Cytotoxic Cholestane and Pregnane Glycosides from *Tribulus macropterus*

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Z. Naturforsch. **62 c**, 319–325 (2007); received November 14, 2006/January 17, 2007

The methanol extract of the whole parts of *Tribulus macropterus* Boiss. (family Zygophyllaceae) showed cytotoxic activity against a human tumour cell line (hepatocyte generation 2, HepG2) (IC_{50} = 2.9 μg/ml). The *n*-butanolic fraction obtained from successive fractionation of the methanolic extract exhibited activity against HepG2 (IC_{50} = 2.6 μg/ml). Therefore, this fraction was subjected to separation using different chromatographic techniques. Five compounds, **1**–**5**, were isolated and identified as: (22*S*,25*S*)-16β,22,26-trihydroxy-cholest-4-en-3-one-16-*O*-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside (**1**), (22*S*,25*S*)-16β,22,26-trihydroxy-cholest-4-en-3-one-16-*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (**2**), sucrose (**3**), D-pinitol (**4**) and 3β-hydroxy-5α-pregn-16(17)en-20-one-3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (**5**) on the basis of spectroscopic and chemical data. The three steroidal compounds **1**, **2** and **5** were also tested against the same cell line HepG2 and their IC_{50} values were 2.4, 2.2 and 1.1 μg/ml, respectively.

Key words: Tribulus macropterus, Cholestane Glycosides, Cytotoxic

Introduction

All cultures from ancient times to the present day have plants as a source of medicine due to their high contents of variable biologically active compounds which play a significant role in control of many diseases. Many researches have been done on searching and discovering new drugs from plants as antimicrobials, antimalarials, antifilarials, anticancers and antischistosomals (Heinrich *et al.,* 2004).

The genus *Tribulus* (family Zygophyllaceae) comprises about 25 species that grow as shrubs or herbs in subtropical areas around the world (Hegnauer, 1973). In Egypt, there are nine species (Boulos, 2000). Four of them had been phytochemically investigated (El-Wakil, 2001; Hamed *et al.,* 2004; Perrone *et al.,* 2005; Temraz *et al.,* 2006). *Tribulus terrestris* is the famous species traditionally used by different cultures for a number of conditions. The extracts or the isolated components of this plant showed various biological activities especially in the treatment of impotence, sexual activity, cardiac diseases, antimicrobial, cytotoxic and anthelminthic activity (Kostova and Dinchev, 2005). Also, there is a number of drugs on the market containing mainly *Tribulus* extract as one of its components, *e.g.* tribulosane used for sexual disorder. Previous phytochemical investigations of *Tribulus* species showed that the major constituents are steroidal glycosides (Mahato *et al.,* 1982; Bedir *et al.,* 2002; Cai *et al.,* 2001; Sun *et al.,* 2002) followed by flavonoid compounds (Louveaux *et al.*, 1998) and alkaloids (Wu *et al.,* 1999).

On continuation of our search for plants having medicinal importance (Abdel Gawad *et al.,* 1998; El-Sayed *et al.,* 2006), the methanolic extract of *Tribulus macropterus* Boiss. showed cytotoxic activity against HepG2 (IC_{50} = 2.9 μg/ml). Although, to the best of our knowledge there is no phytochemical report on this plant, the methanolic extract was subjected to isolation and characterization of some of its chemical constituents.

Experimental

General

Melting points (uncorrected) were measured using the digital melting point apparatus Electrothermal IA 9200 (Electrothermal Engineering LTD, UK). ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD solution containing TMS as internal standard on a Bruker Avance 400 spectro-

