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Mario Delgado Echeverry e Hijos Ltda.

Abogados Consultores – Marcas y Patentes

Bogotá D.C. - Colombia
Avda. Kra. 24 Park Way No 37 – 31 Of. 202
Tels. (571) 2444096 – 3690624
Fax (571) 2442496 A.A. No 6207
E-mail: mde@mdelaw.com
mdelaw@cable.net.co
mdeabogados@cable.net.co

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
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SEÑOR
SUPERINTENDENTE DE INDUSTRIA Y COMERCI
DIVISIÓN DE SIGNOS DISTINTIVOS
E. S. D.

Ref.: **Expediente No.: 05-122.635 Patente de Invención: PROCESOS PARA PRODUCIR EXTRACTOS DE CARALLUMA Y USOS.**

Atentamente se dirige a usted **JAIME EDUARDO DELGADO VILLEGAS**, Abogado, mayor y vecino de Bogotá D.C., e identificado como aparece al pie de mi firma para manifestarle lo siguiente:

A. Como apoderado de RAMASWAMY RAJENDRAN y KAMALA RAJENDRAN, domiciliados en India, solicité ante su Digno Despacho el registro de la Patente de Invención que se enuncia en la referencia.

B. Debido a lo anterior, y con el fin de dar cumplimiento al requerimiento hecho por su Despacho mediante Oficio No. 7103 del 30 de mayo de 2008, me permito aportar fotocopia sencilla de los siguientes artículos:

1 - RAMESH M. ET AL, "Antinociceptive and anti-inflammatory activity of carumbelloside-I isolated from Caralluma umbellata", Journal of Ethnopharmacology, 15.12.1999, Vol 68, No. 1-3, 349-352.

2 - USMANGHANI K., "Characterisation of Chemical Constituents and Toxicity Evaluation of Some Poisonous Plants", James L F et al (Ed) : Poisonous Plants; 3rd International Symposium, Logan, Utah, USA 1988; XV + 661P; Iowa State Univ. Press, Ames, Iowa USA, 1992. Pp 314-326.

3 - RIZWANI G H. ET AL., "Biological Efficacy of the extracts and Constituents of Caralluma tuberculata and C. edulis"; J. Fac. Pharm. Gazi Univ, 11, No. 1 43-53, Dept of Pharmacognosy, Fac. Of Pharmacy, Univ. of Karachi, Karachi 75270, PAKISTAN, 1994.

4 - AHMAD V U ET AL,: "New Pregnane Glycosides from Caralluma tuberculata". J of Nat. Prods (LLOYDIA) Vo. 51, No. 6, 1988, 1092-1097.

5 - RIZWANI G H, ET AL,: "Structures of Caratuberside E and F", Pharemazie, Vol 50, No. 6, 1995, 426-428.

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Fax (571) 2442496 A.A. No. 6207
E-mail: mde@mdelaw.com
mdelaw@cable.net.co
mdecolaborators@cable.net.co

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6 - RIZWANI G H, ET AL,: "Caratuberside A2: a New Pregnane from caralluma tuberculata", Spectroscopy Letters, Vo. 26, No. 8, 1427-1434.

7 - BADER A ET AL,: "Further Constituents from Caralluma negevensis", Phytochem., Vol 62,No 8, Apr. 2003, Pergamon press, GB.April 2003 (2003-04), pags. 1277-1281

8 – BRACA ET AL AL, "New Pregnane Glycosides from Caralluma negevensis", Tetrahedron, Vol. 58, No. 29, 15 Jul 2002, pags. 5837-5848.

9 - QUI S-X, ET AL,: "Acylated C21- Steroidal Bisdesmosidic Glycosides from Caralluma umbellata, Phytochemistry, Vo. 46, No. 2, Sept. 1997, 333-340.

10 - HALIM A F, ET AL, "Pregnane Glycosides from Caralluma retrospiciens, Phytochem". Vol 42, No. 4, 1996, pags. 1135-1139

11- ABDUL-AZIZ AL-YAHYA A M, ET AL, "Pregnane Glycosides from Caralluma russelliana", Journal of Natural Prods, Oct 2000, Vol 63, No. 10, pags. 1451-1453.

