Food Chemistry 125 (2011) 447–455

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Spirostane and cholestane glycosides from the bulbs of *Allium nigrum* L

Aymen Jabrane [a,b], Hichem Ben Jannet [c], Tomofumi Miyamoto [d], Jean-François Mirjolet [e], Olivier Duchamp [e], Féthia Harzallah-Skhiri [b], Marie-Aleth Lacaille-Dubois [a,*]

[a] Laboratoire de Pharmacognosie, Unité de Molécules d'Intérêt Biologique, UMIB UPRES-EA 3660, Faculté de Pharmacie, Université de Bourgogne, F-21079 Dijon Cedex, France
[b] Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, 5000, Monastir, Tunisia
[c] Laboratoire de Chimie des Substances Naturelles et de Synthèse Organique, Faculté des Sciences de Monastir, Université de Monastir, 5000 Monastir, Tunisia
[d] Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan
[e] Oncodesign, 20 Rue Jean Mazen, BP 27627, 21076 Dijon Cedex, France

ARTICLE INFO

Article history:
Received 26 February 2010
Received in revised form 23 July 2010
Accepted 7 September 2010

Keywords:
Allium nigrum
Liliaceae
Cholestane
Steroidal saponins
Nigrosides
NMR

ABSTRACT

A phytochemical investigation of the fresh bulbs of *Allium nigrum* L. led to the isolation of new spirostane-type glycosides as two inseparable isomer mixtures, nigrosides A1/A2 (**1a**/**1b**) and nigrosides B1/B2 (**2a**/**2b**), two new cholestane-type glycosides, nigrosides C and D (**3** and **4**), together with the known compounds, 25(*R*,*S*)-5α-spirostan-2α,3β,6β-trio1-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**5a/5b**) and 25(*R*,*S*)-5α-spirostan-2α,3β,6β-trio1 3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[4-*O*-(3*S*)-3-hydroxy-3-methylglutaryl-β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**6a/6b**), isolated from this plant for the first time. All structures were elucidated mainly by spectroscopic analysis (1D and 2D NMR experiments, FABMS, HRESIMS) and by comparison with literature data. Cytotoxicity of the isolated compounds was assessed against human colon carcinoma (HT-29 and HCT 116) cell lines. Compounds **5a**/**5b** and **6a**/**6b** were found to be the most active with IC_{50} values 1.09 and 2.82 μM against HT-29 and 1.59 and 3.45 μM against HCT 116, respectively.

1. Introduction

Allium is a large, and the most important, representative genus of the Liliaceae family, containing about 500 species, widely distributed in the northern hemisphere (Block, 1985). Since early times, many *Allium* species, such as garlic (*A. sativum* L.), onion (*Allium cepa* L.), chives (*A. schoenoprasum* L.), leeks (*A. porrum* L.) and shallots (*A. ascalonicum* Hort.) have been used as foods, spices, and herbal remedies (Fattorusso, Lanzotti, Taglialatela-Scafati, Di Rosa, & Ianaro, 2000). Previous phytochemical investigations of *Allium sp.* showed that these plants are a rich source of steroidal saponins, as well as sulphur-containing compounds (Hostettmann & Marston, 1995; Iciek, Kwiecien, & Wlodek, 2009; Lanzotti, 2005). The steroidal saponins, an important class of natural products, have been reported to possess various important biological effects, such as haemolytic (Wang et al., 2007), antifungal (Barile et al., 2007; Sautour, Miyamoto, & Lacaille-Dubois, 2007), antidiabetic (Yoshikawa et al., 2007), platelet aggregation inhibitory (Zhang et al., 1999), anti-inflammatory (Shao et al., 2007) and immunomodulatory activities (Lacaille-Dubois, 2005; Zhang et al., 2007).

Recently, steroidal glycosides have attracted serious attention within the scientific community owing to the significant cytotoxic activity against a leukaemia cell line (Yokosuka, Jitsuno, Yui, Yamazaki, & Mimaki, 2009), breast cancer, HeLa cervical cells (Kaskiw et al., 2009) and human colon cancer cell lines (Acharya et al., 2010; Wang et al., 2004).

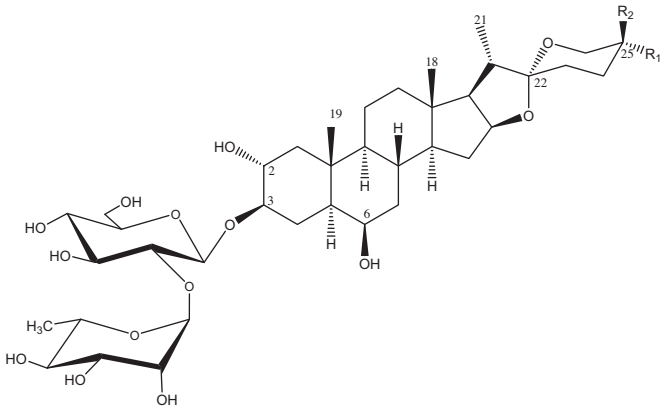
Allium nigrum L. (black garlic, black onion, broad leaved garlic), synonym of *A. bauerianum* Baker., *A. multibulbosum* Jacq., *A. magicum* L., *A. monspessulanum* Gouan., *A. odorum* Ten., is an ornamental plant of the Liliaceae family, first described by Linnaeus in 1762, the name comes from the greenish-black lobed ovaries in the centre of each flower. Some are blacker than others. This species has a large bulb (3–4 cm) with an ovoid-spherical shape and an entire smooth white tunic. It differs from other *Allium* species by having relatively broad leaves (Burnie, 1995). Ethnobotanical reference in Tunisia has indicated that this species was not used in popular medicine (Le Floc'H, 1983) but it was reported to be used as a food spice in Sicily (Lentini & Venza, 2007).

No previous phytochemical or pharmacological study has been reported on this plant. As a part of our continuous search for bioactive saponins from the Liliaceae family (Acharya et al., 2010), we now examine the bulbs of *A. nigrum* (L.). In this paper we describe the isolation and structural elucidation of new steroid saponins, among them two pairs of inseparable epimer mixtures,

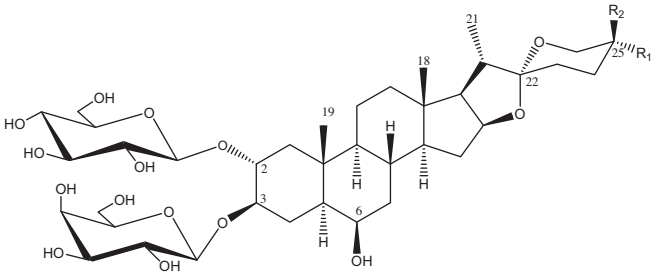
* Corresponding author. Tel.: +33 3 80 39 32 29; fax: +33 3 80 39 33 00.
E-mail address: m-a.lacaille-dubois@u-bourgogne.fr (M.-A. Lacaille-Dubois).

0308-8146/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2010.09.028

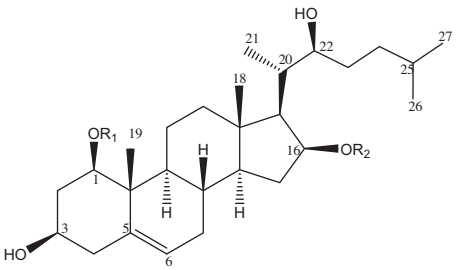
nigrosides A1/A2 (**1a**/**1b**), B1/B2 (**2a**/**2b**) and two cholestane-type saponins, nigrosides C (**3**) and D (**4**), together with the epimers of known compounds (**5a**/**5b**) and (**6a**/**6b**) (Fig. 1). Their structures were determined mainly by spectroscopic methods, including 1D and 2D NMR experiments, FABMS and HRESIMS, and compared with literature data for the known compounds. In addition, the cytotoxicity of all compounds was evaluated against the human colon cancer (HCT 116 and HT-29) cell lines.



