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Chemical Constituents from *Solanum glabratum* Dunal var. *sepicula*

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Abstract: In the course of screening program of Saudi plants for their potential biological activity, the methanolic extract of *Solanum glabratum* Dunal var. *sepicula* as well as its different fractions were tested for its possible cytoxicity in prostate cancer (PC3) and colon cancer (HT29) cell lines using the MTT assay. In the present study, three spirostan saponins and one flavonoid glycoside were isolated from the active *n*-butanol fraction through a bio-guided fractionation approach. Two new saponin glycosides were identified as 23-*β*-D-glucopyranosyl (23*S*, 25*R*)-spirost-5-en-3, 23 diol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**2**) and (25*R*)-spirost-5-en-3-ol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→3)]-*β*-D-galactopyranoside (**3**). In addition, two known compounds were also isolated and identified as isorhamnetin-3-*O*-*α*-L-rhamnopyranosyl (1→6) *β*-D-glucopyranoside (**1**) and (23*S*, 25*R*)-spirost-5-en-3, 23 diol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**4**). The structures of the isolated compounds were elucidated based on their MS, one dimensional and extensive two dimensional NMR spectral data. Among the isolated metabolites, compound **3** showed the highest cytotoxic activity in both PC3 and HT29 cell lines with an IC_{50} values of 14.8 and 19.5 µg/mL, respectively.

Keywords: *Solanum glabratum;* steroidal saponins; cytotoxicity; PC3; HT29. © 2015 ACG Publications. All rights reserved.

1. Introduction

In the field of antitumor chemotherapy, plants have been useful sources of clinically relevant anti-tumor drugs. Indeed, many drug discovery programs have been launched worldwide in order to identify new anticancer drug leads from higher plants. However, the extensive phytochemical investigation necessary to identify novel anti-tumor agents is usually done for only selected number of plants. Traditionally, plants are selected for thorough phytochemical studies using ethnomedical data approach where the selection of a plant is based on prior knowledge of its folk medicinal use. As for the many health disorders classified as cancer, the symptoms and progress of the disease is widely variable, which makes it difficult for diagnosis and treatment by traditional healers [1]. Therefore, ethnomedical information obtained about the use of a certain plant for the treatment of cancer is often unreliable.

An alternative approach in selecting plant candidates for the discovery of anti-cancer agents is through the random screening of many plants extracts followed by bioactivity guided fractionation of the most promising extracts. One leading example of such approach is the discovery of the important anticancer agent "Taxol" from the bark of the Pacific yew, *Taxus brevifolia* through a major screening program of plants, microorganisms and marine animals for anti-tumor activity funded by United States National Cancer Institute (NCI) [2]. Similar research effort identified camptothecins from the Chinese ornamental plant *Camptotheca acuminata* [3]. One may conclude that many new compounds, having promising anti-tumor activities are yet to be discovered especially from the plant flora of under explored regions.

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Solanum glabratum (Solanaceae) is used traditionally as diuretic and for the treatment sof scabies, syphilis, cough, and hemorrhoids [4]. *S. glabratum* methanolic extract exhibited significant antiproliferative activity when tested by Mothana *et al*. (2009) [4] and Almehdar *et al*., (2012) [5]. Mothana et al. (2009) [4] reported the antipoliferative activity of *S. glabratum* methanolic extract with IC_{50} values of 9.4, 8.6 and 9.0 µg/mL against three human cancer cell lines (lung, urinary bladder and breast cancer, respectively). In addition, Abdel-Monem (2009) [6] isolated eight compounds from the *n*-hexane and chloroform fractions of the alcohol extract of *S. glabratum.* These compounds were identified as five sterols, two flavonoid aglycones, and a coumarin. The *n*-hexane and chloroform fractions as well as the major isolated compounds were tested for their antimicrobial activities.

In the present study, *S. glabratum* methanolic extract was subjected to cytotoxicity bioguided fractionation in an attempt to isolate the principle compounds responsible for the *in vitro* cytotoxic activity and elucidate their structures.

2. Materials and Methods

2.1. General experimental procedures

All NMR spectra were recorded on a Varian VNMRS 600 NMR spectrometer operating at a proton NMR frequency of 599.83 MHz using a 5 mm inverse detection cryoprobe. 2D NMR spectra were recorded using standard CHEMPACK 4.1 pulse sequences (gDQCOSY, gHSQCAD, gHMBCAD, ROESY, TOCSY) implemented in Varian VNMRJ 2.2C spectrometer software. Optical rotation, values were measured on a Perkin Elmer 241 polarimeter at 25 °C. Vacuum liquid chromatography (VLC) was carried out on Merck silica gel 60 (65–400 mesh). Reverse-phase medium-pressure column chromatography (MPLC) was performed on a Merck Lobar Lichroprep RP-18 column, equipped with a fluid pump and a fraction collector. Semi-preparative HPLC was conducted on an Agilent 1100 series II HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with a photodiode array detector and chromatographic separation was accomplished using a reversed phase, C18 column (5 µm, 20 × 300 mm, J.T.Baker, Phillipsburg, NJ). Analytical thin-layer chromatography (TLC) was carried out on Merck TLC plates (250µm thickness, KGF Si gel 60 and KGF RP-18 Si gel 60) and spots were visualized by spraying the dried plates with p-anisaldehyde/H_2SO_4 followed by heating at 110 °C.