0939–5075/2007/0500–0319 $ 06.00

meter equipped with a 5 mm normal configuration $^{13}C\{^1H\}$ probe with standard sequences operating at 400 MHz for proton and 100 MHz for carbon-13. The multiplicities of carbon atoms or the number of attached protons for a ^{13}C signal were determined using a DEPT-135 experiment. Mass spectra were recorded using a HPLC-MS instrument equipped with an Agilent G 1978A dual ESI and APCI mode ion source. Preparative HPLC was done using a Waters Model 590 pump with a refractive index detector (RID 6A, Shimadzu) and separating using a reversed phase column Lichroprep® RP-18 column (5 μm, 250 × 21.2 mm). Different sizes of open glass chromatographic columns were used packed with silica gel 60 (70–230 mesh, Merck) and Sephadex LH-20 Sigma. TLC was performed over pre-coated silica plates (GF_{254}, Merck) and the spots were visualized by spraying with 40% sulphuric acid/methanol reagent followed by heating the plate at 110 °C for 15 min.

Plant material

The whole plant was collected from Suez-Ismailia road, Egypt, in June 2004 and identified by Dr. Abdel-Halim Abdel-Motagaly, Horticulture Department, Agriculture Research Center. A voucher specimen was deposited at laboratory of Medicinal Chemistry, TBRI. The plant was dried in shade, finally powdered with an electric mill and kept for biological and chemical investigation.

Extraction and isolation

500 g of the powdered plant were extracted three times with methanol (3 × 5 l) at room temperature. The solvent was evaporated under reduced pressure using a rotatory evaporator affording 70.36 g methanolic extract. The methanolic extract was defatted with petroleum ether (60–80 °C). The defatted methanol extract was dissolved in a small amount of distilled water (1/2 l) and then successively extracted with chloroform (2 × 1 l), ethyl acetate (2 × 1 l) and finally with *n*-butanol (2 × 1 l). The solvents were evaporated to afford chloroform (2.65 g), ethyl acetate (1.39 g) and *n*-butanol (24.63 g) extracts. About 22 g of the butanolic extract were subjected to open glass column chromatography (5 × 120 cm) packed with silica gel 60 (70–230 mesh) as adsorbent material. The elution started with chloroform (100%) followed by a gradient of $CHCl_3$/MeOH till pure methanol. Fractions of 250 ml were collected, analyzed by TLC (pre-coated silica gel GF_{254}; solvent systems: $CHCl_3$/MeOH 8:2, *n*-propanol/EtOAc/H_2O 4:3:1 v/v/v) and grouped into two major groups A (2.36 g) and B (4.89 g). Group A (collected by the eluting system $CHCl_3$/MeOH 9:1) was rechromatographed over an open glass silica gel column (3 × 30 cm) eluting with a gradient of chloroform and methanol. Two subgroups were collected and monitored by TLC using $CHCl_3$/MeOH 9:1 and 85:5. Each of them was purified over a Sephadex LH-20 open glass column (3 × 30 cm, eluting with methanol) to give compounds **1** (421 mg) and **2** (15 mg). Group B (collected by the eluting system $CHCl_3$/MeOH 7:3) was subjected to preparative HPLC [reversed phase C18 column, 50 mg/ml per injection (6 times), isocratic elution (65% MeOH/H_2O) at the flow rate 6 ml/min with refractive index detection]. Three major peaks were detected at R_t = 12.7, 17.3 and 22.5 min, respectively. Similar peaks in all chromatographic HPLC runs were collected affording three compounds: **3** (121 mg), **4** (32 mg) and **5** (12 mg). The structures of the isolated compounds were elucidated from their spectroscopic and chemical analyses.

Acid hydrolysis

5 mg of compounds **1**, **2** and **5** were hydrolyzed by reflux with 2 M HCl/1,4-dioxane (1:1, 2.5 ml), for 3 h. Dioxane was removed under reduced pressure and the remaining reaction mixture was extracted with dichloromethane (3 × 3 ml). After removing the organic layer, the aqueous layer was neutralized by sodium bicarbonate and evaporated till dryness, then extracted with a very small amount of pyridine. The sugar units were detected by means of TLC with authentic sugar samples (solvent system: EtOAc/*n*-BuOH/H_2O 20:70:10 v/v/v).

