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Acylated pregnane glycosides from *Caralluma tuberculata* and their antiparasitic activity

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ABSTRACT

Five pregnane glycosides were isolated from *Caralluma tuberculata* (**1**–**5**), in addition to a known one (russelioside E, **6**). The structures of the isolated compounds were elucidated by the analysis of NMR data and FAB–MS experiments. All the isolated compounds were tested for their antimalarial and antitrypanosomal activities as well as their cytotoxicity against human diploid embryonic cell line (MRC5).

1. Introduction

Malaria and trypanosomiasis are tropical parasitic diseases, which continue as one of the main reasons of suffering for many millions of people in both tropical and subtropical zones of the world.

Malaria kills 1–2 million people each year and 300–500 million new clinical cases of malaria are reported annually (Snow et al., 2005). However, the true number of clinical cases is suggested to be much higher than that officially reported (Rogers et al., 2002).

On the other hand, human African trypanosomiasis (HAT) 'sleeping sickness' is a major cause of morbidity and mortality in sub-Saharan Africa. As measured by Disability-adjusted Life Years unit, HAT poses a major health and economic burden in the regions accounting for about 1.5 million (Gross et al., 1999; Remme et al., 2002), while an estimated 60 million people are at risk of contracting the disease, which is fatal if untreated (WHO, 1998; Barrett, 1999).

The chemotherapy of these tropical diseases has been undermined by the fact that currently used drugs are relatively toxic or to a certain extent ineffective by the spreading of resistance. Therefore, there is an urgent need to discover new effective agents. Results of biological screening showed that many natural products possess antiparasitic properties with high efficacy and good selectivity (Camacho et al., 2000; Tagboto and Townson, 2001; Kayser et al., 2003).

In this study, the methanolic extract of *Caralluma tuberculata* N.E.Br. as well as its petroleum ether, chloroform and *n*-butanol soluble fractions were tested for their antimalarial and antitrypanosomal activities, in addition to their cytotoxicity against human diploid embryonic cell line (MRC5). Six compounds (**1**–**6**) were isolated from the chloroform fraction and identified by spectral means and were also tested for their antimalarial and antitrypanosomal activities.

2. Results and discussion

In a biologically-guided fractionation approach, the methanolic extract of *C. tuberculata* as well as, petroleum ether, chloroform and *n*-butanol fractions were tested for their antiprotozoal activity against malaria and trypanosomiasis (see Table 1). As for the antimalarial activity, only petroleum ether soluble fraction demonstrated moderate inhibitory effect (IC_{50} 7.94 μg/mL), this fraction showed high cytotoxicity on MRC5 (IC_{50} 0.8 μg/mL). As for the

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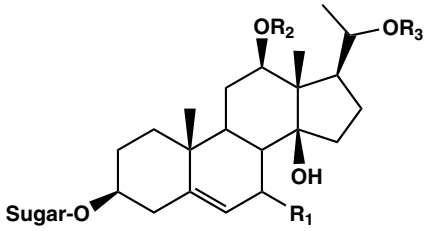
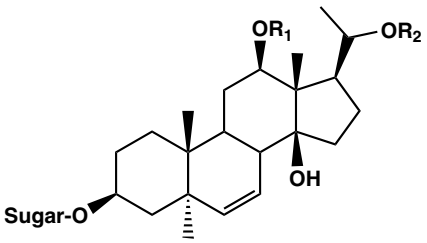
antitrypanosomal activity, the methanolic extract was inactive, while the petroleum ether soluble fraction showed potent activity (IC_{50} 0.5 μg/mL) with a selectivity index of 1.6. On the other hand, the $CHCl_3$ soluble fraction was moderately active (IC_{50} 3.5 μg/mL) with a selectivity index of 17.9 (Table 1). Accordingly, the $CHCl_3$ soluble fraction was chosen for isolating the bioactive compounds. Compounds **1**–**6** were isolated from this fraction and their antimalarial and antitrypanosomal activity were evaluated.

Compounds **1**–**6** gave positive Liebermann–Buchard and Keller–Kiliani reactions, suggesting the presence of a steroidal skeleton and 2-deoxy sugar moieties in their structures.

Compound **6** was identified as 12-*O*-benzoyl-20-*O*-acetyl 3β, 12β, 14β, 20β-tetrahydroxy-(20*S*)-pregn-5-ene β-D-glucopyranosyl-(1 → 4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside, previously isolated from *C. russeliana* and known as russelioside E by comparison of the reported spectral data (Abdel-Sattar et al., 2007).

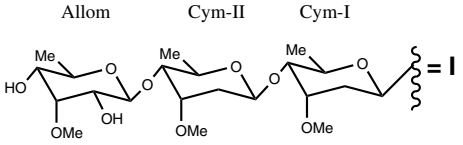
Compounds **1**–**5** were isolated for the first time from nature and their structures were determined as follows.

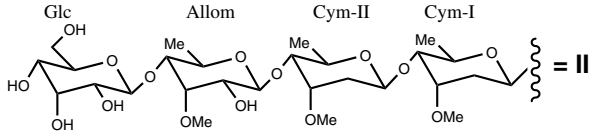




1, sugar=I, R_1=H, R_2=Benzoyl, R_3=Acetyl
2, sugar=I, R_1= -OH, R_2=Benzoyl, R_3=Acetyl
5, sugar=II, R_1=H, R_2=R_3=Benzoyl
6, sugar=II, R_1=H, R_2=Benzoyl, R_3=Acetyl