2.2. LC-MS analysis

The LC-ESI and ESI-MS^n mass spectra were obtained on a LCQ Deca XP MAX system (ThermoElectron, San Jose, USA) equipped with a ESI source (4.0 kV, sheath gas: nitrogen; capillary temperature: 275 ^oC). The Ion Trap MS system is equipped with a HYPERSIL GOLD RP18-column (5 mm, 150 × 1 mm, ThermoScientific). The MS^n spectra were recorded during the HPLC run with starting collision-induced dissociation energy of 20 eV and an isolation width of ± 2 amu.

2.3. ESI-FTICR-MS analysis

The negative and positive ion high resolution ESI mass spectra were obtained from a Bruker Apex III Fourier transform ion cyclotron resonance (FTICR) (Bruker Daltonics, Billerica, USA) equipped with an Infinity cell, a 7.0 Tesla superconducting magnet (Bruker, Karlsruhe, Germany), an RF-only hexapole ion guide and an APOLLO electrospray ion source (Agilent, off axis spray). All data were acquired with a 512 k data points and zero filled to 2048 k by averaging 32 scans. The XMASS 6.1.2 and the Data Analysis Software (DA 4.0) were used for the calculation of the elemental composition.

2.4. Plant Material

S. glabratum was collected from Al-Madinah-Jeddah Road, Saudi Arabia, in March 2011 and identified by the staff members of the Department of Biology (Botany), College of Science, King Abdulaziz University, Saudi Arabia. A voucher specimen (#SG 1144) has been deposited in the Herbarium of the Faculty of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia.

2.5. Extraction and Isolation.

Dried aerial parts of *S. glabratum* (750 g) were extracted with MeOH (3 x 3 L) at room temperature using ultra turrax homogenizer (IKA, Germany) to give 81 g of green semisolid extract on evaporation. The methanolic extract (70 g) was suspended in water (400 mL), then extracted with chloroform, ethyl acetate and n-butanol, each with 4 x 250 mL, to give 20.6 g of chloroform fraction, 6.5 g of ethyl acetate fraction, 26 g of *n*-butanol fraction and 14 g of remaining aqueous fraction. The crude methanolic extract and its fractions were tested for cytotoxicty against PC3 cell line. The most active *n*-butanol fraction (12 g) was subjected to a VLC on silica gel (1500 g) by eluting with gradients of $CHCl_3$/MeOH from 90:10 (3 L), up to 20:80 (by increasing MeOH and reducing $CHCl_3$, each by 10% increments), to generate six sub-fractions (fr.**A**–fr.**F**). The active subfraction **F** (81 % inhibition at 50 µg/mL on PC3) was subjected to chromatographic separation on a MPLC-UV (Phenomenex RP-18 column, 250 × 25 mm, flow = 15 mL/min, fraction volume 10 mL) using a gradient of CH_3CN/H_2O (30:70) to reach (90:10) affording 14 subfractions. The pooled subfractions (9-14) on further purification using RP-HPLC using $CH_3CN:H_2O$ (25:75) yielded compound **1** (5 mg, t_R = 9.3 min) as a yellowish amorphous powder. Subfraction **D** (79 % inhibition at 50 µg/mL on PC3) was subjected to multiple reversed phase column chromatography separations, using CH_3CN/H_2O (70:30) to obtain compounds **2** (7 mg, t_R = 12.97 min) and **3** (5 mg t_R = 20.37 min). Sub-fraction **C** was subjected to MPLC-UV using a gradient of CH_3CN/H_2O (50:50) to afford compound **4** (5 mg, t_R = 16.62 min). All of the MPLC and HPLC fractions were monitored by UPLC/MS.

2.6. Determination of the absolute configuration of the sugar moieties

The *n*-butanol fraction (1 g) was subjected to acid hydrolysis following the method reported by Abdallah *et al*. (2013) [7].