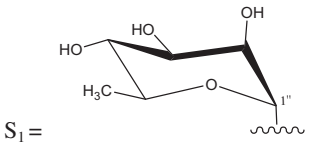
1a R_1= CH_3 R_2 = H
1b R_1=H R_2 = CH_3



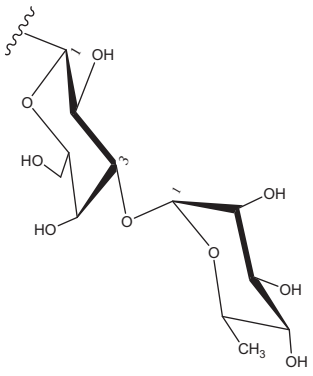
2a R_1= CH_3 R_2 = H
2b R_1=H R_2 = CH_3



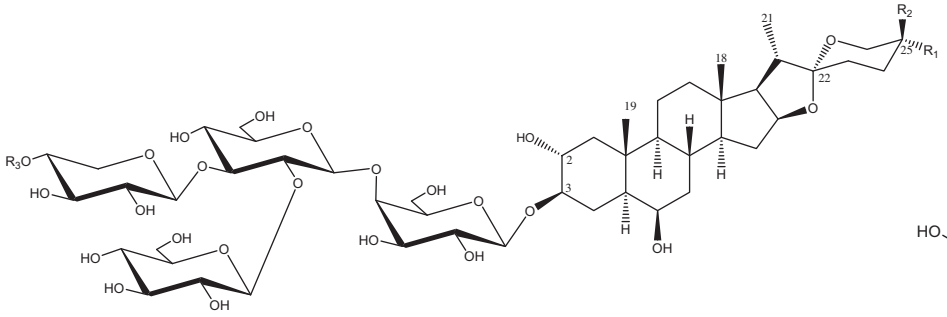
3 R_1= S_1 R_2 = S_2
4 R_1 = OH R_2 = S_2

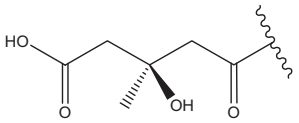


S_1 =



S_2 =





(*S*)-HMG: (*S*)-3hydroxy-3-methylglutaric acid

5a	R_1 = CH_3	R_2 = H	R_3 = H
5b	R_1 = H	R_2 = CH_3	R_3 = H
6a	R_1 = CH_3	R_2 = H	R_3 = (*S*)-HMG
6b	R_1 = H	R_2 = CH_3	R_3 = (*S*)-HMG

Fig. 1. Structures of coupounds **1a/1b, 2a/2b, 3, 4, 5a/5b** and **6a/6b.**

2. Materials and methods

2.1. General experimental procedures

Optical rotations were recorded on an AA-OR automatic polarimeter. The 1D and 2D NMR spectra (^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, TOCSY, NOESY, HSQC and HMBC) were performed using a UNITY-600 spectrometer at the operating frequency of 600 MHz on a Varian INOVA 600 instrument equipped with a SUN 4 L-X computer system (600 MHz for ^{1}H and 150 MHz for ^{13}C spectra). Conventional pulse sequences were used for COSY, HSQC, and HMBC spectra. TOCSY spectra were acquired using the standard MLEV17 spin-locking sequence and a 90 ms mixing time. The mixing time in the NOESY experiment was set to 500 ms. The carbon type (CH_3, CH_2, CH) was determined by DEPT experiments. Chemical shifts are reported as δ values (ppm), referenced with respect to the residual solvent signal of C_5D_5N, and coupling constants (J) were measured in Hz. The samples were solubilised in pyridine-d_5. FABMS (negative-ion mode, glycerol matrix) was conducted on a JEOL-SX-102 mass spectrometer. HRESIMS was carried out on a Q-TOF 1 micromass spectrometer. Compound isolations were carried out using a medium-pressure liquid chromatography (MPLC) system [Gilson M 305 pump, 25 SC head pump, M 805 manometric module, Büchi glass column (460 × 25 mm and 460 × 15 mm), Büchi precolumn (110 × 15 mm)] on silica gel 60 (Merck, 15–40 μm), and reversed-phase RP-18 silica gel (Silicycle 75–200 μm), and with a Gilson Pump Manager C-605, having two pumps (2 × Büchi pump modul C-601) and one fraction collector (Büchi C-660). Vacuum-liquid chromatography (VLC) was performed on a reversed-phase RP-18 silica gel (Silicycle, 75–200 μm) (12 × 6 cm). Thin-layer chromatography (TLC, Silicycle) and high-performance thin-layer chromatography (HPTLC, Merck) were performed on precoated silica gel plates 60 F_{254}. The spray reagent for saponins was the Komarowsky reagent, which is a mixture (5:1) of *p*-hydroxybenzaldehyde (2% in MeOH) and ethanolic H_2SO_4 (50%).

2.2. Plant material

Bulbs of *A. nigrum* L. were collected in the region of Béja (Tunisia), in April, 2008 and identified by Professor Féthia HARZALLAH-SKHIRI, in the Institut Supérieur de Biotechnologie de Monastir, Tunisia. A voucher specimen (*An* 30.4.08) was deposited at the Laboratory of Plant Biology in the above Institute.

2.3. Extraction and isolation of compounds

The fresh bulbs of *A. nigrum* (2.5 kg) were refluxed twice with MeOH–H_2O (7:3, 3 l) for 1 h and evaporated *in vacuo* yielding an aqueous extract that was partitioned successively with $CHCl_3$ (3 l) and H_2O-satd *n*-BuOH (2 l), yielding, after evaporation of the solvents, the corresponding $CHCl_3$ (2.4 g) and *n*-BuOH (18 g) fractions. A 5.5 g aliquot of the *n*-BuOH residue was submitted to VLC on RP-18 silica gel using H_2O (500 ml), MeOH–H_2O mixtures (5:5, 2 × 500 ml), and finally MeOH (2 × 500 ml) as eluents. After evaporation of the solvents, five fractions were obtained: AN I–V. AN-II (1.35 g) was submitted to MPLC [silica gel (15–40 μm), system a: $CHCl_3$-MeOH–H_2O (7/3/1) lower phase)] to give eight fractions (Fr1–Fr8). Fr3 (120 mg), rechromatographed under the same conditions by MPLC (system a), afforded compound **5a**/**5b** (14 mg, 0.0025% w/w of the fresh bulbs). Fr7 was concentrated to dryness, to give compound **6a**/**6b** (29 mg, 0.0052% w/w of the fresh bulbs). AN-IV (3 g) was submitted to VLC using silica gel 60 (15–40 μm), eluted with a gradient of $CHCl_3$–MeOH (9/1–2/8), to give four sub-fractions, sF1–sF4. An aliquot of 250 mg of sF3 (1.24 g) was rechromatographed successively by MPLC on RP-18 silica gel MeOH–H_2O (7/3), using a Gilson Pump Manager, affording the pure compounds **3** (11 mg, 0.0019% w/w of the fresh bulbs) and **4** (13 mg, 0.0023% w/w of the fresh bulbs). Fraction sF4 was submitted to successive chromatographies (MPLC on silica gel, system a) to give compounds **1a**/**1b** (12 mg, 0.0021% w/w of the fresh bulbs) and **2a**/**2b** (15 mg, 0.0027% w/w of the fresh bulbs).

Table 1
^{1}H NMR spectroscopic data of the aglycone moieties of **1a**/**1b** and **2a**/**2b**. (Pyridine-d_5, δ in ppm, J in Hz) [a,b].