12 - ABDEL-SATTAR E, ET AL, "Penicillosides A-C, C-15 Oxypregnane Glycosides from Caralluma penicillata", Phytochem, Vol 57, No. 8, Aug 2001, 1213-1217.

13 - QUI S-X, ET AL, "Bisdesmosidic Pregnane Glycosides from Caralluma lasiantha"; Phytochem. Vol 50, No 3,1999, 485-491

14 - LEE K Y, ET AL, "New Acetylcholinesterase-Inhibitory Pregnane Glycosides of Cynanchum atratum Roots", Helvetica Chimica Acta 2003, Vol 86, No. 2, pags. 474-483.

15 - LEE JEONGHYUNG ET AL, "Multidrug Resistance Reversing and Antiangigenic Activity of Pregnane Glycosides from Cyanchum wilfordii", Proc. of the Amer. Assocn. for Cancer Res. Annual Meeting No 41,Mar. 2000, pag. 751, and 91st Annual Meeting of the Amer. Assen. for Cancer Res, San Francisco, CA USA, Apr. 1-5. 2000.

Los artículos que se mencionan con anterioridad se encuentran en su idioma original, debido a que en el presente Oficio el examinador no requiere de forma explícita las traducciones al español de los documentos solicitados.

En caso de que sea necesario presentar la traducción a la documentación adjunta, solicito comedidamente a su Despacho requerir con posterioridad mediante un Oficio explícito, indicando qué documentos deben ser traducidos -sólo en el caso de que sea necesario- puesto que este proceso implicaría un período de tiempo bastante amplio, así como altos costos.

Debido a la presentación a la contestación del Oficio 7103, solicito se continúe con el trámite normal de la Patente que se enuncia en la referencia, así como el estudio de los artículos que se adjuntan.

J. Fac. Pharm. Gazi
11 (1), 43-53 (1994)

BIOLOGICAL EFFICACY OF THE EXTRACTS AND CONSTITUENTS OF Caralluma tuberculata AND C. edulis

Ghazala H. RIZWANI(*), K. USMANGHANI(*), Mansoor AHMAD(*), Shahid RASHID(**), Viqar Uddin AHMAD(***)

Key Word Index :

Caralluma tuberculata, Caralluma edulis, Asclepiadaceae, toxicity, hypoglycaemic, cardiovascular, antibacterial, platelet activities.

ABSTRACT

Biological activity of the ethanolic and aqueous extracts of both plants as well as caratuberosides were evaluated. Brine shrimp (LC_{50}) bioassay of ethanolic and ethyl acetate extracts of both plants showed toxicity whereas aqueous extract was found to be non-toxic. The aqueous extract of Caralluma tuberculata was found to possess hypoglycaemic activity in alloxan treated rats. However, ^{3}H-ouabain binding assay with caratuberosides A-C, and an inhibitory effect of ethanolic extract of C. edulis on adrenaline induced platelet aggregation essentially exhibited no response.

INTRODUCTION

Caralluma tuberculata N.E. Brown, and C. edulis Edgew (Asclepiadaceae) succulent perennial herbs are widely used as

(*) Department of Pharmacognosy, Faculty of Pharmacy University of Karachi, Karachi 75270 PAKISTAN.

(**) Dept. of Pharmacology.

(***) H.E.J. Research Institute of Chemistry, University of Karachi.

antidiabetic. In the previous communications[1-4], we have already reported the active constituents including the caratubersides from this plant.

RESULTS AND DISCUSSION

The pharmacological activities of the extract and its chemical components were tested. The ethanolic extracts of C. tuberculata, containing pregnane glycosides, were subjected to different biological evaluation tests including the brine shrimp bioassay, hypoglycaemic activity, and cardiovascular profile. Moreover, pregnane-^{3}H-ouabain binding assay of the isolated pregnane glycosides from C. tuberculata, inhibition of, epinephrine-induced platelet aggregation on the ethanolic extract of C. edulis, were also investigated and reported in this communication.