*2.7. Isorhamnetin-3-O-α-L-rhamnopyranosyl (1→6) β-D-glucopyranoside (**1**)*

Yellow, amorphous powder; $[\alpha]_D^{25}$ – 0.95 (*c*=0.10, MeOH); UVλ_{max}: 255, 355 (MeOH); ^{1}H NMR (MeOH, 600 MHz): δ 6.21 (1H, *d*, *J* = 2.0 Hz, H-6), 6.30 (1H, *d, J* = 2.0, H-8), 6.93 (1H, *d*, *J* = 8.1 Hz, H-5′), 7.64 (1H, *dd*, *J*= 2.0, 8.1 Hz, H-6′), 7.95 (1H, *d*, *J* = 2.0 Hz, H-2′), 3.95 (3H, *s,* 3′-OCH3), 5.24 (1H, *d*, *J* = 7 Hz, H-1″),4.50 (1H, *d, J* = 1.8 Hz, H-1‴), 3.23-3.45 (4H, *m*, H-2″, H-3″, H-4″, H-5″), 3.26-3.45 (4H, *m*, H-2‴, H-3‴, H-4‴, H-5‴), 3.81 (1H, *dd*, *J* = 11.0 and 7.0 Hz, H-6″*a*), 3.41 (1H, *dd*, *J* = 11.0 and 4.0 Hz, H-6″*b*), 1.10 (3H, *d*, *J* = 7Hz, CH_3-6‴); ^{13}C NMR (MeOH, 150 MHz): δ 158.8 (C-2), 135.4 (C-3), 179.4 (C-4), 162.9 (C-5), 102.5 (C-6), 166.1 (C-7), 94.6 (C-8),158.4 (C-9), 105.4 (C-10), 123.0 (C-1′), 114.2 (C-2′), 148.3 (C-3′), 150.7 (C-4′), 115.7 (C-5′), 123.5 (C-6′); Glucose 104.1 (C-1″), 77.6 (C-2″), 77.6 (C-3″), 71.3 (C-4″), 77.1 (C-5″), 68.2 (C-6″), 102.2 (C-1‴), 69.5 (C-2‴), 72.0 (C-3‴), 77.9 (C-4‴), 77.1 (C-5‴), 17.7 (C-6‴); negative ion HRESIMS *m/z* 623.16287 $[M-H]^-$ (calc. for 623.16175), $C_{28}H_{31}O_{16}$.

*2.8. 23-β-D-glucopyranosyl (23S, 25R)-spirost-5-en-3, 23 diol 3-O-α-L-rhamnopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**2**)*

White, amorphous powder; $[\alpha]_D^{25}$ - 6.8 (*c*=0.10, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) spectroscopic data of the aglycone and sugar moieties of compound **2** are listed in Tables 1 and 2; (Mol.wt. 1046); negative ion HRESIMS *m/z* 1081.50201 $[M+Cl]^-$ (calc.

for 1081.498633) $C_{51}H_{82}O_{22}Cl$, positive ion HRESIMS m/z 1069.5156 $[M+Na]^+$ (calc. for 1069.5189) $C_{51}H_{82}O_{22}Na$.

*2.9. (25R)-spirost-5-en-3-ol 3-O-α-L-rhamnopyranosyl-(1→2)-O-[β-D-glucopyranosyl-(1→3)]-β-D-galactopyranoside (**3**)*

White, amorphous powder; $[\alpha]_D^{25}$ - 12.9 (*c*=0.10, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) spectroscopic data of the aglycone and sugar moieties of compound **3** are listed in Tables 1 and 2; (Mol wt. 884); negative ion HRESIMS *m/z* 919.4495 $[M+Cl]^-$ (calc. for 919.4463) $C_{45}H_{72}O_{17}$ Cl, positive ion HRESIMS *m/z* 907.4674 $[M+Na]^+$ (calc. for 907.4661); $C_{45}H_{72}O_{17}Na$.

*2.10. (23S, 25R)-spirost-5-en-3, 23 diol 3-O-α-L-rhamnopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (**4**)*

White, amorphous powder; $[\alpha]_D^{25}$ - 8.9 (*c*=0.10, MeOH); ^{1}H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) spectroscopic data of the aglycone and sugar moieties of compound **4** are listed in Tables 1 and 2; (Mol wt. 884); negative ion HRESIMS *m/z* 883.4708 $[M-H]^-$, positive ion HRESIMS *m/z* 907.4661 $[M+Na]^+$ (calc. for 907.4682); $C_{45}H_{72}O_{17}Na$.

2.11. Cell lines and culture conditions

Human prostate PC3 cancer cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (DMSZ ACC# 465) and the HT29 colon cancer cell line was obtained from the medical immunology department at Martin Luther-Universität Halle-Wittenberg (Prof. Seliger). The cells were grown as monolayers in adherent cell lines and were routinely cultured in RPMI 1640 (Roswell Park Memorial Institute) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% L-glutamine in 75 cm^3 polystyrene flasks (Corning Life Sciences, UK) and maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.12. Cytotoxicity assay

The cytotoxicity assay was performed according to the procedure reported by Farag and Wessjohann (2013) [8]. Cells were plated at a density of 1×10^4/well in 96-well plates. They were allowed to attach to the plate for 24 h. After 24 h, the media were replaced with RPMI media 1640 containing either the plant extract or the isolated compounds. The isolated compounds were initially dissolved in DMSO at a concentration of 2 mg/mL and further diluted with RPMI 1640 medium. Three concentrations were used (10, 50 & 100 µg/ mL) for each tested sample. The maximum DMSO concentration in the assay was 0.1%, which was not cytotoxic to the tumor cells. After 72 h, the medium was taken out and assayed for cell viability using MTT-assay. The mean of duplicate experiments for each dose was used to calculate the IC_{50} values. Digitonin was used as a positive drug control.

Table 1. ^{13}C NMR chemical shift assignments for the aglycone moiety of compounds **2–4** in CD_3OD.

