg) and compound **2** (10 mg); further elution with 25% CH_3CN gave compound **3** (8 mg) and **4** (6 mg). Fraction II eluted with 25% CH_3CN yielded compound **5** (5 mg) and **6** (12 mg). After elution with 30% CH_3CN, compound **7** (15 mg) and compound **8** (2 mg) were obtained. Fraction III eluted with 30% CH_3CN gave compound **9** (63 mg) and compound **10** (5 mg); 30% CH_3CN allowed compound **11** (9 mg) to be obtained. Fraction IV eluted with 25% CH_3CN gave compound **12** (6 mg), and elution with 30% CH_3CN gave compound **13** (8 mg), compound **14** (12 mg), compound **15** (10 mg), compound **16** (7 mg), compound **17** (3 mg), compound **18** (4 mg), compound **19** (4 mg), and then compound **20** (12 mg).

4.4. Basic hydrolysis of saponins *1*–*4*

Each individual pure compound (2 mg) was heated at reflux with 10 ml of 5% KOH solution in water for 2 h. The cooled reaction mixture was adjusted to pH 6 by 1 N HCl and then extracted with EtOAc (3 × 10 ml). The saponins were submitted to acid hydrolysis with 2 N HCl in 50% aqueous methanol for 2 h. The aglycones in the EtOAc layer were evaporated to dryness, 1.46 mg of each pure aglycone was obtained (73% yield), dissolved in pyridine-d_5 in a 3-mm tube, and analyzed by ^{1}H NMR and ^{13}C NMR. The aqueous layer was evaporated to dryness and analyzed by TLC on silica gel in the solvent $CHCl_3$–MeOH–H_2O (70:40:5 v/v/v). Glucuronic acid, R_f 0.15, and arabinose, R_f 0.40, were identified by comparison with an authentic samples.

4.5. Acid hydrolysis of saponins *1*–*4*

The individual pure compounds (2 mg) were hydrolyzed with 10 ml of 2 N HCl in 50% aqueous methanol under reflux for 2 h at 100 °C, and thereafter sugars and aglycones were separated and the aqueous solution was extracted with EtOAc (3 × 10 ml). The aglycones in the EtOAc layer were evaporated to dryness, 1.5 mg of each pure aglycone was obtained (75% yield), dissolved in pyridine-d_5 in a 3-mm tube, and analyzed by ^{1}H NMR and ^{13}C NMR. The aqueous layer was evaporated to dryness and analyzed by TLC. The presence of glucuronic acid, glucose and arabinose was established by comparison with authentic samples. The TLC on sil-

ica gel in the solvent system $CHCl_3$–MeOH–H_2O (70:40:5 v/v/v) resulted in the R_f 0.15, 0.26, and 0.40 respectively.

To obtain a quantity of the aglycone of saponin **1,2** and **3**, two grams of the *n*-BuOH fraction of seed coats was hydrolyzed with 400 ml of the acidic solution under reflux for 6 h at 100 °C, and thereafter sugars and aglycones were separated and the aqueous solution was extracted with EtOAc three times (3 × 100 ml). The aglycones in the EtOAc layer were evaporated to dryness (1420 mg), applied to silica gel column chromatography with $CHCl_3$–MeOH (9:1 v/v) as a solvent system to give 3β-hydroxy-23-oxo-olean-12-en-28-oic acid (**I**; 8 mg) and 3β-hydroxy-27-oxo-olean-12-en-28-oic acid (**II**; 15 mg). The mixtures of phytolaccagenic acid and serjanic acid (fractions 17–49; 240 mg) was rechromatographed on silica gel with $CHCl_3$–MeOH (9:1 v/v) and the residue was recrystallized from CH_2Cl_2–*n*-hexane giving serjanic acid (**III**; 20 mg) and phytolaccagenic acid (**V**; 45 mg). All of the aglycones were characterized by comparison of their ^1H and ^{13}C NMR spectral with published data (Bandara et al., 1990). The substances were used as standard compounds for biological activity tests.

4.6. Cell culture and treatment protocols

The experiment was performed according to the method reported by Thongphasuk et al. (2008). Briefly, the human colon adenocarcinoma cell line Caco-2 cells were cultured in DMEM containing 10% FCS, 1% P/S, 1% L-glutamine, and 1% nonessential amino acids in a 5%-CO_2 incubator at 37 °C. For cytotoxicity studies, cells were seeded at 50×10^3 cells per well in 24-well plates. The medium was replaced with serum-free medium containing various concentrations of selected saponins or 0.1% DMSO control. After 24 h of treatment, cells were harvested.

4.7. DNA analysis of apoptosis by flow cytometry

The harvested cells were washed with PBS and then pelleted by centrifugation. Cells were fixed with cold 80% ethanol and kept at 4 °C overnight. After washing with PBS and centrifugation, cells were treated with 100 μg/ml RNase A and incubated at 37 °C for 30 min. Following addition of propidium iodide (PI, 50 μg/ml), DNA contents were analyzed by a BD FACSCalibur™ with CellQuest software (Becton Dickinson) for acquisition and data analysis. Cells gated as % sub-G_1 represent cell populations exhibiting apoptosis.

4.8. Cytotoxic activity

Cytotoxic effects were measured *in vitro* on HeLa (cervix adenocarcinoma) cell line, using MTT assay (Sladowski et al., 1993) with some modification. The cytotoxicity tests were carried out in 96-well plates, using 1×10^4 cells per well in all cases, which were allowed to adhere to the cell culture plate for 48 h before treatment. The supernatant was removed and 100 μl of serially diluted compounds (0.195–100 mg/ml), 100 μl [Dulbecco's Modified Eagle's Medium (DMEM) 500 ml, penicillin–streptomycin 5 ml, NEA 10% 5 ml, FBS 5 ml] were added to each well. After 24 h of incubation, the culture medium was carefully removed, and 20 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well. The plates were incubated at 37 °C for 4 h. The medium was then removed and the resulting formazan crystals were dissolved with 100 μL DMSO. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. The plate was mixed on a microshaker for 10 min and then the samples was measured at 570 nm using Microplate Reader (Tecan). For each compound, the percent viability was plotted against concentration and the IC_{50} (concentration required to reduce viability by 50%) value was calculated as the relative to the untreated controls.

4.9. Statistical analysis

Statistical analysis was performed by Student's *t* test. *P*-values less than 0.05 was considered to be statistically significant. The IC_{50} values were calculated using Excel based program.

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