*Compound **1***: Creamy powder; m.p. 263–264 °C; R_f = 0.57 ($CHCl_3$/MeOH 8:2). – Acid hydrolysis afforded D-xylose and D-glucose as sugar moieties. – ESI-MS (negative ion mode): *m/z* =725 $[M-H]^-$, 563 $[(M-H)-162]^-$, 431 $[(M-H)-(162+132)]^-$. – ^1H NMR (400 MHz, CD_3OD): δ = 0.94 (3H, *d, J* = 6.5 Hz, Me-27), 0.96 (3H, *d, J* = 6.5 Hz, Me-21), 0.98 (3H, *s*, Me-18), 1.27 (3H, *s,* Me-19), 4.22 (1H, *d, J* = 7.5 Hz, Xyl), 4.23 (1H, *ddd, J* = 9, 7.5, 4.6 Hz, H-16), 4.58 (1H, *d, J* = 7.8 Hz, Glc), 5.77

Table I. ^{13}C NMR chemical shifts and ^{13}C-DEPT of the aglycone of compounds **1**, **2** and **5**.

C	**1**		**2**		**5**	
	δ	DEPT	δ	DEPT	δ	DEPT
1	36.74	CH_2	36.75	CH_2	38.22	CH_2
2	33.42	CH_2	33.19	CH_2	30.57	CH_2
3	202.53	C	202.40	C	78.92	CH
4	124.23	CH	124.10	CH	33.27	CH_2
5	175.46	C	175.5	C	46.43	CH
6	34.85	CH_2	34.70	CH_2	29.91	CH_2
7	33.35	CH_2	33.30	CH_2	33.18	CH_2
8	36.84	CH	36.6	CH	35.27	CH
9	55.51	CH	55.51	CH	56.47	CH
10	40.12	C	40.1	C	37.08	C
11	22.05	CH_2	21.93	CH_2	22.24	CH_2
12	41.08	CH_2	41.12	CH_2	36.35	CH_2
13	43.42	C	43.21	C	47.64	C
14	55.52	CH	55.51	CH	57.89	CH
15	37.20	CH_2	37.00	CH_2	32.51	CH_2
16	83.21	CH	82.93	CH	147.20	CH
17	58.87	CH	58.72	CH	156.72	C
18	13.65	CH_3	13.63	CH_3	16.37	CH_3
19	17.81	CH_3	17.63	CH_3	12.29	CH_3
20	36.18	CH	36.73	CH	199.47	C
21	12.03	CH_3	11.92	CH_3	27.17	CH_3
22	74.31	CH	74.32	CH	–	
23	34.01	CH_2	34.05	CH_2	–	
24	31.19	CH_2	31.03	CH_2	–	
25	37.31	CH	37.82	CH	–	
26	68.46	CH_2	68.00	CH_2	–	
27	17.42	CH_3	17.94	CH_3	–	

Table II. ^{13}C NMR chemical shifts and ^{13}C-DEPT of the sugar parts of compounds **1**, **2** and **5**.

C	**1**		**2**		**5**	
	δ	DEPT	δ	DEPT	δ	DEPT
	16-O-Xyl		16-O-Glc		3-O-Gal	
1	106.98	CH	105.92	CH	100.75	CH
2	74.82	CH	75.22	CH	77.06	CH
3	88.42	CH	88.71	CH	76.02	CH
4	70.16	CH	70.32	CH	81.95	CH
5	66.62	CH_2	77.95	CH	74.09	CH
6			62.71	CH_2	60.92	CH_2
	Xyl (1–3) Glc		Glc (1–3) Glc		Gal (1–2) Rha	
1	105.36	CH	105.2	CH	102.05	CH
2	75.63	CH	75.53	CH	72.51	CH
3	78.02	CH	78.21	CH	72.54	CH
4	71.76	CH	71.84	CH	73.23	CH
5	78.33	CH	78.46	CH	69.74	CH
6	62.82	CH_2	62.31	CH_2	17.91	CH_3
					Gal (1–4) Glc	
1					105.53	CH
2					81.85	CH
3					88.44	CH
4					70.54	CH
5					77.55	CH
6					63.31	CH_2
					Glc (1–2) Xyl	
1					105.42	CH
2					75.39	CH
3					78.34	CH
4					70.99	CH
5					67.17	CH_2
6						
					Glc (1–3) Xyl	
1					105.04	CH
2					75.12	CH
3					78.46	CH
4					71.08	CH
5					67.27	CH_2
6						

(1H, *bs,* H-4). – ^{13}C NMR and ^{13}C-DEPT data of aglycone and sugars: see Tables I and II.