No	2		3		4	
	^{13}CNMR	**^{1}HNMR**	**^{13}CNMR**	**^{1}HNMR**	**^{13}CNMR**	**^{1}HNMR**
1	38.6 (CH_2)	1.87 (1H, *m*)	38.4 (CH_2)	1.86 (1H, *m*)	38.6 (CH_2)	1.82 (1H, *m*)
		1.07 (1H, *m*)		1.07 (1H, *m*)		1.06 (1H, *m*)
2	30.8 (CH_2)	1.9 (1H, *m*)	30.6 (CH_2)	1.90 (1H, *m*)	30.8 (CH_2)	1.90 (1H, *m*)
		1.60 (1H, *m*)		1.61 (1H, *m*)		1.59 (1H, *m*)
3	78.1 (CH)	3.57 (1H, *m*)	78.5 (CH)	3.63 (1H, *m*)	78.1 (CH)	3.61 (1H, *m*)
4	39.5 (CH_2)	2.44 (1H, *m*)	39.4 (CH_2)	2.45 (1H, *m*)	38.7 (CH_2)	2.44 (1H, *m*)
		2.3 (1H, *m*)		2.30 (1H, *m*)		2.29 (1H, *m*)
5	141.9 (C)	-	141.8(C)	-	141.9 (C)	-
6	122.7 (CH)	5.39 (1H, *brd*, *J*= 4)	122.34 (CH)	5.36 (1H, *brd*, *J*=4)	122.7 (CH)	5.37 (1H, *br.d*, *J*= 4)
7	33.3 (CH_2)	1.99 (1H, *m*)	32.9 (CH_2)	2.01 (1H, *m*)	33.2 (CH_2)	2.00 (1H, *m*)
		1.53 (1H, *m*)		1.53 (1H, *m*)		1.53 (1H, *m*)
8	32.2 (CH)	1.4 (1H, *m*)	32.3 (CH)	1.5 (1H, *m*)	32.4 (CH)	1.39 (1H, *m*)
9	51.7 (CH)	0.96 (1H, *m*)	51.4 (CH)	0.97 (1H, *m*)	51.7 (CH)	0.95 (1H, *m*)
10	38.1(C)	-	38.0(C)	-	38.1 (C)	-
11	22.1 (CH_2)	1.54 (2H, *m*)	21.8 (CH_2)	1.54 (2H, *m*)	21.7 (CH_2)	1.54 (2H, *m*)
12	41.3 (CH_2)	1.79 (1H, *m*)	40.75 (CH_2)	1.76 (1H, *m*)	41.2 (CH_2)	1.79 (1H, *m*)
		1.19 (1H, *m*)		1.20 (1H, *m*)		1.19 (1H, *m*)
13	41.8(C)	-	41.4(C)	-	41.9 (C)	-
14	57.7 (CH)	1.12 (1H, *m*)	57.7 (CH)	1.15 (1H, *m*)	57.8 (CH)	1.14 (1H, *m*)
15	32.4 (CH_2)	1.94 (1H, *m*)	32.7 (CH_2)	1.97 (1H, *m*)	32.6 (CH_2)	1.96 (1H, *m*)
		1.83 (1H, *m*)		1.89 (1H, *m*)		1.84 (1H, *m*)
16	82.4 (CH)	4.4 (1H, *q*, *J*=7.4)	82.1 (CH)	4.39 (1H, *q*, *J*=7.7)	82.6 (CH)	4.42 (1H, *q*, *J*=7.5)
17	62.8 (CH)	1.77 (1H, *m*)	63.5 (CH)	1.74 (1H, *m*)	62.9 (CH)	1.76 (1H, *m*)
18	17.3 (CH_3)	0.89 (3H, *s*)	16.7 (CH_3)	0.80 (3H, *s*)	16.6 (CH_3)	0.86 (3H, *s*)
19	20.0 (CH_3)	1.03 (3H, *s*)	19.8 (CH_3)	1.04 (3H, *s*)	19.8 (CH_3)	1.03 (3H, *s*)
20	36.7 (CH)	2.65(1H, *dq*, *J*= 7.2)	42.7 (CH)	1.90 (1H, *dq*, *J*= 7.3)	36.6 (CH)	2.55 (1H, *q*, *J*= 7.2)
21	14.4 (CH_3)	0.95 (3H, *d*, *J*= 7.2)	14.9 (CH_3)	0.96 (3H, *d*, *J*= 7.3)	14.4 (CH_3)	0.93 (3H, *d*, *J*=7.2)
22	111.4 (C)	-	110.4 (C)	-	111.9 (C)	-
23	76.7 (CH)	3.64 (1H, *m*)	31.5 (CH_2)	1.88 (1H, *m*)	67.9 (CH_2)	3.45 (2H, *dd*, *J*= 4, 10)
				1.42 (1H, *m*)		
24eq	37.7 (CH_2)	2.13 (1H, *m*)	30.0 (CH_2)	1.91 (1H, *m*)	38.1 (CH_2)	1.83 (1H, *m*)
26ax		1.48 (1H, *m*)		1.47 (1H, *m*)		1.40 (1H, *m*)
25	32.7 (CH)	1.76 (1H, *m*)	32.5 (CH)	1.60 (1H, *m*)	33.0 (CH)	177 (1H, *m*)
26eq	66.7 (CH_2)	3.36 (1H, *m*)	66.5 (CH_2)	3.42 (1H, *m*)	66.7 (CH_2)	3.36 (1H, *m*)
26ax		3.24 (1H, *m*)		3.30 (1H, *m*)		3.24 (1H, *m*)
27	17.0 (CH_3)	0.82 (3H, *d*, *J*= 6.6)	17.5 (CH_3)	0.79 (1H, *d*, *J*= 6.6)	16.9 (CH_3)	0.84 (1H, *d*, *J*= 6.6)

Table 2. ^{13}C NMR chemical shift assignments for the sugar moieties of compounds **2-4** in CD_3OD.