3, sugar=I, R_1=Benzoyl, R_2=Acetyl
4, sugar=II, R_1=Benzoyl, R_2=Acetyl





Compound **1** was isolated as an amorphous powder, and had a molecular formula $C_{51}H_{76}O_{16}$ (*m*/*z* 967.5018 $[M + Na]^+$, calcd 967.5031) on the basis of the HRFAB–MS, ^{13}C NMR, DEPT and quasimolecular ion peaks at *m*/*z* 967 $[M + Na]^+$ and *m*/*z* 945 $[M + H]^+$ in the FAB^+–MS spectra. IR spectrum of **1** showed absorption bands due to hydroxyl (3400 cm^{-1}) and ester (1720 and 1245 cm^{-1}) groups. The carbon and proton signals of **1** in the NMR spectra were assigned by extensive techniques including HMQC, HMBC, ^1H–^1H TOCOSY, ^1H–^1H COSY and ROESY. The ^{13}C NMR and DEPT spectra of **1** showed 51 carbon signals, which consisted of 10 methyls, 9 methylenes, 25 methines, and 7 quaternary carbons. Thirty of those carbon resonances were ascribed to the aglycone moiety. When compared to those in **6**, MS and NMR data suggested the presence of the same aglycone in **1** and **6** (Nikaido et al., 1967; Braca et al., 2002; Abdel-Sattar et al., 2007). Acyl substitution in **1** with an acetyl group [signals at δ_H 1.92 (*s*, $C\underline{H_3}$-C=O), δ_C 173.35 (C=O), 21.68 ($C\underline{H_3}$-C=O)], and a benzoyl group [signals at δ_H 8.11 (2H, *m*, H-3, 7), 7.63 (1H, *m*, H-5), 7.50 (2H, *m*, H-4, 6) and at δ_C 167.90 (C-1), 131.94 (C-2), 130.65 (C-3, 7), 129.55 (C-4, 6) 134.31 (C-5)] was suggested to be at C-12 (δ_C 79.76) and C-20 (δ_C 75.14), respectively (Piacente et al., 1998; Abdel-Sattar et al., 2002; Braca et al., 2002). The HMBC spectrum confirmed the acetylation position at C-20 and benzoylation at C-12 from the long-range correlation between carbonyl carbon signals at δ_C 173.35 and 167.90 and H-20 (δ_H 4.93) and H-12 (δ_H 4.84), respectively. The relative stereochemistry at C-17 was deduced from the ROESY correlations between H-17 (δ_H 2.05) and H-9 (δ_H 1.36), with no evidence of spatial correlation between H-17 and Me-18, which confirmed the α-configuration of H-17. Therefore, the aglycone moiety was determined as 12-*O*-benzoyl-20-*O*-acetyl 3β, 12β, 14β, 20β-tetrahydroxy-(20*S*)-pregn-5-ene as in **6** (Braca et al., 2002; Abdel-Sattar et al., 2007).

The ^1H and ^{13}C NMR spectra of **1** displayed signals for three anomeric protons at δ_H 4.86 (*dd*, *J* = 2, 9.5 Hz), 4.79 (*dd*, *J* = 2, 9.5 Hz) and 4.58 (*d*, *J* = 8 Hz), [anomeric carbons at δ_C 97.30, 101.10 and 104.40, respectively]. The β-configuration of the anomeric protons were evident from their large coupling constants (8–9.5 Hz). Signals for three methyls at δ_H 1.15 (*s*, Me-18),1.05 (*s*, Me-19) and 1.10 (*d*, J = 6.0 Hz, Me-21), three methoxyls at δ_H 3.41, 3.42, 3.60 (each *s*) in the ^1H NMR spectra, with signals for two methylenes at δ_C 36.64 (C-2 of sugar-1) and 36.33 (C-2 of sugar-2) in ^{13}C NMR spectra, suggesting the presence of two 2,6-dideoxy-3-*O*-methylhexose units and one 6-deoxy-3-*O*-methylhexose unit. TLC of the hydrolyzate of **1** (Halim and Khalil, 1996) revealed the identification of cymarose. 6-Deoxy-3-*O*-methyl-β-D-allose (allomethylose) was identified by extensive study of 2D NMR spectra of **1** (HMBC, TOCOSY, ROESY and ^1H–^1H COSY) and by comparison with data reported in the literature (Braca et al., 2002; Abdel-Sattar et al., 2007). The sequential assignments of proton and carbon resonances of the sugar moieties were determined from HMBC, TOCOSY, ROESY and ^1H–^1H COSY experiments. It was evident that C-1 (δ_C 97.3) of the inner cymarose (cym-I) unit linked to C-3 of the aglycone, in contrast to C-1 of outer cymarose (cym-II) which resonate downfield at δ_C 101.1. From the aforementioned data, the structure of compound **1** was established as 12-*O*-benzoyl-20-*O*-acetyl 3β, 12β, 14β, 20β-tetrahydroxy-(20*S*)-pregn-5-ene 6-deoxy-3-*O*-methyl-β-D-allopyranosyl (1 → 4)-β-D-cymaropyranosyl (1 → 4)-β-D-cymaropyranoside.

The stereochemistry of C-20 was determined through a detailed analysis of the conformation of the side-chain at C-17 as *S* in a similar way as in russeleosides A–D (Al-Yahya et al., 2000), penicillosides A–C (Abdel-Sattar et al., 2001) and russeleosides E–H (Abdel-Sattar et al., 2007), and was performed on compounds **1** and **3**. A ROESY cross-peaks were observed between H-17/H-20 and H_3-18/H-20 and was further supported from the absence of any correlation between H_3-21/H_3-18.

Compound **2** was isolated as an amorphous powder, and had a molecular formula $C_{51}H_{76}O_{17}$ (*m*/*z* 583.4977 $[M + Na]^+$, calcd

Table 1
Antiprotozoal activity of the MeOH extract of *C. tuberculata* and its constituents

Sample	Cytotoxicity (MRC_5) IC_{50} μg/mL	Anti-trypanmosomal activity (IC_{50} μg/mL)	SI	Antimalarial activity IC_{50} μg/mL		
				KI	SI	FCR_3
MeOH ext.	5.0	>12.5	–	>12.5	–	ND
Petroleum ether-sol. Fr.	0.8	0.5	1.6	7.94	0.10	ND
$CHCl_3$-sol. Fr.	62.6	3.5	17.9	>12.5	–	ND
n-butanol-sol. Fr.	–	>12.5	–	>12.5	–	ND
Fr. C-**2**	5.0	ND	ND	6.2	0.81	3.0
Fr. C-**3**	ND*	ND*	ND	7.0	ND	5.0
Fr. C-**4**	ND*	ND*	ND	>12.5	ND	11.0
Fr. C-**5**	ND*	ND*	ND	>12.5	ND	4.8
Compound **1**	22.28	1.85, 3.4[a]	12.04	7.0	3.18	ND
Compound **2**	17.75	5.80	3.06	6.2	2.86	ND
Compound **3**	64.11	7.19	8.9	>12.5	ND	ND
Compound **4**	19.20	6.14	3.13	10.8	1.77	ND
Compound **5**	>100	6.38	ND	6.6	ND	ND
Compound **6**	>100	7.03	ND	8.3	ND	ND
Artemisinin	45.1	–	–	0.005	9020	0.005
Chloroquine	18.5	–	–	0.17	108.8	0.011
Melarsoprol	1.4	0.000011	127,273	–	–	–
Pentamidine	5.7	0.0016	3569	–	–	–

[a] IC_{90} μg/mL, ND: not determined.
* Antitrypanosomal program was started after used up of all fractions in chromatographic isolation.