Pos.	**1a**/**1b** δ_H		**2a**/**2b** δ_H	
1	1.19$^{\alpha}$ (1H, t, 11.9), 2.12$^{\beta}$ (1H, m)		1.13, 2.35 (1H, m)	
2	4.08 (1H, m)		4.31 (1H, m)	
3	3.98 (1H, m)		4.25 (1H, m)	
4	2.08 (1H, m), 2.36 (1H, q, 12.8)		2.22, 2.28 (1H, q, 11.9)	
5	1.06		1.01	
6	3.94		3.90 (1H, m)	
7	1.06, 1.96		1.03, 1.95	
8	2.04		2.00 (1H, m)	
9	0.62		0.56 (1H, m)	
11	1.31, 1.45		1.28, 1.48	
12	0.99, 1.58		0.92, 1.55	
14	1.03		1.00	
15	1.38 (1H, m), 1.98		1.38 (1H, m), 1.95	
16	4.45		4.44 (1H, m)	
17	1.74 (1H, m)		1.73 (1H, m)	
18	0.73 (3H, s)		0.70 (3H, s)	
19	1.25 (3H, s)		1.15 (3H, s)	
20	1.85 (1H, dq, 6.6, 6.9)	1.80 (1H, m)	1.84 (1H, m)	1.81 (1H, m)
21	1.06 (3H, d, 6.9)	1.06 (3H, d, 6.9)	1.06 (3H, d, 6.9)	1.06 (3H, d, 6.9)
23	1.63, 1.52	1.32, 1.79	1.64, 1.58	1.35 (1H, m), 1.80
24	1.45, 1.48	1.27, 2.04	1.46, 1.48	1.28 (1H, m), 2.03 (1H, m)
25	1.48	1.53	1.49	1.52
26	3.42 (1H, t, 10.5)	3.33 (1H, d, 10.5)	3.39 (1H, t, 10.5)	3.33 (1H, d, 10.5)
	3.53 (1H, br d, 9.2)	3.97 (1H, m)	3.53 (1H, br d, 9.2)	3.97 (1H, m)
27	0.62 (3H, d, 4.3)	0.98 (3H, d, 6.9)	0.62 (3H, d, 4.3)	0.98 (3H, d, 6.9)

[a] Overlapped signals are reported without designated multiplicity.
[b] $^{\alpha}$axial proton, $^{\beta}$equatorial proton.

Table 2
^{13}C NMR spectroscopic data of the aglycone moieties of **1a**/**1b** and **2a**/**2b**. (Pyridine-d_5, δ in ppm).

Pos.	Dept	**1a**/**1b** δ_C		**2a**/**2b** δ_C	
1	(CH_2)	46.7		44.5	
2	(CH)	70.2		76.4	
3	(CH)	84.5		78.4	
4	(CH_2)	30.8		31.4	
5	(CH)	47.1		46.9	
6	(CH)	69.7		69.4	
7	(CH_2)	39.7		39.6	
8	(CH)	29.5		29.4	
9	(CH)	54.1		53.9	
10	(C)	36.6		36.5	
11	(CH_2)	21.0		20.9	
12	(CH_2)	39.9		39.8	
13	(C)	40.5		40.4	
14	(CH)	55.8		55.7	
15	(CH)	31.4		31.6	
16	(CH_2)	80.9		80.9	
17	(CH)	62.3		62.2	
18	(CH_3)	16.2		16.1	
19	(CH_3)	16.7		16.3	
20	(CH)	41.6	42.1	41.6	42.0
21	(CH_3)	14.6	14.4	14.5	14.3
22	(C)	109.2	109.6	109.1	109.6
23	(CH_2)	31.4	25.9	31.3	25.8
24	(CH_2)	28.7	25.7	28.7	25.6
25	(CH)	30.2	27.0	30.0	27.0
26	(CH_2)	66.5	65.0	66.5	65.1
27	(CH_3)	16.9	15.9	16.8	15.8

2.4. Characterisation of isolated compounds

Nigrosides A1/A2 (compound **1a**/**1b**): white amorphous powder: $[\alpha]_D^{21}$: −39.5 (MeOH, *c* 0.50). Negative FABMS: *m/z* 755 $[M–H]^-$, HRESIMS (positive-ion mode): *m/z* 779.4202 $[M + Na]^+$ (calcd. for 779.4194). ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) data: see Tables 1, 2 and 4.

Nigrosides B1/B2 (compound **2a**/**2b**): white amorphous powder: $[\alpha]_D^{21}$: −28.5 (MeOH, *c* 0.30). Negative FABMS: *m/z* 771 $[M–H]^-$, HRESIMS (positive-ion mode): *m/z* 795.4147 $[M + Na]^+$ (calcd. for 795.4143). ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) data: see Tables 1, 2 and 4.

Nigroside C (compound **3**): yellow amorphous powder: $[\alpha]_D^{21}$: −60.6 (MeOH, *c* 0.20). Negative FABMS: *m/z* 887 $[M–H]^-$, HRESIMS (positive-ion mode): *m/z* 911.4986 $[M + Na]^+$ (calcd. for 911.4980). ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) data: see Tables 3 and 4.

Nigroside D (compound **4**): yellow amorphous powder: $[\alpha]_D^{21}$: −58.2 (MeOH, *c* 0.18). Negative FABMS: *m/z* 741 $[M–H]^-$, HRESIMS (positive-ion mode): *m/z* 765.4409 $[M + Na]^+$ (calcd. for 765.4401). ^{1}H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) data: see Tables 3 and 4.

Compounds **5a**/**5b**: white amorphous powder: $[\alpha]_D^{21}$: −59.8 (MeOH, *c* 0.50). Negative FABMS: *m/z* 1065 $[M–H]^-$, *m/z* 933 $[(M–H)–132]^-$, 771 $[(M–H)–132–162]^-$, 609 $[(M–H)–132–162–162]^-$, HRESIMS (positive-ion mode): *m/z* 1089.5203 $[M + Na]^+$ (calcd. for 1089.5198). ^{1}H NMR and ^{13}C NMR data of aglycone moiety appeared similar to those obtained for **1a/1b**: ^{1}H NMR (pyridine-d_5, 600 MHz) δ_H 2.08 (1H, m, Hα-4), δ_H 2.28 (1H, q, 12.4, Hβ-4), δ_H 0.73 (3H, s, Me-18), δ_H 1.14 (3H, s, Me-19), δ_H 1.06 (3H, d, 6.9, Me-21), δ_H 1.48 (1H, m, H-25) of (25*R*)-isomer, δ_H 1.52 (1H, m, H-25) of (25*S*)-isomer, δ_H 3.40 (1H, t, 11.0, Hα-26) and δ_H 3.52 (1H, m, Hβ-26) of (25*R*)-isomer, δ_H 3.31 (1H, d, 11.0, Hα-26) and δ_H 3.96 (1H, m, Hβ-26) of (25*S*)-isomer, δ_H 0.62 (3H, d, 4.3, Me-27) of (25*R*)-isomer and δ_H 0.98 (3H, d, 7.1, Me-27) of (25S)-isomer. ^{13}C NMR (pyridine-d_5, 600 MHz) δ_C 46.5 (C-1), δ_C 70.2 (C-2), δ_C 84.0 (C-3), δ_C 31.8 (C-4), δ_C 47.3 (C-5), δ_C 69.6 (C-6), δ_C 39.7 (C-7), δ_C 29.6 (C-8), δ_C 54.0 (C-9), δ_C 36.6 (C-10), δ_C 21.0 (C-11), δ_C 40.5 (C-12), δ_C 40.0 (C-13), δ_C 55.8 (C-14), δ_C 31.2 (C-15), δ_C 81.0 (C-16), δ_C 62.3 (C-17), δ_C 16.2 (C-18), δ_C 16.7 (C-19), δ_C 42.1

Table 3
^{1}H and ^{13}C NMR spectroscopic data of the aglycone moieties of **3** and **4**. (Pyridine-d_5, δ in ppm, *J* in Hz)[a,b].