Brine shrimp (LC_{50}) bioassay method was used to testify the efficacy of the extracts of C. tuberculata. Three fractions (i.e., ethanolic, ethyl acetate and aqueous) were subjected to brine shrimp bioassay as shown in Table-1. Ethanolic and ethyl acetate fractions displayed 35.36 LC_{50} at a concentration of 10, 100 and 1000µg/ml, while non-toxic effect (>1000 µg/ml) was observed in aqueous fraction. In other words we can say that it is a neutral fraction of C. tuberculata, the brine shrimp (LC_{50}) bioassay of ethanolic and ethyl acetate extract showed toxicity whereas aqueous extract was found to be non-toxic.

Hypoglycaemic activity of C. tuberculata ethanolic and aqueous extract was tested by determining the blood sugar lowering effect. The ethanolic and aqueous extracts of C. tuberculata showed hypoglycaemic activity at a dose of 71.42 mg/kg in alloxan fed diabetic male albino rats. The blood sugar level was reduced from 278.61 mg/100 ml to 248.37 mg/100 ml in ethanolic extract treated diabetic rats, while in aqueous extract treated rats blood sugar level was lowered from 317.63 mg/100 ml to 295.64 mg/100 ml after 30 to 60 minutes of extract administration, and these extracts raised the blood sugar after 3 hours. In aqueous extract treated rats the blood sugar level was significantly reduced from 314.63 mg/100 ml to 233.62 mg/100 ml after 24 hours. The fasting blood sugar in normal ethanolic extract treated rats was raised from

Table 1. Brine Shrimp Bioassay of Ethanolic, Ethyl Acetate And Aqueous Extracts of Caralluma tuberculata.

Types of extract	10 µg/ml		100 µg/ml		1000 µg/ml		Concentration LC_{50} µg/ml
	alive	death	alive	death	alive	death	
1 Ethanolic	16	14	5	25	—	30	35.3693
2 Ethyl acetate	26	4	5	25	—	30	8.9389
3 Aqueous	32	—	30	—	26	4	>1000

85.85 mg/100 ml to 138.46 mg/100 ml at 3-3.5 hours and 177.63 mg 100 ml after 24 hours.

The aqueous extract treated normal rats also showed similar effects at the same time intervals. Results of the mean blood glucose level in normal and alloxan fed rats are shown in Table-2. Among the two models used, the plant extracts showed a significant (<0.005) blood sugar lowering potency in fasted and alloxan fed models.

Akhtar[5] worked on the antidiabetic activity of C. edulis, and found that it does not lower the blood sugar level in rabbits. Whereas Ahmad et al[6]. reported that C. tuberculata aqueous extract significantly reduced the blood glucose level. However, the results of our experiments proved that it possess an antidiabetic activity in albino rats.

The results of the effects of ethanolic extract of C. edulis (i) on force of contraction on left ventricle, (ii) heart rate, (iii) coronary flow on isolated perfused rabbit heart were (in doses between 0.12 - 1000 µg) found to possess no effect on the cardiovascular system in terms of change of the contractile force, cardiac frequency and coronary out flow during one minute after administration (Table 3). It seems that the over all effect of the crude extract of C. edulis on these parameters is rather insignificant.

Table 2. Mean Blood Glucose Levels mg/100 ml ± S.E.M. of Normal And Alloxan-Diabetic Rats At Various Time Intervals After Oral Administration of Caralluma tuberculata N.E. Brown.