983.4980 and *m/z* 984.5006 $[M + Na + H]^+$, calcd 984.5014) on the basis of the HRFAB–MS, ^{13}C NMR, DEPT, and quasimolecular ion peaks at *m/z* 983 $[M + Na]^+$ and *m/z* 984 $[M + Na + H]^+$ in the FAB^+–MS spectra. IR spectrum of **2** showed absorption bands similar to those of **1**. The analysis of ^1H and ^{13}C NMR spectra of **2** showed great similarity to those of **1**. The FAB^+–MS of **2** showed 16 mass units more than **1**, indicating the probable presence of an extra oxygen atom in the molecule. The analysis of 1D and 2D NMR spectra of compound **2** showed similar sugar moieties with the same connectivity as in **1**, and was identified as 6-deoxy-3-*O*-methyl-β-D-allopyranosyl (1→4)-β-D-cymaropyranosyl (1 → 4)-β-D-cymaropyranose. In addition, compound **2** showed the presence of acyl groups (acetyl and benzoyl) and acylation positions were similar to those **1**, as confirmed by comparison of their spectral data. The only difference was in the spectral data of the aglycone part, which showed a new proton signal appeared at δ_H 4.48 (*dt*, *J* = 2, 6 Hz), which showed coupling with H-6 at δ_H 5.83 (*dd*, *J* = 2, 6 Hz) and H-8 at δ_H 1.95 (m) in ^1H–^1H COSY and ^1H–^1H TCOSY spectra and directly connected to carbon resonated at δ_C 77.18 (HMQC), which indicated the presence of OH group at C-7 (Nomura et al., 1979; Li et al. 1999; Lu et al., 2004). The α-configuration of the hydroxyl group at C-7 was confirmed by the splitting pattern and coupling constant of H-6 (*dd*, *J* = 2, 6 Hz), which appears at δ_H ~5.3–5.8 as a doublet of doublet (*J* = ~2 and ~5.5 Hz) and not at δ_H ~5.3 as a triplet (*J* = 2 Hz) or a triplet of doublet (*J* = 2, 0.5 Hz) if it acquired β-configuration (Nomura et al., 1979; Li et al. 1999; Lu et al., 2004). From the previous discussion, compound **2** is identified as 12-*O*-benzoyl-20-*O*-acetyl 3β, 7α, 12β, 14β, 20β-tetrahydroxy-(20*S*)-pregn-5-ene 6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyrano side.

Compound **3** was isolated as an amorphous powder, and had a molecular formula $C_{51}H_{76}O_{17}$ (*m/z* 983.4944 $[M + Na]^+$, calcd 983.4976 and *m/z* 984.5046 $[M + Na + H]^+$, calcd 984.5054) on the basis of the HRFAB–MS, ^{13}C NMR, DEPT, and quasimolecular ion peaks at *m/z* 983 $[M + Na]^+$ and *m/z* 961 $[M + H]^+$ in the FAB^+–MS spectra. The analysis of ^1H and ^{13}C NMR spectra of **3** showed great similarity to those of **1** and **2**. The FAB^+–MS of **3** showed 16 mass units more than **1**, indicating the probable presence of an extra oxygen atom in the molecule. The analysis of 1D and 2D NMR spectra of compound **3** showed similar sugar moieties with the same connectivity as in **1** and **2** and the same acyl groups (acetyl and benzoyl) with similar acylation positions, as deduced from the comparison of their spectral data. The only difference was in the spectral data of the aglycone part, which showed the disappearance of the broad doublet of H-6 (δ_H 5.46) in **1** or the doublet of doublet of H-6 (δ_H 5.83, *J* = 2, 6 Hz) and the triplet of doublet of H-7 (δ_H 4.48, *J* = 2, 6 Hz) in **2**, with subsequent appearance of two coupled olefinic proton signals (^1H–^1H COSY) appeared at δ_H 5.74 (*dd*, *J* = 10, 3.2 Hz, H-6) and δ_H 6.01 (*dd*, *J* = 10, 2 Hz, H-7). Those protons showed direct connection to carbons resonated at δ_C 132.55 (C-6) and 131.82 (C-7), which indicated the presence of an olefinic double bond at C-6/C-7 (Warashina and Noro (2003); Lin et al. (1999)). The presence of an additional quaternary oxycarbon signal at δ_C 83.65 indicated the presence of OH group at C-5 as indicated from long-range correlation between C-5 (δ_C 83.65) and each of H-7 (δ_H 6.01) and H_3-19 (δ_H 0.97) in HMBC spectrum. From the aforementioned discussion, compound **3** could be identified as 12-*O*-benzoyl-20-*O*-acetyl 3β, 5α, 12β, 14β, 20β-pentahydroxy-(20*S*)-pregn-6-ene 6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **4** was isolated as an amorphous powder, and had a molecular formula $C_{57}H_{86}O_{22}$ (*m/z* 1145.5494 $[M + Na]^+$, calcd 1145.5508 and *m/z* 1146.5658 $[M + Na + H]^+$, calcd 1146.5543) on the basis of the HRFAB–MS, ^{13}C NMR, DEPT, and quasimolecular ion peaks at *m/z* 1145 $[M + Na]^+$ and *m/z* 1123 $[M + H]^+$ in the FAB^+–MS spectra. The analysis of ^1H and ^{13}C NMR spectra of **4** showed a great similarity to those of **3**. The analysis of 1D and 2D NMR spectra of compound **4** revealed a similar aglycone moiety with the same acylation groups (benzoyl and acetyl) at the same positions as in **3**, identifying the aglycone part as 12-*O*-benzoyl-20-*O*-acetyl 3β, 5α, 12β, 14β, 20β-pentahydroxy-(20*S*)-pregn-6-ene. The molecular formula and FAB^+–MS of **4** showed 162 mass units more than **3**, indicating the probable presence of an additional hexose unit in the molecule. The only difference between **3** and **4**, was in the sugar moiety. The ^1H and ^{13}C NMR spectra of **4** displayed four anomeric protons instead of three in **3**, which appeared at δ_H 4.85 (*dd*, *J* = 2, 9.5 Hz), 4.79 (*dd*, *J* = 2, 9.5 Hz), 4.58 (*d*, *J* = 8 Hz) and 4.35 (*d*, *J* = 8.5 Hz), and corresponding anomeric carbons resonated at δ_C 98.19, 101.17, 103.95 and 106.24, respectively. The β-configuration of the anomeric protons were revealed from their large coupling constants (8–9.5 Hz). TLC of the hydrolyzate of **4** revealed the identification of cymarose and glucose, in addition to the identification of allomethylose in a similar way as in **1**. The sequential assignments of proton and carbon resonances of the sugar moieties and their connectivity to each other or to aglycone part were determined from HMBC, TOCOSY, ROESY and ^1H–^1H COSY experiments. Direct evidence of the sugars sequence and their linkage sites were determined from HMBC experiment which showed unequivocal correlation peaks between $H\text{-}1_{cym\text{-}I}$–C-3, $H\text{-}1_{cym\text{-}II}$–$C\text{-}4_{cym\text{-}I}$, $H\text{-}1_{allom}$–$C\text{-}4_{cym\text{-}II}$ and $H\text{-}1_{glc}$–$C\text{-}4_{allom}$. From the aforementioned discussion, compound **4** could be identified as 12-*O*-benzoyl-20-*O*-acetyl 3β, 5α, 12β, 14β, 20β-pentahydroxy-(20*S*)-pregn-6-ene β-D-glucopyranosyl-(1 → 4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