Pos.	Dept	**3**		**4**	
		δ_C	δ_H	δ_C	δ_H
1	(CH)	81.4	3.60 (1H, br d, 11)	77.7	3.65 (1H, dd, 11.7, 4.0)
2	(CH_2)	35.2	1.80^{α}, 2.68^{β} (1H, br d, 11)	42.1	2.20, 2.56
3	(CH)	67.6	3.75 (1H, m)	67.7	3.83 (1H, m)
4	(CH_2)	42.8	2.50, 2.58 (1H, t, 12.3)	43.0	2.53, 2.58
5	(C)	138.6		139.6	
6	(CH)	125.1	5.42 (1H, br d, 5.0)	124.3	5.44 (1H, br d, 5.0)
7	(CH_2)	31.1	1.32, 173	31.6	1.35, 1.78
8	(CH)	33.1	1.24	32.6	1.28
9	(CH)	50.4	1.18	50.8	1.19
10	(C)	42.6		43.0	
11	(CH_2)	24.4	1.51, 2.47	23.8	1.60, 2.61
12	(CH_2)	40.1	1.42, 2.02 (1H, br d, 6.7)	40.1	1.20, 1.91 (1H, m)
13	(C)	41.8		41.7	
14	(CH)	54.8	0.81	54.8	0.80
15	(CH_2)	36.1	1.69, 2.22 (1H, m)	36.8	1.67, 2.25 (1H, m)
16	(CH)	82.0	4.50	82.0	4.51
17	(CH)	58.0	1.91 (1H, t, 8.8)	57.6	1.87 (1H, br d, 10.9)
18	(CH_3)	13.4	0.91 (3H, s)	13.2	0.92 (3H, s)
19	(CH_3)	14.1	1.07 (3H, s)	13.5	1.21 (3H, s)
20	(CH)	35.3	2.42	35.4	2.40
21	(CH_3)	12.1	1.08 (1H, d, 6.9)	12.1	1.08 (1H, d, 6.9)
22	(CH)	73.0	4.22	73.5	4.19
23	(CH_2)	33.2	1.64, 1.75	33.1	1.63, 1.75
24	(CH_2)	36.7	1.55, 1.78	36.1	1.54, 1.78
25	(CH)	28.3	1.50	28.4	1.49
26	(CH_3)	22.6	0.79 (3H, d, 6.2)	22.6	0.80 (3H, d, 6.2)
27	(CH_3)	22.7	0.81 (3H, d, 6.4)	22.7	0.83 (3H, d, 6.4)

[a] Overlapped signals are reported without designated multiplicity.
[b] $^{\alpha}$axial proton, $^{\beta}$equatorial proton.

Table 4
^{1}H and ^{13}C NMR spectroscopic data of the sugar moieties of **1a**/**1b**, **2a**/**2b**, **3** and **4**. (Pyridine-d_5, δ in ppm, J in Hz)[a].

	1a/1b		**2a/2b**		**3**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
		Glc		*Glc*				
1	100.3	4.90 (1H, d, 7.8)	101.3	4.99 (1H, d, 7.6)				
2	78.3	4.06 (1H, t, 8.1)	73.8	4.50 (1H, t, 9.0)				
3	78.6	4.15 (1H, t, 9.0)	77.2	4.07				
4	71.4	3.89 (1H, t, 9.5)	70.6	4.36				
5	77.7	3.91 (1H, m)	77.9	3.98 (1H, m)				
6a	62.1	4.47 (1H, dd, 12.3, 3.4)	61.9	4.34				
6b		4.10		4.19 (1H, dd, 10.9, 5.1)				
		Rha				*Rha I*		*Rha*
1	101.9	6.11 (1H, br s)			103.2	5.86 (1H, br s)	103.5	5.86 (1H, br s)
2	71.7	4.70 (1H, br s)			71.8	4.63 (1H, br s)	71.5	4.63 (1H, br s)
3	71.9	4.51 (1H, dd, 3.0, 9.0)			71.9	4.52 (1H, dd, 3.1, 9.5)	71.9	4.52 (1H, dd, 3.1, 9.5)
4	73.5	4.21 (1H, t, 9.5)			72.9	4.21	72.8	4.21
5	69.1	4.81 (1H, dq, 6.4, 6.1)			69.4	4.51	69.5	4.51
6	18.1	1.63 (3H, d, 5.9)			18.1	1.50 (3H, d, 6.1)	18.1	1.50 (3H, d, 6.1)
				Gal		*Gal*		*Gal*
1			101.8	4.97 (1H, d, 7.6)	106.5	4.62 (1H, d, 6.7)	106.7	4.62 (1H, d, 6.7)
2			71.1	4.03 (1H, t, 8.1)	71.5	4.38 (1H, t, 8.6)	71.5	4.38 (1H, t, 8.6)
3			74.2	4.16 (1H, dd, 10.2, 5.9)	81.5	4.06 (1H, dd, 9.7, 2.6)	81.5	4.06 (1H, dd, 9.5, 2.6)
4			69.4	4.05	69.0	4.60	69.1	4.61
5			76.5	3.89 (1H, m)	75.9	3.88 (1H, m)	76.0	3.88 (1H, m)
6a			61.7	4.37 (1H, br d, 10.0)	61.3	4.33 (1 h, br d, 9.8)	61.2	4.35 (1H, br d, 9.8)
6b				4.14		4.24		4.25
						Rha II		
1					97.4	5.48 (1H, br s)		
2					72.2	4.41 (1H, br s)		
3					72.3	4.35 (1H, t, 6.6)		
4					73.5	4.18		
5					70.2	4.10		
6					18.20	1.55 (3H, d, 5.9)		

[a] Overlapped signals are reported without designated multiplicity.

(C-20), δ_C 14.1 (C-21), δ_C 109.6 (C-22), δ_C 26.0 (C-23), δ_C 25.7 (C-24), [δ_C 28.8 (C-25), δ_C 66.6 (C-26), δ_C 16.8 (C-27) of (25*R*)-isomer], [δ_C 27.0 (C-25), δ_C 64.8 (C-26), δ_C 15.9 (C-27) of (25*S*)-isomer], ^{1}H NMR and ^{13}C NMR data of sugar moieties are shown in Table 5.

Compounds **6a**/**6b**: white amorphous powder: $[\alpha]_D^{21}$: −61.6 (MeOH, *c* 0.50). Negative FABMS: *m/z* 1209 $[M–H]^-$, *m/z* 1065 $[(M–H)–144]^-$, 933 $[(M–H)–144–132]^-$, 771 $[(M–H)–144–132–162]^-$, 609 $[(M–H)–144–132–162–162]^-$. HRESIMS (positive-ion mode): *m/z* 1233.5621 $[M + Na]^+$ (calcd. for 1233.5617). ^{1}H NMR and ^{13}C NMR data of aglycone moiety appeared similar to those obtained for **5a/5b.** ^{1}H NMR and ^{13}C NMR data of sugars moieties and acyl group are shown in Table 5.

2.5. Acid hydrolysis

A solution of each saponin (2 mg) in H_2O (2 ml) was treated with 2 N aq. CF_3COOH (5 ml) and refluxed for 3 h at 95 °C. After extraction with CH_2Cl_2 (3 × 5 ml), the aq. layer was repeatedly concentrated with MeOH until neutral, and then analysed by TLC (SiO_2, $CHCl_3$/MeOH/H_2O 8:5:1) with authentic samples. Furthermore, a silylated derivative of the sugars was prepared according to the previously described procedure (Haddad, Miyamoto, Laurens, & Lacaille-Dubois, 2003) and analysed by GC. The following sugars were characterised: D-glucose, L-rhamnose for (**1a**/**1b**), D-galactose, D-glucose for (**2a**/**2b**), L-rhamnose, D-galactose for (**3** and **4**), D-glucose, D-galactose and D-xylose for (**5a**/**5b**) and (**6a**/**6b**).