uralluma berculata xtract o. of perimants = 4	Normal albino rats				No. of experiments n = 4	Alloxan-Diabetic albino rats			
	B.Adm.	0 h	3 h	24 h		B.Adm.	0 h	3 h	24 h
ntrol	92.12 (±3.13)	100.75 (±1.71)	80.75 (±1.56)	115.16 (±1.87)		608.65 (±7.62)	488.25 (±10.19)	544.48 (±3.47)	742.86 (±6.34)
hanolic tract	85.85 (±1.99)	138.46** (±3.18)	142.63*** (±1.83)	177.63* (±1.90)		278.61*** (±4.34)	248.37*** (±3.27)	270.51*** (±2.60)	285.72*** (±2.83)
ueous tract	111.92* (±2.61)	142.54** (±2.19)	138.29*** (±2.14)	146.60 (±2.63)		317.63*** (±3.57)	295.64*** (±5.48)	314.22*** (±3.00)	233.62*** (±4.10)

Adm. = Before drug administration

, 3 h, 24 h = After 0, 3, 24 hours drug administration

l values represent glucose level in mg/100 ml as mean ± S.E.M.

gnificant relative to control *P<0.1, **P<0.01, ***P<0.005, n/ group = 4.

Table 3. **Effect of Aqueous Extract of Caralluma Edulis On Force of Cardiac Contraction, Cardiac Frequency And Coronary Out Flow of Isolated Perfused Rabbit Herat.**

	Percent change from the control		
Dose (μg)	Inotropic effect	Chronotropic effect	Effect on coronary out flow
0.1-1000	8.5 ± 1.54	18.2 ± 1.17	19.25 ± 2.46

n = 4

The results were expressed as mean ± standard error (S.E.M.).

Table 4. **^{3}H-Qubain-Binding Assay With Caratuberosides.**

Compound	IC_{50} (μM)
Caratuberoside A	260
Caratuberoside B	270
Caratuberoside C	580
5A	7600
Caratuberoside A + B	91
Progestrone	56
Quabain	0.012

The strength to this contention has been kindly provided by Professor LaBella, who very kindly tested the preguane glycosides isolated from C. tuberculata and C. edulis as shown in Table 4. The isolated pregnanes caratuberoside A, caratuberoside B, caratu-

Table 5. Effects On Aqueous Extract of Caralluma Edulis On Adrenaline Induced Aggregation of Human Platelets.

	% inhibition of adrenaline induced platelet aggregation			IC_{50} mg/ml
	Dose A (0.625 mg/ml)	Dose B (1.25 mg/ml)	Dose C (2.50 mg/ml)	
[aqueous (n = 4)]	-8.22 ± 5.96*	-0.82 ± 7.33*	7.81 ± 8.88*	

The results were expressed as mean ± standard error (S.E.M.).

*Not significantly different from control (P>0.05).

beroside C, and a mixture of caratuberoside A and B in ^{3}H-Ouabain-binding assay were found to be essentially inactive.

The aqueous extract of C. edulis was tested on adrenaline induced aggregation of human platelets. In n = 4 experiments, the dose A (0.625 mg/ml) showed -8.22 ± 5.96 % inhibition of adrenaline induced platelets aggregation, while dose B (1.25 mg/ml) -0.82 ± 7.33, and dose C (2.50 mg/ml) 7.81 ± 8.88. All these results were not significantly different from control (P>0.05). The extract alone, however, have no direct aggregatory effect (Table 5).

On these biological evaluation findings it is therefore concluded that caratuberoside molecules are basically inactive. Nonetheless the aqueous extract of C. tuberculata showed hypoglycaemic activity, and that is why this herb drug is used for lowering blood sugar level in folkloric medicine.

EXPERIMENTAL

The different extracts and caratuberosides were obtained according to the methods as given in the literature[1-4].

i) Brine Shrimp LC_{50} Bioassay Method :

Three different extracts of C. tuberculata, i.e. ethanolic, ethyl acetate and aqueous extracts were prepared for the estimation of LC_{50} activity in brine shrimp. The procedure described by Meyer et al[7]. and followed by McLaughlin et al[8]. and Alkofahi et al[9]. was adopted for this work.