Compound **5** was isolated as an amorphous powder, and had a molecular formula $C_{62}H_{88}O_{21}$ (*m/z* 1191.5677 $[M + Na]^+$, calcd 1191.5716 and *m/z* 1192.5725 $[M + Na+H]^+$, calcd 1192.5750) on the basis of the HRFAB–MS, ^{13}C NMR, DEPT, and quasimolecular ion peaks at *m/z* 1191 $[M + Na]^+$ in the FAB^+–MS spectra. Analysis of the ^1H and ^{13}C NMR spectral data of **5** in addition to the result of acid hydrolysis confirmed the saccharide moiety in **5** to be β-D-glucopyranosyl-(1 → 4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside, similar to that in **4**. The aglycone of **5** revealed a similar

aglycone moiety to that of **1** except that both hydroxyl groups at C-12 and C-20 were acylated with two benzoyl groups. This was confirmed by the presence of two groups of aromatic proton signals in ^{1}H NMR spectrum corresponding to two benzoyl groups. The structure of **5** could be elucidated as 12, 20-dibenzoyl 3β, 12β, 14β, 20β-tetrahydroxy-(20*S*)-pregn-5-ene β-D-glucopyranosyl-(1 → 4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1 → 4)-β-D-cymaropyranosyl-(1 → 4)-β-D-cymaropyranoside.

The absolute configuration of the sugars were determined to be in the D-form on the basis of their optical rotation values (Abe et al., 1999), after acid hydrolysis of pregnane glycosides and chromatographic separation of the sugar mixture.

2.1. Antimalarial activity

All fractions tested (CT-2, -3, -4, and -5) were active against the drug-sensitive strain (FCR3) with IC_{50} < 12.5 μg/mL, with CT-2 and CT-5 as the most active fractions; IC_{50} 3.0 and 4.8 μg/mL, respectively. However, fractions CT-2 and CT-3 were only active against the drug-resistant strain (K1); IC_{50} 6.2 and 7.0 μg/mL, respectively (Table 1).

The result of the antiprotozoal activity of isolated compounds was shown in Table 1, relative to that shown by artemisinin and chloroquine; the positive controls for antimalarial activity, and melarsoprol and pentamidine, the positive controls for antitrypanosomal activity.

The isolated compounds (**1**–**6**) were tested for their *in vitro* antimalarial activity against *P. falciparum* (K1, drug resistant) and for their cytotoxicities against MRC5 human cell line (see Table 1). Compounds **1, 2** and **4**–**6** showed weak *in vitro* antimalarial activity (IC_{50} < 12.5 and >6.25 μg/mL), while compound **3** was inactive (IC_{50} > 12.5 μg/mL).

2.2. Antitrypanosomal activity

The $CHCl_3$ extract of *C. tuberculata* showed a moderate activity (IC_{50} of 3.5 μg/mL) and a good selectivity index, Si = 17.9.

The isolated compounds (**1**–**6**) were tested for their *in vitro* antitrypanosomal activity. From the results in Table 1, compound **1** was the most active with IC_{50} 1.85 μg/mL (IC_{90} 3.4 μg/mL), followed by compounds **2**, **4**, **5**, **6** and **3** (see Table 1).

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a DIP-360 automatic polarimeter (Jasco Co., Tokyo). IR spectra were taken out on a JASCO FT/IR-230 IR spectrometer. Mass spectra were measured on JEOL JMS-700 Mstation (or MS; JEOL JMS-AX505HA), matrix: thioglycerol-glycerol (1:1 by vol.), acceleration voltage: 8 KV, emitter current: 3 mA, gun High Voltage: 6 KV, gas: Xe, samples were dissolved in MeOH. NMR spectra measured on Varian Unity-400 machine using MeOH-d_4. HPLC specification (pump: Shimadzu LC-6A liquid chromatograph, UV Detector: Shimadzu SPD-6A spectrophotometric detector, column: senshu pak pegasil ODS 20 × 250 mm, UV detector used at 206 nm, and the flow rate was 4 mL/min.