2.6. MTT cytotoxicity assay

The bioassay was carried out according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method (Carmichael, Degraff, Gazdar, Minna, & Mitchell, 1987) with two human colon cancer cells (HT-29 and HCT 116). Isolated compounds and Paclitaxel (positive control) were dissolved in DMSO to make stock solutions, which were diluted to a working solution before use (the final DMSO concentration was 0.1% v/v). HT-29 and HCT-116 cells were seeded at an initial density of 5000 or 10,000 cells/well in 96-well plates and treated with medium having various concentrations of the test compounds. DMSO controls (0.1%) did not affect cell proliferation. After 96 h, 20 μl of MTT solution (5 mg/ml in PBS) were added to the culture medium and the reaction mixture was incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. The MTT solution was aspired and 200 μl of DMSO were added. The optical density (OD) was measured spectrophotometrically at 570 nm. The results were expressed as concentrations of compound producing 50% toxicity (IC_{50} value). The experiments were repeated twice.

3. Results and discussion

3.1. Phytochemical investigation

The *n*-BuOH fraction from the MeOH:H_2O (7:3) extract of the *A. nigrum* bulbs was submitted to several chromatographic steps (VLC and MPLC on normal and reversed RP-18 silica gel), yielding new saponins, two of them as inseparable mixtures named nigrosides A1/A2 (**1a**/**1b**), B1/B2 (**2a**/**2b**), and the pure compounds nigrosides C (**3**) and D (**4**), together with the known saponins, also as inseparable mixtures (**5a**/**5b**) and (**6a**/**6b**). The structures of the new saponins were elucidated by 600 MHz NMR analyses, including 1D and 2D NMR experiments, and compared with literature data for the known compounds. These latter were identified as 25(*R,S*)-5α-spirostan-2α,3β,6β-trio1-3-*O*-β-D-glucopyranosyl-(1 → 2)-O- [β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (aginoside/turoside A) (**5a**/**5b**) and 25

Table 5
^{1}H and ^{13}C NMR spectroscopic data of the sugar moieties of **5a**/**5b** and **6a**/**6b** (Pyridine-d_5, δ in ppm, J in Hz)[a].

	5a/5b		**6a/6b**	
	δ_C	δ_H	δ_C	δ_H
		Gal		*Gal*
1	102.4	4.89 (1H, d, 7.9)	102.4	4.89 (1H, d, 7.9)
2	72.0	4.44	72.0	4.44
3	75.0	4.08	75.0	4.07
4	79.1	4.48	79.1	4.45
5	75.3	4.01	75.3	4.01
6a	60.6	4.20	60.6	4.20
6b		4.44		4.44
		Glc I		*Glc I*
1	103.8	5.06 (1H, d, 7.9)	103.8	5.06 (1H, d, 7.9)
2	80.4	4.18	80.4	4.18
3	86.7	3.98	86.7	3.97
4	69.6	3.65	69.6	3.65
5	76.9	3.70	76.9	3.69
6a	62.1	3.94	62.1	3.93
6b		4.35		4.35
		Xyl		*Xyl*
1	104.3	5.10 (1H, d ,7.9)	104.3	5.15 (1H, d,7.9)
2	74.8	3.85	74.8	3.85
3	77.7	4.01	77.7	4.01
4	70.8	3.97	72.9	4.00
5	66.6	3.56	66.6	3.56
		4.12		4.12
		Glc II		*Glc II*
1	103.8	5.50 (1H, d, 7.6)	103.8	5.50 (1H, d, 7/6)
2	75.4	3.94	75.4	3.94
3	77.4	4.11	77.4	4.11
4	71.0	4.00	71.0	4.00
5	77.7	3.85	77.7	3.85
6a	62.1	4.30	62.1	4.30
6b		4.40		4.40
			Acyl	
1			171.2	–
2			48.3	2.68, 2.91
3			70.2	–
4			48.7	2.74, 3.02
5			173.4	–
6			28.4	1.48

[a] Overlapped signals are reported without designated multiplicity.

(*R*,*S*)-5α-spirostan-2α,3β,6β-trio1–3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[4-*O*-(3*S*)-3hydroxy-3-methylglutaroyl-β-D-xylopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (**6a**/**6b**), previously isolated in *Allium* ssp (Kawashima, Mimaki, & Sashida, 1991; Kawashima, Mimaki, & Sashida, 1993), but reported for the first time in *A. nigrum*.

After acid hydrolysis of each compound, the sugar moieties were characterised by TLC with authentic samples as: rhamnose and glucose (in the case of **1a**/**1b**), galactose and glucose (in the case of **2a**/**2b**), rhamnose and galactose (in the case of **3**, **4**), and galactose, glucose and xylose for **5a**/**5b**, **6a**/**6b**. The absolute configuration was determined by GC of the chiral derivatives of the hydrolysate of each compound (see expr part) as D for glucose, xylose and galactose and L for rhamnose. In the ^{1}H NMR spectra of the compounds, the relatively large $^3J_{H\text{-}1,H\text{-}2}$ values of the glucose, xylose, and galactose (between 7.0 and 8.0 Hz) moieties indicated a β anomeric proton. The broad singlet of the anomeric proton of the rhamnose unit indicated an α-orientation (Avunduk et al., 2008).

Saponins **1a**/**1b** and **2a**/**2b** were isolated as amorphous white powders. The ^{1}H and ^{13}C NMR data obtained from 1D and 2D spectroscopic experiments revealed that **1a**/**1b** and **2a**/**2b** were spirostanol derivatives, existing as mixtures of C-25 *R* and *S* epimers. As far as we know, the isolation of a steroidal mixture of C-25 *R* and *S* epimers is sometimes difficult (Zheng, Zhang, Li, & Yang, 2004). Thus, their structures were elucidated as C-25 epimeric mixtures.

Compound **1a**/**1b**, in high-resolution electrospray ionisation mass spectroscopy (HR-ESIMS) (positive-ion mode), exhibited a pseudo-molecular ion peak at m/z = 779.4202 $[M + Na]^+$, consistent with a molecular formula $C_{39}H_{64}O_{14}$. Its fast-atom bombardment mass spectrum (FABMS) (negative-ion mode) displayed a pseudo-molecular ion peak at m/z 755 $[M–H]^-$, indicating a molecular weight of 756. A fragment-ion peak was observed at m/z 609 ($[M–H–146]^-$), corresponding to the loss of one deoxyhexosyl unit. The ^{1}H and ^{13}C NMR spectra, in combination with DEPT and HSQC spectra of **1a/1b,** displayed the presence of two angular methyl proton signals at δ_H 0.73 (3H, s), 1.25 (3H, s), three secondary methyl proton signals at δ_H 1.06 (3H, d, J = 7.0 Hz), 0.62 (3H, d, J = 4.3 Hz, (*R*)-form), 0.9 8 (3H, d, J = 7 Hz, (*S*)-form) and characteristic acetal carbon signal at δ_C 109.6 (*S*)-form and at δ_C 109.2 (*R*)-form, indicating the presence of a steroidal spirostanol skeleton (Agrawal, Jain, & Pathak, 1995). Analysis of NMR data showed ^{13}C NMR signals at δ_C 47.1 (C-5), 54.1 (C-9), and 16.7 (C-19), characteristic of A/B *trans*-ring fusion, indicating that **1a**/**1b** is a 5α-steroidal spirostanol derivative. This was confirmed by the NOESY correlations observed between δ_H 3.98 (Agly H-3) and 1.06 (Agly H-5). The HMBC correlations between δ_H 4.08 (Agly H-3), and δ_C 70.2 (Agly C-2) and between δ_H 1.06 (Agly H-5) and δ_C 69.7 (Agly C-6), indicated a secondary alcoholic function at positions C-2 and C-6, respectively. The 2α orientation of the hydroxyl unit at (C-2) was proved by the NOESY cross-peak between δ_H 1.25 (s, ax H_3-19) and δ_H 4.08 (m, H-2), proving the axial orientation of this proton, while the NOESY cross-peak between δ_H 1.06 (s, H-5) and δ_H 3.94 (m, H-6) indicated the 6β orientation of the hydroxyl at (C-6). The above aglycone was identified as 25(*R*,*S*)-5α-spirostan-2α,3β,6β-triol (agigenin/neoagigenin) by comparison of ^{13}C and ^{1}H NMR chemical shifts of **1a**/**1b** obtained from the 2D NMR data (Tables 1 and 2) with those reported in the literature (Carotenuto et al., 1997; Sata et al., 1998). The 25 (*R*,*S*) stereochemistry of the Me-27 group was deduced from the resonance of protons and carbons from the ring F portion (C-22–C-27) (Tables 1 and 2) (Agrawal, 2003). The NOESY correlations between H_3-19/H-2, H_3-19/H-8, H_3-18/H-8, H_3-18/H-20, H-5/H-9, H-9/H-14, H-14/H-17 and H-16/H-17 confirmed the conventional A/B *trans*, B/C *trans*, C/D *trans*, D/E *cis*, and C-20α stereochemistry of the aglycone of **1a**/**1b**.