No	2		3		4	
	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR	^{13}CNMR	^{1}HNMR
Glc- C-23						
1'	105.8 (CH)	4.30 (1H, *d*, *J*= 7.6)				
2'	75.39 (CH)	3.16 (1H, *dd*, *J*= 8.8, 7.7)				
3'	78.5 (CH)	3.31 (1H, *m*)				
4'	71.6 (CH)	3.25 (1H, *m*)				
5'	77.9 (CH)	3.26 (1H, *m*)				
6'	62.8 (CH_2)	3.86 (1H, *d*, *J*= 10.7) 3.65 (1H,*dd*, *J*=10.7, 5.7)				
Glc C-3			**Gal at C-3**		**Glc C-3**	
1''	100.5 (CH)	4.49 (1H, *d*, *J*= 7.6)	100.4 (CH)	4.48 (1H, *d*, *J*=7.3)	100.3 (CH)	4.48 (1H, *d*, *J*= 7.6)
2''	79.3 (CH)	3.38 (1H, *m*)	75.4 (CH)	3.77 (1H, *m*)	79.3 (CH)	3.38 (1H, *m*)
3''	79.3 (CH)	3.58 (1H, *t*, *J*= 8.8)	85.4 (CH)	3.74 (1H, *m*)	79.3 (CH)	3.58(1H, *t*, *J*= 8.8)
4''	80.0 (CH)	3.51 (1H, *t*, *J*= 9.1)	75.9 (CH)	3.52 (1H, *m*)	80.0 (CH)	3.51 (1H, *t*, *J*= 9.1)
5''	76.6 (CH)	3.31 (1H, *m*)	77.2 (CH)	3.31 (1H, *m*)	76.6 (CH)	3.31 (1H, *m*)
6''	62.0 (CH_2)	3.78 (1H, *d*, *J*= 11.6) 3.65 (1H, *dd* , *J*= 11.6, 5.9)	62.3 (CH_2)	3.85 (1H, *m*) 3.68 (1H, *m*)	62.0 (CH_2)	3.78 (1H, *d*, *J*= 11.6) 3.65 (1H, *dd* , *J*= 11.6, 5.9)
Rh C-2''			**Rh at C-2`**		**Rh at C-2`**	
1'''	102.4 (CH)	5.2 (1H, *brd*, *J*=1.2)	102.3 (CH)	5.2 (1H, *brd*, *J*=1.2)	101.4 (CH)	5.2 (1H, *brd*, *J*=1.2)
2'''	72.2 (CH)	3.92 (1H, *m*)	71.9 (CH)	3.93 (1H, *m*)	72.2 (CH)	3.92 (1H, *m*)
3'''	72.5 (CH)	3.82 (1H, *m*)	72.2 (CH)	3.65 (1H, *m*)	72.5 (CH)	3.82 (1H, *m*)
4'''	73.8 (CH)	3.39 (1H, t, *J*= 8.5)	73.8 (CH)	3.38 (1H, *m*)	73.8 (CH)	3.39 (1H, *t*, *J*= 8.5)
5'''	69.8 (CH)	4.12(1H, *dq*, *J*= 8.5, 6.3)	69.5 (CH)	4.16 (1H, *dq*, *J*=8.5,6.3)	69.8 (CH)	4.12, dq, *J*= 8.5, 6.3
6'''	18.0 (CH_3)	1.23(3H, *d*, *J*=6.3)	18.6 (CH_3)	1.23 (3H, *d*, *J*= 6.3)	18.0 (CH_3)	1.23 (3H, *d*, *J*=6.3)
Rh C-4''			**Glc at C-3`**		**Rh at C-4`**	
1''''	103.1 (CH)	4.83(1H, *brd*, *J*=1.2)	105.9 (CH)	4.47 (1H, *d*, *J*=7.3)	103.0 (CH)	4.84 (1H, *d*, *J*=7.3)
2''''	72.4 (CH)	3.82 (1H, *m*)	75.0 (CH)	3.27 (1H, *m*)	72.4 (CH)	3.82 (1H, *m*)
3''''	72.2 (CH)	3.63 (1H, *m*)	78.0 (CH)	3.34 (1H, *m*)	72.2 (CH)	3.64 (1H, *m*)
4''''	74.0 (CH)	3.39 (1H, *t*, *J*= 8.5)	71.0 (CH)	3.32 (1H, *m*)	73.9 (CH)	3.39 (1H, *t*, *J*= 8.5)
5''''	70.7 (CH)	3.92 (1H, *dq*, *J*= 8.5, 6.3)	78.9 (CH)	3.25 (1H, *m*)	70.7 (CH)	3.93 (1H, *dq*, *J*= 8.5, 6.3)
6''''	17.9 (CH_3)	1.24 (3H, *d*, *J*=6.3	62.0 (CH_3)	3.81 (1H, *m*) 3.64 (1H, *m*)	17.9 (CH_3)	1.24 (3H, *d*, *J*=6.3)

3. Results and Discussion

3.1. Structure elucidation

In a continuation of our interest in the chemical and biological investigation of medicinal plants used in Saudi traditional medicine [7, 9-11], the methanolic extracts of forty species were tested *in vitro* for their potential cytotoxicity in different human cancer cell lines [4]. The methanolic extract of *S. glabratum* showed good *in vitro* cytotoxic activity against human breast cancer, hepatocellular carcinoma and cervix cancer cell lines.