3.2. Plant material

The whole plant of *Caralluma tuberculata* N.E.Br. (syn. *Boreallu-ma tuberculata* (N.E.Br.) Plowes, *Apteranthes tuberculata* (N.E.Br.) Meve & Liede) was collected from Al-Sheffa area, Al-Taif governorate, Saudi Arabia, in May 2005 and were dried in shade. A specimen was deposited in the herbarium of College of Pharmacy, King Abdulaziz University, Jeddah, Saudi Arabia (#CT1027).

3.3. Extraction and isolation

The dried aerial parts of *C. tuberculata* (500 g) were extracted with MeOH on cold using ultra turrax homogenizer. The methanolic extract (100 g) was fractionated into petroleum ether (6 g), chloroform (28 g) and *n*-butanol (33 g) fraction. The chloroform fraction (5 g) was chromatographed on a Si gel column (5 × 25 cm) using 2–10% MeOH/$CHCl_3$ and fractions of 150 mL each were collected to give five main fractions, CT-1 (0.21 g), CT-2 (0.8 g), CT-3 (0.4 g), CT-4 (0.5 g) and to CT-5 (0.9 g). Fraction CT-2 (0.8 g) was further chromatographed on a Si gel column (3 × 14 cm) using *n*-hexane-EtOAc-acetone (5:3:2) as eluting solvent and 30 fractions were collected (40 mL each). Subfraction CT-2a (90 mg) eluted between fraction 440 and 490 mL, on concentration gave 21 mg of compound **1**. Subfraction CT-2b (105 mg) eluted between 840 mL and 960 mL (50 mg/mL, injection volume 300 μL) was purified by chromatography on RP-18 HPLC (Senshu Pak pegasil ODS, 20 × 250 mm,) using 70% CH_3CN/H_2O (4 mL/min, UV; 206 nm), to give compounds **2** (4 mg) and **3** (10 mg). Fraction CT-5 (50 mg/mL, injection volume 300 μL) was purified by chromatography on RP-18 HPLC using 65% CH_3CN/H_2O (flow rate 4 mL/min, UV detection at 206 nm), to give compounds **4** (9 mg), **5** (4 mg) and **6** (19 mg).

3.4. Acid hydrolysis of compounds *1*–*6*

Compounds **1**–**6** were hydrolysed according to procedure reported by Halim and Khalil (1996).

Table 2
^{13}C NMR data of aglycone moieties of compounds **1–6** (100 MHz, CD_3OD)

No	1	2	3	4	5	6
1	38.3, *t*	37.9, *t*	29.6, *t*	29.6, *t*	38.3, *t*	38.3, *t*
2	30.7, *t*	30.5, *t*	29.7, *t*	29.7, *t*	30.6, *t*	30.7, *t*
3	79.0, *d*	78.7, *d*	75.5 *d*	75.6, *d*	79.0, *d*	79.0, *d*
4	39.7, *t*	39.9, *t*	33.9, *t*	33.9, *t*	39.8, *t*	39.8, *t*
5	140.5, *s*	146.3, *s*	83.7, *s*	83.7, *s*	140.6, *s*	140.5, *s*
6	122.9, *d*	123.1, *d*	132.6, *d*	132.6, *d*	123.1, *d*	123.0, *d*
7	28.2, *t*	77.2, *d*	131.8, *d*	131.8, *t*	28.1, *t*	28.2, *t*
8	38.0, *d*	39.5, *d*	44.9, *d*	44.9, *d*	37.7, *d*	38.0, *d*
9	44.6, *d*	43.3, *d*	36.5, *d*	36.5, *d*	44.7, *d*	44.6, *d*
10	38.5, *s*	39.3, *s*	39.5, *s*	39.5, *s*	38.4, *s*	38.5, *s*
11	27.2, *t*	27.1, *t*	27.1, *t*	27.3, *t*	27.4, *t*	27.2, *t*
12	79.8, *d*	79.1, *d*	80.8, *d*	80.8, *d*	79.0, *d*	79.8, *d*
13	53.3, *s*	54.5, *s*	54.0, *s*	54.0, *s*	53.6, *s*	53.2, *s*
14	87.3, *s*	86.1, *s*	85.9, *s*	85.9, *s*	87.5, *s*	87.3, *s*
15	32.9, *t*	32.1, *t*	33.1, *t*	33.1, *t*	32.8, *t*	32.9, *t*
16	25.9, *t*	26.5, *t*	27.3, *t*	27.1, *t*	25.2, *t*	25.9, *t*
17	51.2, *d*	49.7, *d*	51.6, *d*	51.6, *d*	51.0, *d*	51.2, *d*
18	10.2, *q*	10.2, *q*	10.6, *q*	10.6, *q*	10.1, *q*	10.2, *q*
19	19.8, *q*	19.1, *q*	15.4, *q*	15.4, *q*	19.8, *q*	19.8, *q*
20	75.1, *d*	75.3, *d*	74.9, *d*	74.9, *d*	75.7, *d*	75.2, *d*
21	19.3, *q*	19.5, *q*	19.5, *q*	19.5, *q*	19.7, *q*	19.4, *q*
	Benzoyl	Benzoyl	Benzoyl	Benzoyl	Benzoyl	Benzoyl
1	167.9, *s*	167.9, *s*	167.8, *s*	167.8, *s*	167.5, *s*	167.9, *s*
2	131.9, *s*	131.9, *s*	131.9, *s*	132.0, *s*	132.0, *s*	131.9, *s*
3, 7	130.7, *d*	130.7, *d*	130.7, *d*	130.7, *d*	130.3, *d*	130.6, *d*
4, 6	129.6, *d*	129.6, *d*	129.6, *d*	129. 6, *d*	129.5, *d*	129.6, *d*
5	134.3, *d*	134.3, *d*	134.3, *d*	134.3, *d*	134.0, *d*	134.3, *d*
	Acetyl	Acetyl	Acetyl	Acetyl	Benzoyl	Acetyl
1	173.4, *s*	172.4, *s*	172.4, *s*	173.4, *s*	168.0, *s*	172.3, *s*
2	21.7, *q*	21.7, *q*	21.6, *q*	21.6, *q*	132.0, *s*	21.8, *q*
3, 7					130.7, *d*	
4, 6					129.8, *d*	
5					134.4, *d*	