The ^{1}H NMR spectrum showed the presence of two anomeric proton signals at δ_H 4.90 (1H, d, J = 7.2 Hz) and 6.11 (1H, br *s*), giving correlation of the HSQC spectrum with two anomeric carbons at δ_C 100.3 and 101.9, respectively. The evaluation of chemical shifts and spin–spin couplings allowed the identification of one β-glucopyranosyl unit (Glc) and one α-rhamnopyranosyl unit (Rha). The absolute configuration of Glc was determined as D and that of Rha as L, as described above. COSY and TOCSY experiments allowed the sequential assignments of all of the proton resonances to the individual monosaccharides, as shown in (Table 4). The sequence of the disaccharide chain of **1a**/**1b** was determined by HMBC and NOESY experiments. The correlations observed in the HMBC spectrum between the ^{1}H NMR signals at δ_H 4.90 (d, J = 7.7 Hz, Glc H-1) and the ^{13}C NMR signal at δ_C 84.5 (Agly C-3), and in the NOESY spectrum between the signal at δ_H 4.90 (Glc H-1) and 3.98 (Agly H-3), suggested that the glucose was linked at C-3 of the aglycone. The correlation in the HMBC spectrum, between the ^{1}H NMR signal at δ_H 6.11 (Rha H-1) and the ^{13}C NMR signal at δ_C 78.3 (Glc C-2), revealed a (1 → 2) linkage between these two sugars, which was confirmed by the cross-peak in the NOESY spectrum between δ_H 6.11 (Rha H-1) and δ_H 4.06 (Glc H-2), On the basis of the above data, compound **1a**/**1b** was elucidated as 25 (*R*,*S*)-5α-spirostan-2α,3β,6β-triol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside, called nigrosides A1/A2.

Compound **2a**/**2b**, in high-resolution electrospray ionisation mass spectroscopy (HR-ESIMS) (positive-ion mode), exhibited a pseudo-molecular ion peak at *m/z* = 795.4147 $[M + Na]^+$, consistent with a molecular formula $C_{39}H_{64}O_{15}$. Its fast-atom bombardment mass spectrum (FABMS) (negative-ion mode) showed a pseudo-molecular ion peak at *m/z* 771 $[M–H]^-$, indicating a molecular weight of 772. Significant fragment-ion peaks were observed at *m/z* 609 ($[M–H–162]^-$), suggesting the elimination of a one hexosyl moiety. A detailed comparison of the ^1H, ^{13}C NMR chemical shift (Tables 1 and 2), and the 2D NMR spectral analysis of the aglycone part of **1a**/**1b** and **2a**/**2b,** revealed that all signals were superimposable, except those of the carbon signals C-2 and C-3 in **2a**/**2b**. The downfield shift of C-2 by 6.2 ppm at δ_C 76.4 and the upfield shift of C-3 by 6.1 ppm at δ_C 78.4 indicated that the aglycone of **2a**/**2b** (agigenin/neoagigenin) was substituted at those two positions (Mimaki, Kuroda, Fukasawa, & Sashida, 1999). Full assignment of the ^1H and ^{13}C NMR signals of the aglycone part of **2a**/**2b** showed glycosylation shifts for C-2 and C-3, suggesting a bidesmosidic spirostane-type saponin. The ^1H NMR spectrum showed the presence of two anomeric proton signals at δ_H 4.97 (1H, d, *J* = 7.6 Hz) and 4.99 (1H, d, *J* = 7.6 Hz), giving correlations in the HSQC spectrum with two anomeric carbon signals at δ_C 101.8 and 101.3, respectively. The complete assignment of the glycosidic NMR signals was achieved by analysis of COSY, TOCSY, NOESY, HSQC, and HMBC experiments (Table 4). Evaluation of the spin–spin couplings and chemical shift from the 2D NMR spectra of **2a**/**2b** allowed the identification of one β-glucopyranosyl (Glc) and one β-galactopyranosyl (Gal) unit. The site of attachment of the sugar was determined by combination of the HMBC and NOESY experiments. In the HMBC spectrum, the anomeric proton signals at δ_H 4.99 (Glc H-1) and 4.97 (Gal H-1) showed correlation with the carbon resonances at δ_C 76.3 (C-2) and 78.4 (C-3), respectively. On the other hand, the NOESY cross-peaks, δ_H 4.99 (Glc H-1)/δ_H 4.31 (Agly H-2), δ_H 4.97 (Gal H-1)/δ_H 4.25 (Agly H-3), δ_H 4.99 (Glc H-1)/δ_H 1.13 (Agly H-1α) and δ_H 4.97 (Gal H-1)/δ_H 2.22 (Agly H-4β) confirmed Glc and Gal to be located at C-2 and C-3, respectively. Thus, compound **2a**/**2b** was determined to be 25(*R,S*)-5α-spirostan-2α,3β,6β-trio1-2-*O*-[β-D-glucopyranosyl]-3-*O*-β-D-galactopyranoside, called nigrosides B1/B2.

Compound **3**, was obtained as an amorphous yellow powder, and showed, in high-resolution electrospray ionisation mass spectroscopy (HR-ESIMS) (positive-ion mode), a pseudo-molecular ion peak at *m/z* = 911.4986 $[M + Na]^+$, consistent with the molecular formula $C_{45}H_{76}O_{17}$. The fast-atom bombardment mass spectrum (FABMS) (negative-ion mode) exhibited a quasi-molecular ion peak at *m/z* 887 $[M–H]^-$ indicating a molecular weight of 888. A fragment-ion peak was observed at *m/z* 741 ($[M–H–146]^-$), corresponding to the elimination of one desoxyhexosyl moiety. The ^1H NMR spectrum of **3** showed signals of the aglycone part and an oligosaccharidic part. The ^1H and ^{13}C NMR spectral data for the aglycone moiety of **3** (Table 3) showed five methyl groups at δ_H 0.91 (3H, s), δ_H 1.07 (3H, s), δ_H 1.08 (3H, d, *J* = 6.9), δ_H 0.79 (3H, d, *J* = 6.2 Hz), δ_H 0.81 (3H, d, *J* = 6.4 Hz) and an olefinic proton at δ_H 5.42 (1H, br d, *J* = 5.0), which gave correlations, in the HSQC spectrum, with five methyl carbons at δ_C 13.4, 14.1, 12.1, 22.6, 22.7 and one olefinic carbon signal at δ_C 125.1, respectively. Four secondary alcoholic carbons at δ_C 82.0 (C-16), 81.4 (C-1), 73.0 (C-22), 67.6 (C-3), and three quaternary carbon signals at δ_C 43.0 (C-10), 41.7 (C-13), 139.6 (C-5), were also observed. These signals suggested that the aglycone of **3** was of a $\Delta^{5,6}$-cholestane type (Inoue et al., 1995; Mimaki, Kawashima, Kanmoto, & Sashida, 1993;). The aglycone stereochemistry of **3** was proposed by analysis of the NOESY data. Comparison of NMR data of **3** (Table 3) with literature values allowed the identification of the aglycone as the previously reported (22S)-cholest-5-ene-1β, 3β, 16β, 22-tetrol, the aglycone of schubertoside D, isolated from some *Allium sp* (Mimaki et al., 1993).