Samples and discs of three different concentrations, 10, 100, 1000 µg/ml were prepared according to the direction as given in the literature[7-9]. Brine shrimp (Artemia salina) nauplii were hatched in a specific tank. Ten shrimps were transferred to each sample vial, and then sea water was added to make the volume 5 ml. Later on dry yeast suspension was added as food to each vial including control. The vials were kept for 24 hours, thereafter the active nauplii were counted, and death percentage was calculated at each dose.

ii) Hypoglycaemic Effects :

The ethanolic (EE) and aqueous (AE) extracts of C. tuberculata were diluted in distilled water, respectively, and then were evaluated for the hypoglycaemic activity in both normal and alloxan treated male albino rats of 200-250 g weight. The hyperglycaemia was induced by administering alloxan monohydrate (Ogawa Seiki Co. Ltd., Tokyo, Japan) (150 mg/kg, i.p. body weight). Rats were maintained on diet soaked with hydrogenated oil.

The ethanolic (EE) and aqueous (AE) extracts were administered at a dose of 100 mg/kg dissolved in water. The blood glucose levels were estimated by the method of Nelson and Somogyi as given by Varley[10] on 0.1 ml of blood drawn from animals at 0,3 and 24 hours' interval. The results were recorded as shown in the Table 2.

iii) Cardiovascular Profile :

The whole plant material of C. edulis was collected from Karachi in September 1988. Fresh plant material after chopping was soaked in EtOH for about four weeks. The alcoholic extract was evaporated under reduced pressure and the syrupy residue so obtained

was dissolved in small quantity of H_2O and subjected to freeze drying. 25 g powder of the crude extract so obtained was used for cardiovascular screening.

In this study, isolated perfused rabbit's heart method as described by Langendroff[11] with modification was used[12]. Briefly healthy rabbits (1-2 kg) of either sex were used. A blow on the neck of the rabbit made them unconcious. The chest was open and the heart was carefully removed in a petridish filled with McEwen's solution (NaCl : 7.60 g/l, KCl : 0.42 g/l, $CaCl_2$: 0.24 g/l, $NaHCO_3$: 2.10 g/l, glucose : 2.00 g/l, sucrose 4.50 g/l), and bubbled with oxygen. Isolated heart was mounted on the apparatus with the help of glass cannula and perfused with McEven's solution at 37°C. Recordings were made by the help of isotonic transducer (Harvard) on chart recorder (Eyla). Parameter measured were : (1) force of contraction (height in mm), (2) heart rate (beats/min), (3) coronary flow (ml/min). Atmospheric pressure on the heart was kept at 45 mm Hg, approximately. No changes weer introduced during these experiments (Fig. 1, Table 3).

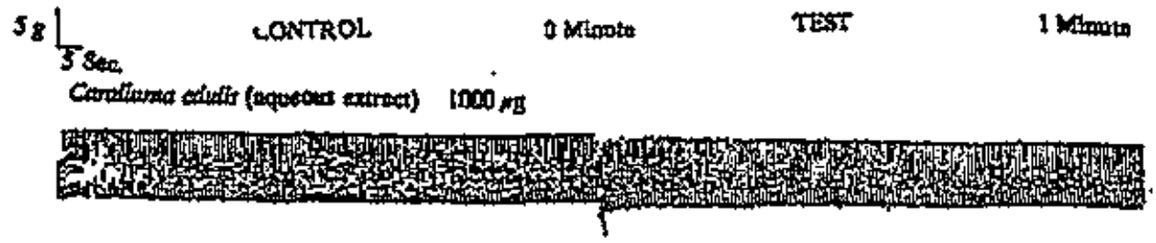

Fig. 1 : Effects of aqueous plant extract (Caralluma edulis) on spontaneous contractions of isolated perfused rabbit heart. A constant dose of 1000 µg was used for all experiments. Dose administration occurred at 0 minute.

iv) Pregnane ^{3}H-Ouabain Binding Assay :

Prof. Dr. LaBella[13] was kind enough to carry out the isolated pregnanes caratuberoside A caratuberoside B caratuberoside C and a mixture of caratuberoside A and B in ^{3}H-Ouabain binding assay (Table 4).

v) Inhibition of Epinephrine Induced Platelet Aggregation :

The experiments on the ethanolic extract of C. edulis for its inhibition of platelet aggregation property were performed by the method of adrenaline (epinephrine) induced platelet aggregation[14, 15].