Table 3
^{13}C NMR data of sugar moieties of compounds **1–6** (100 MHz, CD_3OD)

No	1	2	3	4	5	6
	Cym-I	Cym-I	Cym-I	Cym-I	Cym-I	Cym-I
1	97.3, *d*	97.3, *d*	98.2, *d*	98.2, *d*	97.3, *d*	97.3, *d*
2	36.6, *t*	36.6, *t*	36.6, *t*	36.6, *t*	36.6, *t*	36.6, *t*
3	78.6, *d*	78.6, *d*	78.7, *d*	78.7, *d*	78.7, *d*	78.7, *d*
4	83.9, *d*	83.8, *d*	83.9, *d*	83.8, *d*	83.8, *d*	83.8, *d*
5	70.1, *d*	70.1, *d*	70.1, *d*	69.9, *d*	69.9, *d*	69.9, *d*
6	18.3, *q*	18.3, *q*	18.5, *q*	18.5, *q*	18.5, *q*	18.5, *q*
MeO	58.5, *q*	58.5, *q*	58.5, *q*	58.4, *q*	58.4, *q*	58.4, *q*
	Cym-II	Cym-II	Cym-II	Cym-II	Cym-II	Cym-II
1	101.1, *d*	101.2, *d*	101.3, *d*	101.2, *d*	101.2, *d*	101.2, *d*
2	36.3, *t*	36.3, *t*	36.3, *t*	36.4, *t*	36.4, *t*	36.4, *t*
3	78.6, *d*	78.5, *d*	78.7, *d*	78.6, *d*	78.6, *d*	78.6, *d*
4	83.8, *d*	83.9, *d*	84.0, *d*	84.0, *d*	83.8, *d*	83.8, *d*
5	70.0, *d*	70.0, *d*	70.0, *d*	70.0, *d*	70.0, *d*	70.0, *d*
6	18.8, *q*	18.8, *q*	18.3, *q*	18.2, *q*	18.2, *q*	18.2, *q*
MeO	58.5, *q*	58.4, *q*	58.4, *q*	58.5, *q*	58.5, *q*	58.6, *q*
	Allom	Allom	Allom	Allom	Allom	Allom
1	104.4, *d*	104.0, *d*	104.0, *d*	104.0, *d*	103.9, *d*	103.9, *d*
2	73.3, *d*	73.3, *d*	73.3, *d*	72.6, *d*	72.6, *d*	72.6, *d*
3	84.0, *d*	84.0, *d*	84.0, *d*	83.2, *d*	83.2, *d*	83.2, *d*
4	74.9, *d*	75.0, *d*	75.0, *d*	84.1, *d*	84.1, *d*	84.1, *d*
5	70.9, *d*	70.9, *d*	70.9, *d*	70.1, *d*	70.1, *d*	70.1, *d*
6	18.5, *q*	18.5, *q*	18.8, *q*	18.8, *q*	18.8, *q*	18.8, *q*
MeO	62.6, *q*	62.6, *q*	62.6, *q*	62.0, *q*	62.0, *q*	62.0, *q*
				Glc	Glc	Glc
1				106.2, *d*	106.2, *d*	106.2, *d*
2				75.5, *d*	75.5, *d*	75.5, *d*
3				77.9, *d*	78.0, *d*	77.9, *d*
4				71.9, *d*	71.9, *d*	71.9, *d*
5				78.1, *d*	78.1, *d*	78.1, *d*
6				63.1, *t*	63.1, *t*	63.1, *t*

3.5. Determination of the absolute configuration of the sugar moieties

The chloroform extract (2 g) was hydrolysed according to procedure reported by Halim and Khalil (1996). The H_2O layer was then concentrated and passed through a silica gel column using same procedure reported by Abe et al. (1999). The column afforded three sugars identified as D-cymarose, 6-deoxy-3-*O*-D-methylallose and D-glucose by comparison with the authentic samples on TLC and by comparison of their optical rotation values with those reported in the literature (Abe et al., 1999).

3.6. Caratuberside C (**1**)

Amorphous powder $[\alpha]_D^{25}$ +18.0° (MeOH, *c*. 0.05); IR (KBr) υ_{max} 3400, 1720 and 1245 cm^{-1}; FAB^+–MS *m*/*z* (rel. int): 967 $[M + Na]^+$ (50), 945 $[M + H]^+$ (20); HRFAB–MS (positive) *m*/*z*: 967.5018 (calcd for $[M + Na]^+$: 967.5031) and *m*/*z* 968.5121 (calcd for $[M + Na + H]^+$: 968.5065). ^{1}H and ^{13}C NMR (in CD_3OD, 100 and 400 MHz, respectively): see Tables 2–4.

3.7. Caratuberside D (**2**)

Amorphous powder $[\alpha]_D^{25}$ +12.0° (MeOH, *c*. 0.05), IR (KBr) υ_{max} 3400, 1720 and 1240 cm^{-1}; FAB^+–MS *m*/*z* (rel. int): 983 $[M + Na]^+$ (100), 984 $[M + Na + H]^+$ (57); HRFAB–MS (positive) *m*/*z*: 583.4977 (calcd for $[M + Na]^+$: 983.4980) and *m*/*z* 984.5006 (calcd for $[M + Na + H]^+$: 984.5014). ^{1}H and ^{13}C NMR (in CD_3OD, 100 and 400 MHz, respectively): see Tables 2–4.