The ^1H NMR spectrum of **3** displayed three anomeric proton signals at δ_H 5.86 (1H, br s), 5.48 (1H, br s) and 4.62 (1H, d, *J* = 6.7), which gave correlation with three anomeric carbon signals at δ_C 103.2, 97.4 and 106.5, respectively, in the HSQC spectrum. Complete assignments of each glycosidic proton system were achieved by 2D NMR spectroscopic experiments allowing the identification of two rhamnopyranosyl units (Rha I and Rha II) and one β-galactopyranosyl (Gal) unit (Table 4).

The site of attachment of sugar moieties on the aglycone was determined by HMBC and NOESY experiments. The cross-peak in the HMBC spectrum between δ_H 4.62 (Gal I H-1) and δ_C 82.0 (Agly C-16), between δ_H 5.48 (Rha II H-1) and δ_C 81.4 (Agly C-1), together the cross-peak observed in NOESY spectrum between the proton signal at δ_H 4.62 (Gal H-1) and δ_H 4.50 (Agly H-16) and between the proton signal at δ_H 5.48 (Rha II H-1) and δ_H 3.60 (Agly H-1), confirmed this glycosylation at positions C-1 and C-16 by Rha II and Gal, respectively. The deshielded value at δ_C 81.5 (Gal C-3) suggested the point of linkage of Rha I. The correlation in the HMBC spectrum between the signals at δ_H 5.86 (Rha I H-1) and δ_C 81.5 (Gal C-3) and a NOESY cross-peak between a signal at δ_H 5.86 (Rha I H-1) and δ_H 4.06 (Gal H-3), revealed a (1 → 3) glycosidic linkage between Rha I and Gal. Therefore, the structure of **3** was determined as (22*S*)-cholest-5-ene-1β, 3β, 16β, 22-tetraol 1-*O*-[α-L-rhamnopyranosyl] 16-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranoside, named nigroside C.

Compound **4**, was obtained as an amorphous yellow powder; in high-resolution electrospray ionisation mass spectroscopy (HR-ESIMS) (positive-ion mode), it showed a pseudo-molecular ion peak at *m/z* = 765.4409 $[M + Na]^+$, consistent with the molecular formula $C_{39}H_{66}O_{13}$. The negative-ion mode FABMS spectrum showed an $[M–H]^-$ ion at *m/z* 741, corresponding to a molecular weight of 742. Another fragment-ion peak was observed at *m/z* 595 ($[M–H–146]^-$), suggesting the elimination of a terminal desoxyhexosyl unit. The NMR data of this compound revealed that its aglycone differs from that of compound **3** in the NMR signals of ring A (Table 3). The main difference was the observation of an upfield ^{13}C NMR chemical shift at δ_C 77.7 (C-1), showing a secondary alcoholic function at this position and the downfield ^{13}C NMR chemical shift of C-2 at δ_C 42.1, instead of 35.2 as in compound **3**. The molecular weight of **4** was 146 mass units smaller than that of **3**, indicating that **4** had two sugar units. This was ascertained by observation of two anomeric proton signals at δ_H 4.62 (1H, d, *J* = 6.7) and 5.86 (1H, br s), giving correlation in the HSQC spectrum with two anomeric carbons at δ_C 106.7 and 103.5, respectively. The identification of one α-rhamnopyranosyl (Rha) and one β-galactopyranosyl (Gal) unit was achieved as described above (Table 4).

In the HMBC spectrum, the cross-peaks between δ_H 4.62 (Gal H-1) and δ_C 82.0 (Agly C-16) and between δ_H 5.86 (Rha H-1) and δ_C 81.5 (Gal C-3) provided the sequence of the oligosaccharide chain at C-16 to be Rha (1 → 3) Gal. This sequence was confirmed by cross-peaks in the NOESY spectrum between δ_H 4.62 (Gal H-1) and δ_H 4.51 (Agly H-16), and between δ_H 5.86 (Rha H-1) and δ_H 4.06 (Gal H-3). Accordingly, the structure of **4** was determined as (*22S*)-cholest-5-ene-1β,3β,16β,22-tetraol 16-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-galactopyranoside, called nigroside D.

3.2. Cytotoxic activities

The cytotoxicity of the isolated compounds was evaluated against the HT-29 and HCT 116 human colon cancer cell lines in comparison with Paclitaxel, using the MTT assay (Carmichael et al., 1987). According to the results (Table 6), the most active compounds were the spirostanol glycosides **5a**/**5b** (aginoside/turoside A) and **6a**/**6b** having four sugar units with IC_{50} values of 1.59 and 3.45 μM, respectively against HCT 116 and 1.09 and 2.82 μM,

Table 6
IC_{50} (μM) values of isolated saponins towards HCT-116 and HT-29 cell lines.

Compounds	Cell lines	
	HCT-116	HT-29
1a/1b	47.8	70.8
2a/2b	>100	>100
3	>100	>100
4	>100	>100
5a/5b	1.59	1.09
6a/6b	3.45	2.82
Paclitaxel	0.00321	0.00140

respectively, against HT 29. Compounds **2a**/**2b**, **3** and **4** were considered inactive on both cell lines with $IC_{50} > 100$ μM and compound **1a/1b** showed moderate cytotoxicity with IC_{50} 47.8–70.5 μM on both cell lines. However, the above mentioned activity is three times less active than Paclitaxel (IC_{50} 0.00321 and 0.00140 μM against HCT 116 and HT-29, respectively). These results are in good agreement with reported literature data. Namely, the cytotoxicity of aginoside (**5a**) was previously reported in the literature against P388 (murine leukaemia), A549 (lung cancer), DLD-1(colon cancer) and WS1 (normal skin fibroblast) human cell lines with IC_{50} values of 1.9, 5.8, 7.9, and 3.6 μM, respectively (Mskhiladze et al., 2008; Sata et al., 1998). The same compound exhibited considerable activity at a concentration of 50 $\mu g ml^{-1}$, against a Hela cell line (Inoue et al., 1995). The remaining compounds (**1–4** and **6a**/**6b**) were tested for the first time against HCT 116 and HT–29 cell lines and their cytotoxic activity against other cells has not been previously mentioned. Since few compounds have been isolated and tested in this study, it is difficult to derive structure–activity relationships. However, these results suggest that special consideration should be given to the steroid saponins **5a/5b** and **6a/6b** in biological and pharmacological studies of garlic and its preparations.

4. Conclusions

The phytochemical study of *A. nigrum* L. has resulted in the isolation and characterisation of new saponins **1a**/**1b**, **2a**/**2b**, **3** and **4**, together with the two known ones **5a**/**5b** and **6a**/**6b**. Their structures were elucidated by chemical and spectroscopic means, including NMR and HRESIMS. To the best of our knowledge, this is the first phytochemical analysis of this plant. The cytotoxicities of all isolated compounds were evaluated with Paclitaxel as positive control against HT-29 and HCT 116 human colon cancer cells, for the first time, by using the MTT assay. Among them, compounds **5a**/**5b** and **6a**/**6b** were found to be most active on both cell lines. The information presented here could be used as preliminary data. Moreover, further biological experiments are needed in order to examine the therapeutic potential of *A. nigrum*.

Acknowledgement

The authors are thankful to the IFC-Tunisie (Institut Français de Coopération en *Tunisie)* for a scholarship to A.J.

References

Acharya, D., Mitaine-Offer, A. C., Kaushik, N., Miyamoto, T., Paululat, T., Mirjolet, J. F., et al. (2010). Steroidal saponins from *Chlorophytum orchidastrum*. *Journal of Natural Products, 73*(1), 7–11.

Agrawal, P. K. (2003). 25*R*/25*S* stereochemistry of spirostane-type steroidal sapogenins and steroidal saponins via chemical shift of geminal protons of ring-F. *Magnetic Resonance in Chemistry, 41*, 965–968.