Preparation of the test solution :

Plant extract was dissolved in 0.2 % w/v sodium carbonate (E. Merck, Germany) to provide a stock solution of 60 mg/ml (c). Subsequent dilutions of 30 mg/ml^{-1} (b) and 15 mg/ml (a) were prepared. About 10 µl of each solution was added to 225 µl of platelet rich plasma, sot hat the final concentrations were 2.5 mg/ml (Conc. C) 1.25 mg/ml (Conc. B) and 0.625 mg/ml (Conc. A), respectively. The pH of the solution C was noted using a pH paper (Machery-Nagel Co., Germany). The test solutions A, B and C were freshly prepared for all experiments.

Preparation of aggregating agent :

A 4 mM solution of adrenaline bitartarate (MW = 333.33, Sigma, St. Louis, USA) was prepared in 0.9 % w/v sodium chloride in distiled water. After use, it was stored at -20°C.

Platelet rich plasma (PRP) and Platelet poor plasma (PPP) :

Platelet rich plasma (PRP) was prepared from whole blood taken from a healthy person, who has denied receiving any medicine within 14 days, by centrifugation for 10 minutes at 1200 rpm at room temperature (20-25°C) in HIMAC centrifuge (Hitachi, Japan) HIMAC and was used within 3 hours after blood collection. The settled portion of blood, from which PRP had been removed, was then centrifuged at 3200 rpm for 15 minutes and the supernatant PPP was obtained.

Platelet aggregation :

Platelet aggregation was perfomed in NKK Hema Tracer (Model PAT 4M, Niko Bioscience Inc., Japan) equipped with a four channel chart recorder. The instrument was first calibrated with PRP at zero % and PPP at 100 % light transmission.

Incubation with test substances and aggregations were carried out at 37°C with continuous stirring at 1000 rpm. About 10 µl of test solution 'A' (15 mg/ml), 'B' (30 mg/ml) and 'C' (60 mg/ml) were added to cuvettes containing 225 µl PRP and allowed to incubata for about 2 minutes before challenging with 5 µl of 4 mM adrenaline solution. The control response was also measured along with the response of conc. A (0.625 mg/ml), conc. B (1.25 mg/ml) and conc. C (2.5 mg/ml) for a period of 5 minutes (Fig. 2, Table 5).

For each experiment the height in chart units was measured at the end of the fifth minute and determined and the results were expressed as percentage inhibition from the control, according to the following formula :