Compound ***2***: White powder; m.p. 268–269 °C; R_f = 0.52 ($CHCl_3$/MeOH 8:2). – Acid hydrolysis afforded only D-glucose as sugar moiety. – ESI-MS (negative ion mode): *m/z* = 755 $[M-H]^-$, 593 $[(M-H)-162]^-$, 431 $[(M-H)-(162+162)]^-$. – ^1H NMR (400 MHz, CD_3OD): δ = 0.94 (3H, *d, J* = 6.4 Hz, Me-27), 0.96 (3H, *d, J* = 6.4 Hz, Me-21), 0.98 (3H, *s,* Me-18), 1.28 (3H, *s*, Me-19), 4.31 (1H, *d, J* = 7.5 Hz, Glc), 4.42 (1H, *ddd, J* = 9, 7.7, 4.2 Hz, H-16), 4.62 (1H, *d, J* = 7.5 Hz, Glc), 5.77 (1H, *bs,* H-4). – ^{13}C NMR and ^{13}C-DEPT data of aglycone and sugars: see Tables I and II.

Compound ***3***: Crystals from methanol; m.p. 171–172 °C; R_f = 0.36 (*n*-propanol/EtOAc/H_2O 4:3:1 v/v/v). – ^1H NMR and its melting point (alone and mixing with authentic sample) revealed that it is sucrose.

Compound ***4***: Crystals from methanol; m.p. 180–182 °C, R_f = 0.49 (*n*-propanol/EtOAc/H_2O 4:3:1 v/v/v). – ^{13}C NMR (100 MHz, CD_3OD): δ_C = 83.79 (C-3), 72.63 (C-5), 72.44 (C-1), 71.99 (C-6), 70.97 (C-2), 70.12 (C-4) and 59.66 (MeO). – ^1H and ^{13}C NMR data were found identical to D-pinitol.

Compound ***5***: Amorphous powder; m.p. 253–254 °C; R_f = 0.58 (*n*-propanol/EtOAc/H_2O 4:3:1 v/v/v). – Acid hydrolysis afforded D-xylose, D-galactose, D-glucose and L-rhamnose as sugar moieties. – ESI-MS (negative ion mode): *m/z* = 1049.2 $[M-H]^-$. – ^1H NMR (400 MHz, CD_3OD): δ = 0.85 (3H, *s,* Me-18), 0.88 (3H, *s,* Me-19), 1.24 (3H, *d, J* = 6.2 Hz, Me-Rha), 2.23 (3H, *s*, MeCO), 4.43 (1H, *d, J* = 7.6 Hz, Glc), 4.47 (1H, *d, J* = 7.6 Hz, Gal), 4.61 (1H, *d, J* = 7.6 Hz, Xyl), 4.78 (1H, *d, J* = 7.8 Hz,

Xyl), 5.18 (1H, *d, J* = 1.5 Hz, Rha), 6.62 (1H, *dd, J* = 3.4, 1.4 Hz, H-16). – ^{13}C NMR and ^{13}C-DEPT data of aglycone and sugars: see Tables I and II.

Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of the methanol extract and the isolated compounds of *Tribulus macropterus* was tested at the National Cancer Institute of Egypt using the method of Skehan and Strong (1990). Cells were plated in a 96-well plate (10^4 cells/well) for 24 h before treatment to allow the attachment of cells to the wall of the plate. Different concentrations of the fraction under test (0, 1, 2.5, 5 and 10 μg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and they were incubated for 48 h at 37 °C in 5% CO_2. After 48 h cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer and the colour intensity was measured in an ELISA reader. The survival curve of the tumour cell line was plotted for each tested fraction.