To follow up on these results, the methanolic extract of *S. glabratum* Dunal var. *sepicula* as well as, chloroform, ethyl acetate, *n*-butanol and remaining aqueous fractions were tested for their cytotoxic activity against human prostate cancer cell line (PC3) using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay [12]. Four compounds were isolated from the active *n*-butanol fraction through chromatographic separation on normal, reversed phase (RP-18) Si gel columns and Prep HPLC. The isolated compounds (**1–4**), were identified as a flavonoid glycoside (**1**) and three steroidal saponin glycosides (**2-4**).

Compounds **2-4** (Fig.1) were isolated as amorphous powder and gave positive test for steroids (Liebermann–Burchard) and carbohydrates and/or glycosides. The identification of the sugar moieties and determination of their absolute configuration were determined by hydrolysis of the *n*-butanol fraction [7]. The monosaccharides obtained by acid hydrolysis of **2-4** were identified by comparison on TLC with authentic sugar samples and GC analysis as D-glucose, L-rhamnose for compounds **2** and **4**, and D-glucose, L-rhamnose and D-galactose for compound **3**.

Compound **2** was obtained as an amorphous powder with $[\alpha]_D^{25}$ - 6.8 (*c*=0.10, MeOH) and exhibited a HRESIMS $[M+Na]^+$ peak at m/z 1069.5156 (calc. for 1069.5189), consistent with the molecular formula $C_{51}H_{82}O_{22}$. Its negative-ion ESIMSMS displayed a quasimolecular ion peak at m/z 1081.50201 $[M+Cl]^-$ (calc. for 1081.498633). The ^1H and ^{13}C NMR spectra in combination with DEPT and gHSQCAD spectra of **2** showed four methyl groups at δ_H 0.89 (*s*, H-18), 1.03 (*s*, H-19), 0.95 (*d*, *J* =7.2 Hz, H-21) and 0.82 (*d*, *J*=6.6 Hz, H-27), a broad doublet at δ_H 5.39 (*brd, J*= 4.0, H-6) and a characteristic quaternary carbon atom at δ_C 111.4 (C-22), indicating the presence of a steroidal $\Delta^{5(6)}$-spirostanol skeleton [13, 14]. The ^{13}C-NMR and DEPT spectra displayed a total of 51 carbon signals arising from 6 methyls, 11 methylenes, 30 methines and 4 quaternary carbons corresponding to spirostan aglycone and 4 sugar moieties (hexoses). The presence of a hydroxyl group at C-23 (δ_C76.7 and δ_H 3.64) was evident from the long range HMBC correlation of H-23/C-22 (δ_H 3.64/δ_C 111.4) and H-20/C-23 (δ_H 2.65/δ_C 76.7). Complete ^1H and ^{13}C NMR assignments of the aglycone moiety of **2** (Table 1), were established by analysis of the ^1H-^1H DQCOSY spectrum combined with the gHSQCAD, and gHMBCAD spectra. Based on the previous spectral analysis and by comparison with the published data [15], the aglycone of **2** was identified as (23*S*, 25*R*)-spirost-5-en-3, 23 diol. The absolute configuration of spirostan skeleton as 22α,23S and 25R was deduced from ROESY correlations between the protons of H-26_{ax} (δ_H 3.24) and H-16 (δ_H 4.4), H-26_{eq}(δ_H 3.36) and H_3-27 (δ_H 0.82), and H-26_{eq} and H-25 (δ_H 1.76). The ^{13}C-NMR spectrum of **2** showed signals due to four anomeric carbons (100.5 H-1_{Glc}, 105.8 H-$1_{Glc'}$, 102.4 H-1_{Rh}, 103.1 H-$1_{Rh'}$) corresponding to two D-glucose and two D-rhamnose moieties. In addition, two secondary methyl groups at δ_H 1.23 (*d*, *J*=6.3 Hz) and 1.24 (*d*, *J*=6.3 Hz) were detected in the ^1H-NMR spectrum and could be unequivocally assigned through their ^1H-^1H COSY correlations to two H_3-6 of two rhamnosyl residues. The ^1H-^1H DQCOSY, and ROESY experiments allowed the sequential assignment of the resonances for all the sugar residues, starting from the easily distinguished anomeric protons. In the gHMBCAD spectrum of **2**, the attachment of the two glucose moieties to C-3 and C-23 was confirmed from the long range correlations of their anomeric carbons (δ_C 100.5, H-1_{Glc} and 105.8, H-$1_{Glc'}$) to the respective protons (δ_H 3.57, H-3 and 3.64, H-23), respectively. In similar way the attachment of the two D-rhamnose moieties to carbons of C-2_{Glc} and C-4_{Glc} was confirmed from the long range correlations of their anomeric carbons (δ_C 102.4, H-1_{Rh} and 103.1, H-$4_{Rh'}$) to the respective protons (δ_H 3.38, H-2_{Glc} and 3.51, H-4_{Glc}), respectively. The full assignment of the protons and carbons of compound **2** was further confirmed by comparison to the published data of the related glycosides [13, 14, 16-18]. From the

aforementioned data compound **2** was identified as 23-β-D-glucopyranosyl (23*S*, 25*R*)-spirost-5-en-3, 23 diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside.