Table 4
^{1}H NMR data of sugar moieties of compounds **1–6** (400 MHz, CD_3OD)

No	1	2	3	4	5	6
	Cym-I	Cym-I	Cym-I	Cym-I	Cym-I	Cym-I
1	4.86 (*dd*, 2, 9.5)	4.89 (*dd*, 2, 9.5)	4.86 (*dd*, 2, 9.5)	4.85 (*dd*, 2, 9.5)	4.85 (*dd*, 2, 9.5)	4.85 (*dd*, 2, 9.5)
2	2.05 (*m*), 1.52 (*m*)	2.06 (*m*), 1.54 (*m*)	2.05 (*m*), 1.54 (*m*)	2.05 (*m*), 1.54 (*m*)	2.05 (*m*), 1.54 (*m*)	2.05 (*m*), 1.54 (*m*)
3	3.85 (*m*)	3.85 (*m*)	3.85 (*m*)	3.82 (*m*)	3.83 (*m*)	3.83 (*m*)
4	3.22 (*dd*, 3, 9.5)	3.22 (*dd*, 3, 9.5)	3.22 (*dd*, 3, 9.5)	3.34 (*dd*, 3, 9.5)	3.34 (*dd*, 3, 9.5)	3.34 (*dd*, 3, 9.5)
5	3.83 (*m*)	3.80 (*m*)	3.83 (*m*)	3.80 (*m*)	3.82 (*m*)	3.82 (*m*)
6	1.18 (*d*, 6)	1.18 (*d*, 6)	1.19 (*d*, 6)	1.20 (*d*, 6)	1.18 (*d*, 6)	1.18 (*d*, 6)
MeO	3.41 (*s*)	3.41 (*s*)	3.41 (*s*)	3.41 (*s*)	3.43 (*s*)	3.43 (*s*)
	Cym-II	Cym-II	Cym-II	Cym-II	Cym-II	Cym-II
1	4.79 (*dd*, 2, 9.5)	4.80 (*dd*, 2, 9.5)	4.79 (*dd*, 2, 9.5)	4.79 (*dd*, 2, 9.5)	4.78 (*dd*, 2, 9.5)	4.78 (*dd*, 2, 9.5)
2	2.15 (*m*), 1.53 (*m*)	2.12 (*m*), 1.56 (*m*)	2.15 (*m*), 1.60 (*m*)	2.13 (*m*), 1.60 (*m*)	2.13 (*m*), 1.60 (*m*)	2.13 (*m*), 1.60 (*m*)
3	3.85 (*m*)	3.85 (*m*)	3.85 (*m*)	3.82 (*m*)	3.83 (*m*)	3.83 (*m*)
4	3.23 (*dd*, 3, 9.5)	3.23 (*dd*, 3, 9.5)	3.24 (*dd*, 3, 9.5)	3.22 (*dd*, 3, 9.5)	3.24 (*dd*, 3, 9.5)	3.24 (*dd*, 3, 9.5)
5	3.87 (*m*)	3.68 (*m*)	3.87 (*m*)	3.85 (*m*)	3.82 (*m*)	3.82 (*m*)
6	1.29 (*d*, 6)	1.29 (*d*, 6)	1.29 (*d*, 6)	1.29 (*d*, 6)	1.29 (*d*, 6)	1.29 (*d*, 6)
MeO	3.42 (*s*)	3.42 (*s*)	3.42 (*s*)	3.42 (*s*)	3.43 (*s*)	3.43 (*s*)
	Allom	Allom	Allom	Allom	Allom	Allom
1	4.58 (*d*, 8)	4.58 (*d*, 8)	4.58 (*d*, 8)	4.58 (*d*, 8)	4.57 (*d*, 8)	4.57 (*d*, 8)
2	3.35 (*dd*, 3, 9)	3.36 (*dd*, 3, 9)	3.35 (*dd*, 3, 8)	3.36 (*dd*, 3, 8)	3.32 (*dd*, 3, 8)	3.32 (*dd*, 3, 8)
3	3.63 (*t*, 3)	3.63 (*t*, 3)	3.63 (*t*, 3)	3.76 (*t*, 3)	3.94 (*t*, 3)	3.94 (*t*, 3)
4	3.18 (*dd*, 3, 9.5)	3.18 (*dd*, 3, 9.7)	3.18 (*dd*, 3, 9.5)	3.23 (*dd*, 3, 9.5)	3.21 (*dd*, 3, 9.5)	3.21 (*dd*, 3, 9.5)
5	3.67 (*dq*, 6, 9.5)	3.68 (*dq*, 6, 9.5)	3.67 (*dq*, 6, 9.5)	3.85 (*dq*, 6, 9.5)	3.82 (*dq*, 6, 9.5)	3.82 (*dq*, 6, 9.5)
6	1.22 (*d*, 6)	1.22 (*d*, 6)	1.22 (*d*, 6)	1.28 (*d*, 6)	1.28 (*d*, 6)	1.28 (*d*, 6)
MeO	3.60 (*s*)	3.60 (*s*)	3.60 (*s*)	3.60 (*s*)	3.59 (*s*)	3.59 (*s*)
				Glc	Glc	Glc
1				4.35 (*d*, 8.5)	4.35 (*d*, 8.5)	4.35 (*d*, 8.5)
2				3.18 (*t*, 9)	3.18 (*t*, 9)	3.18 (*t*, 9)
3				3.37 (*t*, 9)	3.32 (*t*, 9)	3.32 (*t*, 9)
4				3.25 (*t*, 9)	3.24 (*t*, 9)	3.24 (*t*, 9)
5				3.26 (*m*)	3.29 (*m*)	3.29 (*m*)
6A				3.64 (*dd*, 11,6)	3.65 (*dd*, 11,6)	3.65 (*dd*, 11,6)
6B				3.85 (brd, 11)	3.90 (brd, 11)	3.90 (brd, 11)

3.8. Caratuberside E (**3**)

Amorphous powder $[\alpha]_D^{25}$ +10.4° (MeOH, *c*. 0.5); IR (KBr) υ_{max} 3400, 1725 and 1235 cm^{-1}; FAB^+–MS *m*/*z* (rel. int): 983 $[M + Na]^+$ (20), 961 $[M + H]^+$ (6); HRFAB–MS (positive) *m*/*z*: 983.4944 (calcd for $[M + Na]^+$: 983.4976) and *m*/*z* 984.5046 (calcd for $[M + Na + H]^+$: 984.5054). ^1H and ^{13}C NMR (in CD_3OD, 100 and 400 MHz, respectively): see Tables 2–4.