Results and Discussion

The defatted methanolic extract of the whole parts of *T. macropterus* was successively fractionated using chloroform, ethyl acetate and *n*-butanol. The *n*-butanolic fraction was chromatographed and purified using a combination of silica gel column chromatography, Sephadex LH-20 column chromatography and reversed phase (C_{18}) preparative HPLC to afford five compounds. Compounds **1**, **2** and **5** were identified as steroidal glycosides whereas compounds **3** and **4**, which represent the major constituents of this plant, were simply identified as the very known compounds sucrose (**3**) and D-pinitol (**4**) from their spectroscopic analysis and comparison with authentic samples over TLC (Achenbach *et al.,* 1994, 1996; Rohini *et al.,* 2005). The two compounds **3** and **4** were previously isolated from the aerial and root parts of Mexican *Tribulus cistoides* (Achenbach *et al.,* 1994, 1996).

Compound **1** gave a positive reaction with the Libermann Burchard test. The molecular formula and molecular weight was determined from ^{13}C, ^{13}C-DEPT NMR and ESI-MS spectra as $C_{38}H_{62}O_{13}$ and 726, respectively. The ^{13}C and ^{13}C-DEPT NMR (CD_3OD) spectra showed 38 carbon signals (4 methyl, 12 methylene, 18 methine and 4 quaternary carbon atoms) comprising 27 carbon atoms for the aglycone and 11 carbon atoms for the sugar moiety. The ESI-MS spectrum in negative ion mode exhibited the main ion peak at *m/z* 725 $[M\text{-}H]^-$ and fragments at 563 $[(M\text{-}H)\text{-}162]^-$, 431 $[(M\text{-}H)\text{-}(162+132)]^-$ attributable to the sequential loss of a hexose and a pentose residue. Also, acid hydrolysis of **1** afforded D-xylose and D-glucose as sugar moieties. This gave the consideration that the sugar portion consists of terminal D-glucose and D-xylose connected with the aglycone part. The downfield shift of carbon atom C-3 of the xylose at δ 88.42 revealed that it is the site of connection between the two sugar units (Minpei *et al.,* 2001, 2002). The ^{1}H NMR spectrum displayed two doublet signals of anomeric protons at δ 4.58 and 4.22 with 7.8 and 7.5 Hz coupling constant, respectively, diagnostic for the β-configuration of the sugar moiety (Mahato *et al.,* 1982). From this data the sugar part can be assigned as β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside. The ^{13}C NMR analysis of the remaining 27 carbon signals (4 methyl, 10 methylene, 9 methine and 4 quaternary carbon atoms) suggested that the aglycone has a cholestane moiety with two secondary alcohols (δ_C 74.31 and 83.21), one primary alcoholic function (δ_C 68.46) and an α,β-unsaturated carbonyl group (δ_C 124.23, 175.46 and 202.53) (Mimaki *et al.,* 1993; Achenbach *et al.,* 1996; Kuroda *et al.,* 2001). By comparing the NMR data of the aglycone with data of *Tribulus* species previously isolated (Achenbach *et al.,* 1996; Hamed *et al.,* 2004; Temraz *et al.,* 2006) it appeared that the aglycone part was established as (22*S*,25*S*)-16β,22,26-trihydroxy-cholest-4-en-3-one. The downfield shift exhibited by C-16 of the aglycone (δ 83.21) allowed us to deduce that this carbon atom was the site of glycosidation. From all the above data, this compound was identified as (22*S*,25*S*)-16β,22,26-trihydroxy-cholest-4-en-3-one-16-*O*-β-D-glucopyranosyl-(1→3)-β-D-xylopyranoside (Fig. 1). This compound was firstly isolated from *T. macropterus* but it was isolated previously from other *Tribulus* species (Achenbach *et al.,* 1996; Hamed *et al.,* 2004; Temraz *et al.,* 2006).