Compound **3** was isolated as an amorphous powder with $[\alpha]_D^{25}$ – 12.9 (*c*=0.10, MeOH) and exhibited a HRESIMS $[M+Na]^+$ peak at *m/z* 907.4674 (calc. for 907.4661), consistent with the molecular formula $C_{45}H_{72}O_{17}$. Its negative-ion ESIMSMS displayed a quasimolecular ion peak at *m/z* 919.4495 $[M+Cl]^-$ (calc. for 919.4463). The positive ESIMSMS experiment resulted in an abundant fragment ion at *m/z* 415 corresponding to spirost-5-ene-3-ol aglycone.

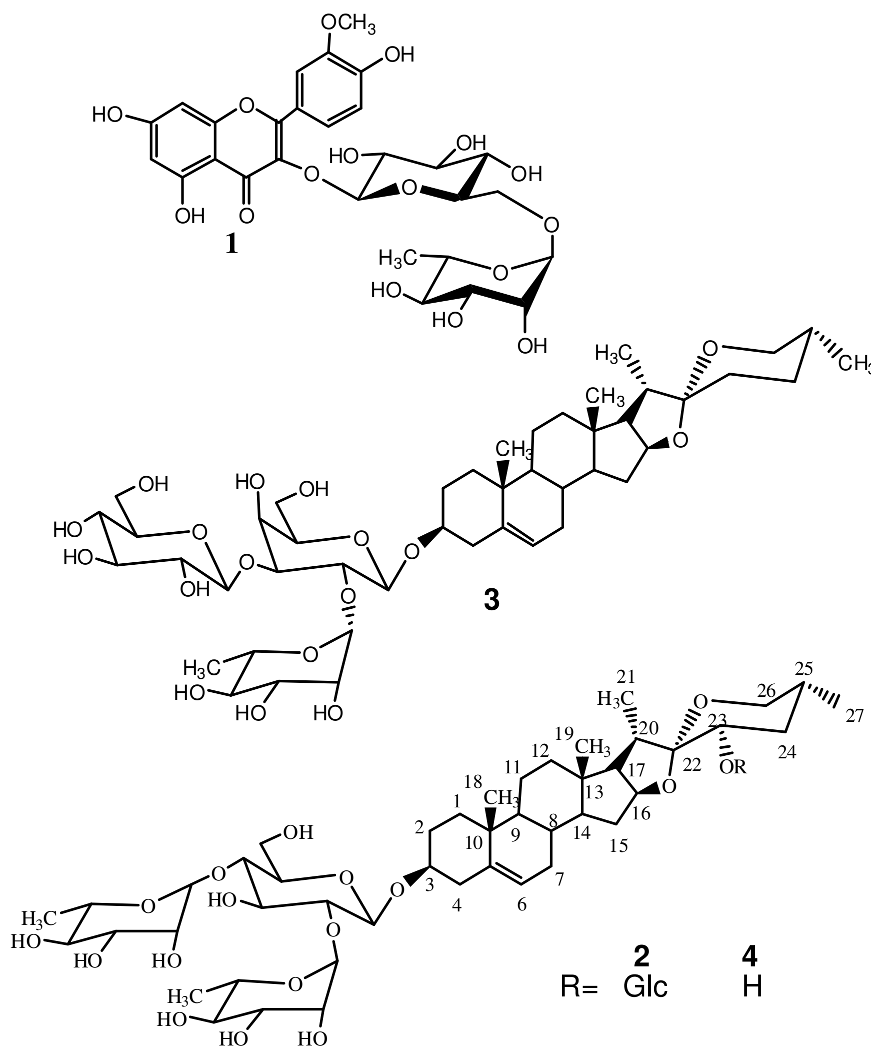

Figure 1. Structures of Compounds 1-4

The ^{13}C NMR spectral data (Table 1 and 2) in combination with DEPT and gHSQCAD spectra of **3** revealed the presence of three anomeric carbons (100.4 H-1_{Gal}, 105.9 H-1_{Glc}, 102.3 H-1_{Rh}) indicated the presence of three sugar units which were subsequently identified as D-galactose, D-glucose and L-rhamnose based on results of acid hydrolysis (TLC and GC). Similar to compound **2**, ^1H NMR spectrum of compound **3** showed four methyl groups resonate at δ_H 0.80 (*s*, H-18), 1.04 (*s*, H-19), 0.96 (*d*, *J* =7.3 Hz, H-21) and 0.79 (*d*, *J*=6.6 Hz, H-27) and a characteristic quaternary carbon atom resonance at δ_C 110.4 (C-22) in ^{13}C NMR spectrum, indicating the presence of steroidal spirostanol skeleton [13, 14]. The ^{13}C NMR spectrum of compound **3** showed the replacement of the oxy carbon signal at δ_C 76.7 (C-23) in **2** by a methylene signal at δ_C 31.49. Thus the aglycone of **3** was identified as (25*R*)-spirost-5-en-3-ol. The 25R configuration was deduced from the intensity of the absorptions (898 > 915 cm−1 band) in its IR spectrum [19, 20] which was also confirmed by the -ring