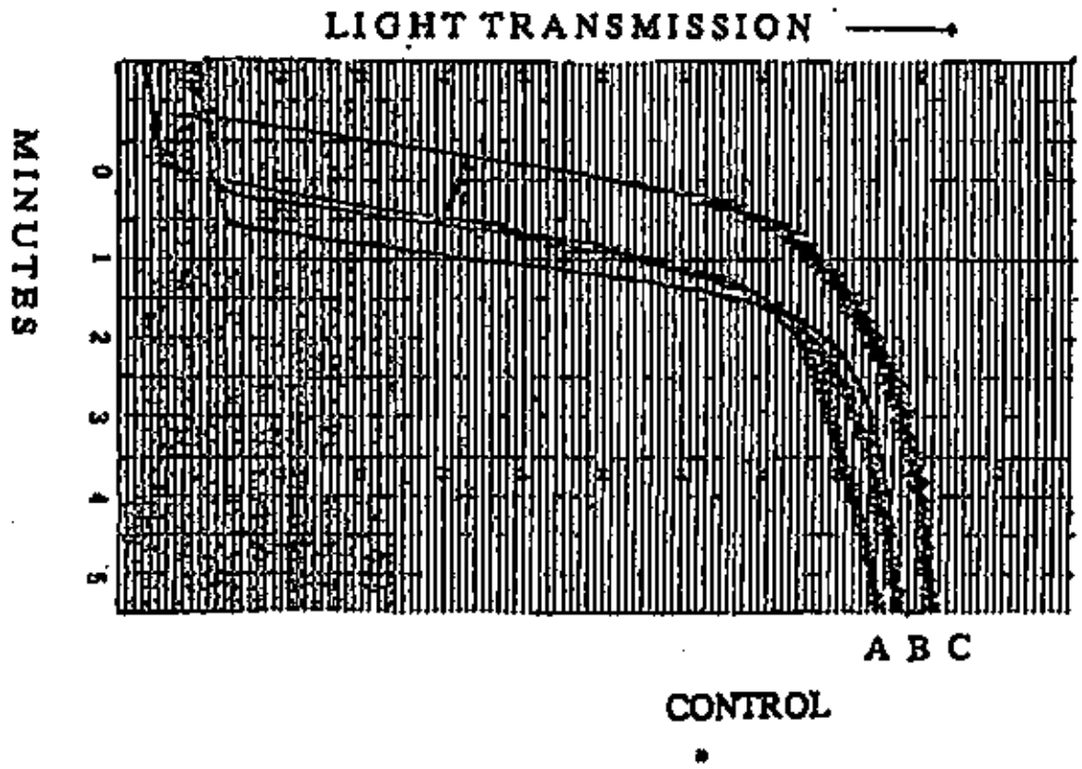

Fig. 2 : Representative tracing showing the effect of concentration A (0.625 mg/ml), B (1.25 mg/ml) and C (2.5 mg/ml) of aqueous extract of Caralluma edulis (upper panel) on adrenaline-induced aggregation of human platelets. RRP was incubated for 2 minutes with the plant extract before adding adrenaline (4 mM).

$$\% \text{ inhibition} = \frac{C - T}{3} \times 100\ \%$$

where C = % aggregation of the control.

T = % Aggregation after 2 minutes incubation with test solution.

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NEW PREGNANE GLYCOSIDES FROM *CARALLUMA TUBERCULATA*

VIQAR UDDIN AHMAD,*

H.E.J. Research Institute of Chemistry

KHAN USMANGHANI, and GHAZALA H. RIZWANI

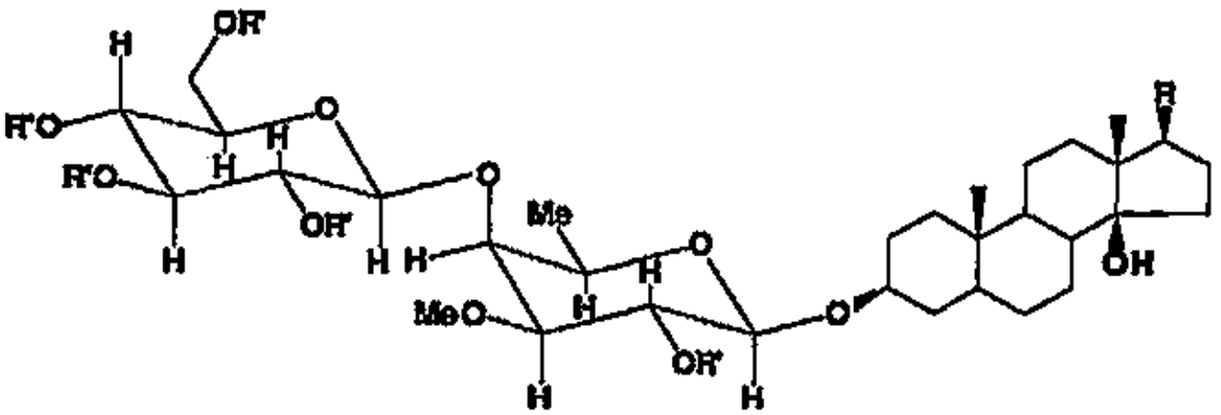
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi-32, Pakistan

ABSTRACT.—Two new pregnane glycosides, named caratubersides A and B, were isolated from the whole plant of *Caralluma tuberculata*. Their structures were determined as 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)-galactopyranosyl]-14-hydroxy-14β-pregnane-20-one [1] and 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxygalactopyranosyl)]-14,20-dihydroxy-14β-pregnane [2] mainly on the basis of spectroscopic studies. Their stereochemistry has been determined by nOe difference, COSY 45, and 2D *J*-resolved NOESY ^{1}H-nmr and DEPT ^{13}C-nmr experiments.