Agrawal, P. K., Jain, D. C., & Pathak, A. K. (1995). NMR spectral investigation. Part 37. NMR spectroscopy of steroidal sapogenins and steroidal saponins: an update. *Magnetic Resonance in Chemistry, 33*(12), 923–953.

Avunduk, S., Mitaine-Offer, A. C., Alankus-Caliskan, O., Miyamoto, T., Senol, S. G., & Lacaille-Dubois, M. A. (2008). Triterpene glycosides from the roots of *Astragalus flavescens*. *Journal of Natural Products, 71*(1), 141–145.

Barile, E., Bonanomi, G., Antignani, V., Zolfaghari, B., Sajjadi, S. E., Scala, F., et al. (2007). Saponins from *Allium minutiflorum* with antifungal activity. *Phytochemistry, 68*(5), 596–603.

Block, E. (1985). The chemistry of garlic and onions. *Science American, 252*, 114–119.

Burnie, D. (1995). *Wild flowers of the Mediterranean*. London: Dorling Kindersley Book. p. 270.

Carmichael, J., Degraff, W. G., Gazdar, A. F., Minna, J. D., & Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of radiosensitivity. *Cancer research, 47*(4), 943–946.

Carotenuto, A., Fattorusso, E., Lanzotti, V., Magno, S., De Feo, V., Carnuccio, R., et al. (1997). Porrigenins A and B, novel cytotoxic and antiproliferative sapogenins isolated from *Allium porrum*. *Journal of Natural Products, 60*(10), 1003–1007.

Fattorusso, E., Lanzotti, V., Taglialatela-Scafati, O., Di Rosa, M., & Ianaro, A. (2000). Cytotoxic saponins from bulbs of *Allium porrum* L. *Journal of Agricultural and Food Chemistry, 48*(8), 3455–3462.

Haddad, M., Miyamoto, T., Laurens, V., & Lacaille-Dubois, M. A. (2003). New biologically active triterpenoidal saponins acylated with salicylic acid from *Albizia adianthifolia*. *Journal of Natural Products, 66*(3), 372–377.

Hostettmann, K., & Marston, A. (1995). *Saponins*. Cambridge: Cambridge University Press.

Iciek, M., Kwiecien, I., & Wlodek, L. (2009). Biological properties of garlic and garlic-derived organosulfur compounds. *Environmental and Molecular Mutagenesis, 50*(3), 247–265.

Inoue, T., Mimaki, Y., Sashida, Y., Nishino, A., Satomi, Y., & Nishino, H. (1995). Steroidal glycosides from allium macleanii and a steroidal glycosides from *Allium macleanii* and *A. senescens*, and their inhibitory activity on tumour promoter-induced phospholipid metabolism of hela cells. *Phytochemistry, 40*(2), 521–525.

Kaskiw, M. J., Tassotto, M. L., Moka, M., Tokar, S. L., Pycko, R., Th'ng, J., et al. (2009). Structural analogues of diosgenyl saponins: Synthesis and anticancer activity. *Bioorganic and Medicinal Chemistry, 17*(22), 7670–7679.

Kawashima, K., Mimaki, Y., & Sashida, Y. (1991). Steroidal saponins from *Allium giganteum* and *A. Aflatunense*. *Phytochemistry, 30*(9), 3063–3067.

Kawashima, K., Mimaki, Y., & Sashida, Y. (1993). Steroidal saponins from the bulbs of *Allium schubertii*. *Phytochemistry, 32*(5), 1267–1272.

Lacaille-Dubois, M. A. (2005). Bioactive saponins with cancer related and immunomodulatory activity: Recent developments. In Atta-Ur-Rahman (Ed.). *Studies in natural products chemistry series* (Vol. 32, pp. 209–246). Amsterdam, The Netherlands: Elsevier Science.

Lanzotti, V. (2005). Bioactive saponins from *Allium* and *Aster* plants. *Phytochemistry Reviews, 4*(2–3), 95–110.

Le Floc'H, E. (1983). Contribution à une étude ethnobotanique de la flore tunisienne. Publications Scientifiques Tunisiennes, programme flore et végétation tunisienne (p.402). Imprimerie Officielle de la République Tunisienne.

Lentini, F., & Venza, F. (2007). Wild food plant of popular use in Sicily. *Journal of Ethnobiology and Ethnomedicine, 3*, 15.

Mimaki, Y., Kawashima, K., Kanmoto, T., & Sashida, Y. (1993). Steroidal glycosides from *Allium albopilosum* and asteroidal glycosides from *Allium albopilosum* and *A. ostrowskianum*. *Phytochemistry, 34*(3), 799–805.

Mimaki, Y., Kuroda, M., Fukasawa, T., & Sashida, Y. (1999). Steroidal saponins from the bulbs of *Allium karataviense*. *Chemical and Pharmaceutical bulletin, 47*(6), 738–743.

Mskhiladze, L., Legault, J., Lavoie, S., Mshvildadze, V., Kuchukhidze, J., Elias, R., et al. (2008). Cytotoxic steroidal saponins from the flowers of *Allium leucanthum*. *Molecules, 13*, 2925–2934.

Sata, N., Matsunaga, S., Fusetani, N., Nishikawa, H., Takamura, S., & Saito, T. (1998). New antifungal and cytotoxic steroidal saponins from the bulbs of an elephant garlic mutant. *Bioscience, Biotechnology and Biochemistry, 62*(10), 1904–1911.

Sautour, M., Miyamoto, T., & Lacaille-Dubois, M. A. (2007). Steroidal saponins from *Asparagus acutifolius*. *Phytochemistry, 68*(20), 2554–2562.

Shao, B., Guo, H., Cui, Y., Ye, M., Han, J., & Guo, D. (2007). Steroidal saponins from *Smilax china* and their anti-inflammatory activities. *Phytochemistry, 68*(5), 623–630.

Wang, S. L., Bing, C., Cui, C. B., Liu, H. W., Wu, C. F., & Yao, X. S. (2004). Diosgenin-3-*O*-α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranoside obtained as a new anticancer agent from *Dioscorea futschauensis* induces apoptosis on human colon carcinoma HCT-15 cells via mitochondria-controlled apoptotic pathway. *Journal of Asian Natural Products Research, 6*(2), 115–125.

Wang, Y., Zhang, Y., Zhu, Z., Zhu, S., Li, Y., Li, M., et al. (2007). Exploration of the correlation between the structure, hemolytic activity, and cytotoxicity of steroid saponins. *Bioorganic and Medicinal Chemistry, 15*(7), 2528–2532.

Yokosuka, A., Jitsuno, M., Yui, S., Yamazaki, M., & Mimaki, Y. (2009). Steroidal glycosides from *Agave utahensis* and their cytotoxic activity. *Journal of Natural Products, 72*(8), 1399–1404.

Yoshikawa, M., Xu, F., Morikawa, T., Pongpiriyadacha, Y., Nakamura, S., Asao, Y., et al. (2007). Medicinal Flowers. XII.(1) New spirostane-type steroid saponins

with antidiabetogenic activity from *Borassus flabellifer*. *Chemical and Pharmaceutical Bulletin, 55*(2), 308–316.
Zhang, X. F., Cui, Y., Huang, J. J., Zhang, Y. Z., Nie, Z., Wang, L. F., et al. (2007). Immuno-stimulating properties of diosgenyl saponins isolated from *Paris polyphylla*. *Bioorganic and Medicinal Chemistry Letters, 17*(9), 2408–2413.
Zhang, J., Meng, Z., Zhang, M., Ma, D., Xu, S., & Kodama, H. (1999). Effect of six steroidal saponins isolated from *Anemarrhenae rhizoma* on platelet aggregation and hemolysis in human blood. *Clinica Chimica Acta, 289*(1–2), 79–88.
Zheng, Q. A., Zhang, Y. J., Li, H. Z., & Yang, C. R. (2004). Steroidal saponins from fresh stem of *Dracaena cochinchinensis*. *Steroids, 69*(2), 111–119.