F resonances of C-23 (δ_C 31.5), C-24 (δ_C 30.0), C-25 (δ_C 32.5), C-26 (δ_C 66.5), and C-27 (δ_C 17.5) [21]. Similar to compound **2**, the attachment of the D-galactose moiety to C-3 of the aglycone was evident from the long range correlation between H-1_{Gal}/C-3(δ_H 4.48/δ_C 78.5) as revealed from the gHMBCAD spectrum and from the correlation between δ_H 4.48 (*d*, *J*=7.3) and 3.63 (*m*) in ROESY spectrum (Fig. 2). The attachment of D-rhamnose and the second D-glucose moieties to carbons C-2_{Gal} and C-$3_{Gal,}$ respectively, was confirmed from the long range correlations of their anomeric carbons (δ_C 102.3, H-1_{Rh} and 105.9, H-1_{Glc}) to the respective protons (δ_H 3.77, H-2 $_{Gal}$ and 3.74, H-3_{Gal}), respectively. The full assignment of the protons and carbons of compound **3** was further confirmed by the analysis of 2D NMR spectra (^{1}H-^{1}H gDQCOSY, gHSQCAD, gHMBCAD, ROESY and zTOCSY) and by comparison to the published data of the related glycosides [17, 18, 22, 23]. Thus, the structure of compound **3** was identified as (25*R*)-spirost-5-en-3-ol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-glucopyranosyl-(1→3)]-β-D-galactopyranoside.

The known compounds **1** and **4** were identified by comparison of their spectral data with those reported in the literature as isorhamnetin-3-O-α-L-rhamnopyranosyl (1→6) β-D-glucopyranoside (**1**) [24] and (23*S*, 25R)-spirost-5-en-3, 23 diol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**4**) [13,14, 25].

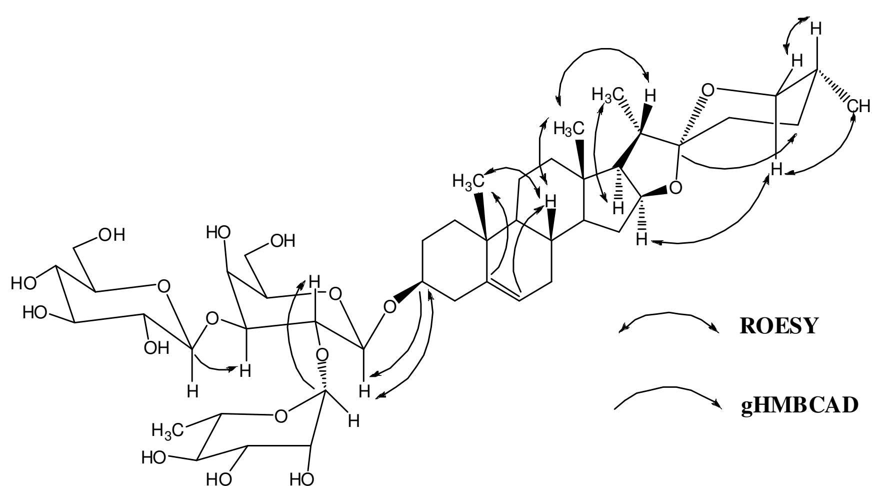

Figure 2. Some selected HMBC and ROESY correlations for compound **3**

3.2 Cytotoxic activity

The methanolic extract of *S. glabratum* showed 90 % cell growth inhibition when tested against PC3 cell line (human prostate cancer) at 10 µg/mL, in a dose-dependent manner. Subsequently, the isolated compounds (**1-4**) were tested for their cytotoxicity in two different human cancer cell lines PC3 and HT29. The cytotoxicity of these compounds are listed in Table **3** as the concentration that inhibits cell growth by 50% relative to cells incubated in the presence of 0.1% DMSO (IC_{50} value) using digitonin as a reference drug. Among the tested compounds (Table 3), compound **3** showed the best cytotoxic activity against both PC3 and HT29 cell lines with IC_{50} of 14.0 and 16.7µM, respectively.

Table 3. Cytotoxic activity of *S. glabratum* fraction and isolated compounds against human prostate PC3 and colon HT29 cancer cell lines (IC_{50} in µg/mL).

Fractions and isolated compounds	PC3	HT29
Chloroform	> 20	n.d.
Ethyl acetate	11.0	n.d.
n- butanol	9.0	n.d.
Aqueous	10.0	n.d.
Compound **1** (µM)	> 32	> 32
Compound **2** (µM)	> 32	> 32
Compound **3** (µM)	14.0	16.7
Compound **4** (µM)	> 32	> 32
Digitonin (positive drug)	1.8	3.0

n.d. not determined

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