3.9. Caratuberside F (**4**)

Amorphous powder $[\alpha]_D^{25}$ +12.7° (MeOH, *c*. 0.45); IR (KBr) υ_{max} 3410, 1725 and 1230 cm^{-1}; FAB^+–MS *m*/*z* (rel. int): 1145 $[M + Na]^+$ (25), 1123 $[M + H]^+$ (10); HRFAB–MS (positive) *m*/*z*: 1145.5494 (calcd for $[M + Na]^+$: 1145.5508) and *m*/*z* 1146.5658 (calcd for $[M + Na + H]^+$: 1146.5543). ^1H and ^{13}C NMR (in CD_3OD, 100 and 400 MHz, respectively): see Tables 2–4.

3.10. Caratuberside G (**5**)

Amorphous powder $[\alpha]_D^{25}$ +12.0° (MeOH, *c*. 0.2); IR (KBr) υ_{max} 3405, 1725 and 1240 cm^{-1}; FAB^+–MS *m*/*z* (rel. int): 1191 $[M + Na]^+$ (12); HRFAB–MS (positive) *m*/*z*: 1191.5677 (calcd for $[M + Na]^+$: 1191.5716) and *m*/*z* 1192.5725 (calcd for $[M + Na + H]^+$: 1192.5750). ^1H and ^{13}C NMR (in CD_3OD, 100 and 400 MHz, respectively): see Tables 2–4.

3.11. Antimalarial activity

Samples were screened by *in vitro* anti-malarial assay system by monitoring parasite lactate dehydrogenase (pLDH) activity using Malstat reagent (Makler et al., 1993) on drug-resistant K1 strain and drug sensitive FCR3 strain of *Plasmodium falciparum*. Chloroquine and artemisinin will be used as positive controls.

Type A^+ human plasma and erythrocytes were obtained from healthy volunteers in the Research Center for Clinical Pharmacology of Kitasato Institute. *P. falciparum* strains K1 (drug resistant) and FCR3 (drug sensitive) and were generous gifts of Prof. K. Kita (The University of Tokyo). The parasites were grown based on a method described by Trager and Jensen (1976). *P. falciparum* strains were cultured in the human erythrocytes in RPMI medium (RPMI-1640 with 25 mM HEPES buffer, 24 mM $NaHCO_3$, 0.2% glucose, 0.05% L-glutamine, 50 μg/mL hypoxanthine, and 25 μg/mL gentamicin) supplemented with 10% human plasma at 37 °C, under 93% N_2, 4% CO_2 and 3% O_2. Antimalarial activity of the test sample was achieved by a dose response curve using the parasite lactate dehydrogenase (pLDH) assay according to the method of Makler et al. (1993). One hundred ninety microliter of asynchronous parasites (2.0% hematocrit and 0.5 or 1% parasitaemia) was seeded in a 96-well microplate, and 10 μL of a test material solution was added. After incubation at 37 °C for 72 h under 93% N_2, 4% CO_2 and 3% O_2, the plate was immediately frozen at −20 °C for 18 h. The plate was then thawed at 37 °C, and 20 μL of the haemolyzed parasite suspension was transferred to another plate containing 100 μL of Malstat reagent. The plate was further incubated for 15 minutes at room temperature, and 20 μL of a 1:1 mixture of nitroblue tetrazolium and phenazine ethosulfate (2 mg and 0.1 mg/mL, respectively) was added to each well. After incubation for 2 h at room temperature in the dark condition, the blue formazan product was measured at 655 nm by an iEMS microplate reader MF (Labosystems, Helsinki, Finland). The 50% inhibitory concentration (IC_{50}) value was estimated from a dose response curve, and evaluated the sample as potent (IC_{50}; <1.56 μg/mL), intermediate (IC_{50}; 1.56–6.25 μg/mL), weak (IC_{50}; 6.25–12.5 μg/mL), and no activity (IC_{50}; >12.5 μg/mL).

3.12. Antitrypanosomal activity

Trypanosoma brucei brucei GUTat 3.1 strain was a generous gift of Dr. Y. Yabu (Nagoya City University, Japan). *T. brucei brucei* GUTat 3.1 bloodstream-form trypomatigotes were maintained in Isocove's modified Dulbecco's medium (IMDM) with various supplements containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C, under 5% CO_2-95% air according to the method of Yabu et al. (1998).

In vitro antitrypanosomal activity of test sample has been estimated by a dose response curve using Alamar Blue sensitivity assay according to the method of Räz et al. (1997): bloodstream forms of *T. brucei brucei* were inoculated into 96-well microtiter plates (Costar, USA). The trypanosomes were incubated with the serial sample dilutions for 70 h at 37 °C in 5% CO_2. Serial dilutions of the standard drugs (see Table 1) were prepared and tested in the same manner. Control experiment was performed as previously mentioned, containing medium only. Then 10 μL Alamar Blue were added and after two hours of incubation, the fluorescence was determined at an excitation wavelength of 530 nm and an emission wavelength of 590 nm using a Gemini Plate Reader (Molecular Devices). Fluorescence development was expressed as the percentage of that of the control, and IC_{50s} were determined using a dose response curve. The criteria for the evaluation were the same as in antimalarial activity.

3.13. Cytotoxicity Test on MRC5 Cells

A human diploid embryonic cell line, MRC5 was used to test the cytotoxicity of samples. The cytotoxicity of the test material was measured by the colorimetric MTT assay (Mossman, 1983; Otoguro et al., 1991) in 96-well microplates. In brief, 100 μL of MRC5 cell suspension was added in 96-well microplates at 1×10^3 cells/well, and cultivated for 24 h. Then 90 μL of standard culture medium (MEM + 10% FCS) with or without 10 μL of test material solutions were added to each well. The cultures were further incubated at 37 °C under 5% CO_2-95% air for 7 days, and 20 μL of MTT–PBS solution (5 mg/mL) was added to each well. The plate was then incubated at 37 °C for 4 h under 5% CO_2-95% air. Then the incubation medium was aspirated, and 100 μL of DMSO was added to solubilize the MTT formazan product. After mixing, absorbance at 540 nm was measured with an iEMS microplate reader ME. The 50% inhibitory concentration (IC_{50}) value was estimated from a dose response curve.

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