Compound **2** gave a positive reaction with the Libermann Burchard test. The molecular formula and molecular weight was determined as $C_{39}H_{64}O_{14}$ and 756 from the ^{13}C, ^{13}C-DEPT NMR and ESI-MS spectra, respectively. The ^{13}C and ^{13}C-DEPT NMR (CD_3OD) spectra showed 39

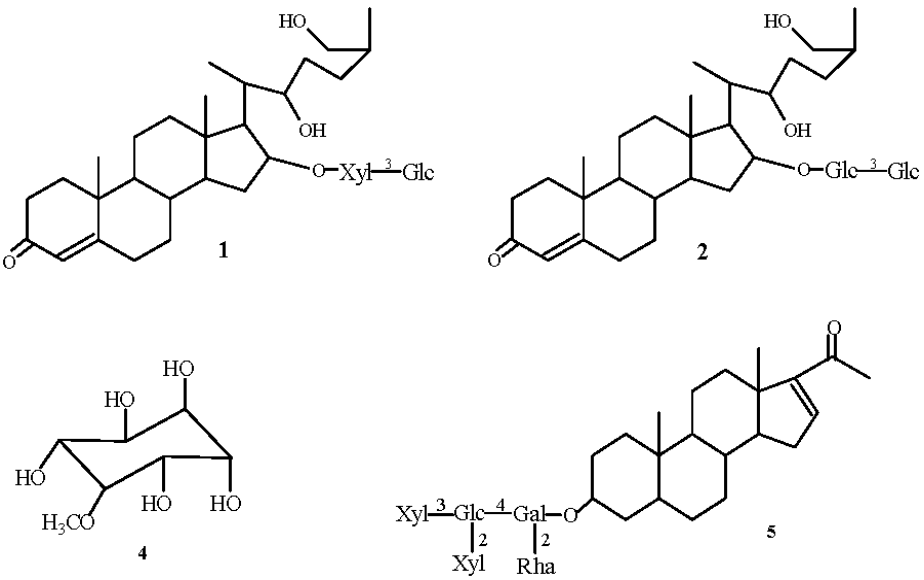

Fig. 1. Structures of the cytotoxic compounds **1**, **2** and **5** and of the known compound **4** (D-pinitol).

carbon signals (4 methyl, 12 methylene, 19 methine and 4 quaternary carbon atoms) comprising 27 carbon atoms for the aglycone and 12 carbon atoms for the sugar moiety. The ESI-MS spectrum exhibited a prominent ion peak in negative ion mode at *m/z* 755 $[M\text{-}H]^-$ and fragments at 593 $[(M\text{-}H)\text{-}162]^-$, 431 $[(M\text{-}H)\text{-}(162+162)]^-$. This may be attributed to the sequential loss of two hexose units. Acid hydrolysis of **2** afforded only D-glucose as sugar moiety. The downfield shift of carbon atom C-3 of the inner glucose unit at δ 88.71 revealed that it is the site of connection with the outer glucose unit. The ^{1}H NMR spectrum displayed two doublet signals of anomeric protons at δ 4.31 and 4.62 with 7.5 and 7.5 Hz coupling constant, respectively, diagnostic for the β-configuration of the sugar moiety. Therefore, the sugar part can be assigned as β-D-glucopyranosyl (1→3)-β-D-glucopyranoside. The NMR spectrum of the aglycone part of this compound was found identical to the aglycone of **1**. From the above data, this compound was identified as (22*S*,25*S*)-16β,22,26-trihydroxy-cholest-4-en-3-one-16-*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranoside (Fig. 1). This compound was firstly isolated from the *Tribulus* genus.

Compound **5** gave a positive reaction with the Libermann Burchard test. The molecular formula and molecular weight were determined as $C_{49}H_{78}O_{24}$ and 1050 from the spectral data (^{13}C, ^{13}C-DEPT NMR and ESI-MS in negative ion mode). The ^{13}C and ^{13}C-DEPT NMR (CD_3OD) spectra showed 49 carbon signals (4 methyl, 12 methylene, 29 methine and 4 quaternary carbon atoms) of which 21 carbon atoms were from the aglycone and 28 carbon atoms from the sugar moiety. By comparison of the ^{1}H-^{13}C NMR spectrum of the aglycone part with literature data it was confirmed that this aglycone has a pregnane skeleton with only one secondary hydroxy group (δ_C 78.92) and one acyl group (δ_C 199.47 and 27.17; δ_H *s*, 2.23) connected with a double bond (δ_C 147.20 and 156.72; δ_H 6.62) forming an α,β-unsaturated ketone (Mohamed *et al.,* 2000; Akihito *et al.,* 2002; Yoshihiro *et al.,* 2002; Vijay *et al.,* 2003). This means that the aglycone part of this compound could be identified as 3β-hydroxy-5α-pregn-16(17)en-20-one. The downfield shift of the only hydroxylated carbon atom C-3 at δ 78.92 revealed that this is the site of glycosidation (Akihito *et al.,* 2002; Vijay *et al.,* 2003). The ESI-MS spectrum exhibited a prominent ion peak in negative ion mode at *m/z* 1049.2 $[M\text{-}H]^-$ and fragments at 917 $[(M\text{-}H)^-\text{-}132]$, 785 $[(M\text{-}H)^-\text{-}2\times132]$ attributable to the sequential loss of 2 pentose sugar units. Acid hydrolysis of **5** gave D-xylose, D-galactose, D-glucose and L-rhamnose which were identified by direct comparison with authentic sugars. ^{1}H NMR spectrum displayed four doublets at δ_H 4.43, 4.47, 4.61 and 4.78 with 7.6, 7.6, 7.6 and 7.8 Hz coupling con-

stant, respectively, diagnostic for the β-configuration of the glucose, galactose and xylose units. Also, there is a doublet with narrow coupling constant 5.18, *d*, J = 1.5 Hz, along with a doublet of a methyl group (δ_H 1.24, 3H, *d*, J = 6.2 Hz, Me-Rha, δ_C 17.91) indicating α-configuration of the L-rhamnose. The ^{13}C NMR peaks at δ 81.85 and 88.44 indicated a 2,3 biglycosidic glucose unit according to the chemical shift of methyl glucopyranoside and the role of glycosidation shifts (Agrawal *et al.,* 1985; Agrawal, 2004). Also, peaks at δ 77.06 and 81.95 were assigned to a 2,4 biglycosidic galactose unit (Agrawal *et al.,* 1985; Agrawal, 2004). By comparing the ^{13}C NMR data of the sugar moiety with the data of previously isolated glycosides from *Tribulus* species, it was found that compound **5** has the same sugar moiety, β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside, but is different in the aglycone portion (Cai *et al.,* 2001; Bedir *et al.,* 2002; Sun *et al.,* 2002; Kostova and Dinchev, 2005). From all the above data compound **5** can be identified as 3β-hydroxy-5α-pregn-16(17)en-20-one-3-*O*-β-D-xylopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-galactopyranoside (Fig. 1). To our knowledge, this compound has not been identified before.

The methanol extract was tested against the human tumour cell line HepG2 (IC_{50} = 2.9 μg/ml). Although, the *n*-butanolic fraction obtained from successive fractionation showed activity at IC_{50} = 2.6 μg/ml, the isolated three steroidal compounds **1**, **2** and **5** were tested against the same cell line HepG2 and their IC_{50} values were 2.4, 2.2 and 1.1 μg/ml, respectively. These results are in good accordance with the cytotoxic activity of steroidal glycosides isolated from other plant species including *Tribulus* (Kuroda *et al.*, 2001; Bedir *et al.,* 2002; Minpei *et al.,* 2001, 2002).

Acknowledgement

The authors are deeply grateful to Prof. Dr. Jerzy W. Jaroszewski, Natural Products Research, Department of Medicinal Chemistry, Danish University of Pharmaceutical Sciences, Denmark, for his great help in running NMR and mass spectra.

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