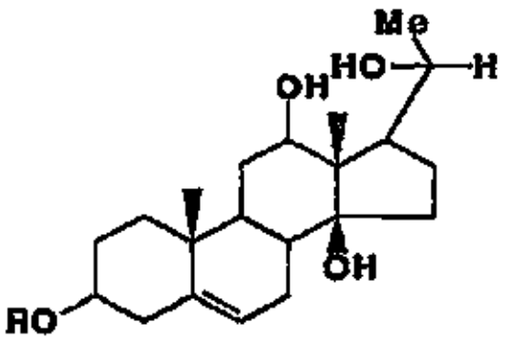
Caralluma tuberculata N.E. Brown (Asclepiadaceae) (syn. *Boucerosia aucheriana* Decn.) is a succulent perennial herb occurring wild and also cultivated throughout Pakistan (1–3). This plant is eaten raw or cooked as a vegetable and is also reputed to be a cure for diabetes and rheumatism (4). In 1967, Nikaido *et al.* (5) reported the isolation and structure elucidation of two genins, boucerin [4] and dihydroboucerin [5], from *B. aucheriana* Decn., a synonym of *C. tuberculata*. After hydrolysis of the glycosides, these authors also identified cymarose, sarmentose, oleandrose, and digitoxose in the hydrolysate. Apart from this work no other chemical investigation on this species is reported in the literature. This paper presents the isolation and characterization of two new pregnane glycosides.

RESULTS AND DISCUSSION

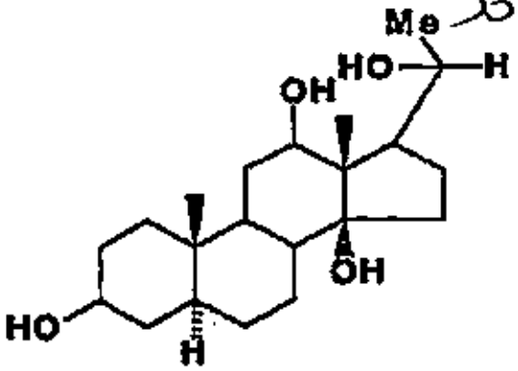
The EtOH extract of the whole plant of *C. tuberculata* yielded, after evaporation, fractionation, and repeated chromatographic separation on Si gel columns, a mixture of 1 and 2. The mixture gave positive tests with Liebermann-Burchard reagent (for sterols), vanillin sulfate (for genins), and Molisch reagent (for sugars). Glycosides 1 and 2 were separated by preparative hplc employing a Bond-Pak C-18 reversed-phase column, with MeOH-H_2O (80:20) as the mobile phase.



1 R=-CCH$_3$, R'=H
2 R=-CH(OH)CH$_3$, R'=H



4



5

Caratubeside A [1] was obtained as a white, crystalline substance, mp 170–171°, $[\alpha]^{20}D$ +60° (c = 0.66 in MeOH). On acidic hydrolysis with 1% HCl, 1 yielded a crystalline genin as well as glucose and another sugar. Glucose was identified by tlc as well as hplc. The structure of the second sugar was determined as 6-deoxy-3-*O*-methyl-β-D-galactose on the basis of extensive nmr studies including ^{1}H nmr (COSY-45, *J*-resolved, NOESY, nOe difference) and ^{13}C nmr (broad band and DEPT experiments) on **1** as described below.

Caratubeside A [1] displayed no absorption in the uv region indicating the absence of any conjugated double bonds in the molecule. The ir spectrum (KBr) revealed peaks at max 3300 (OH), 2900 (CH_2), 1690 (C=O), 1100 (C-O) cm^{-1}. In the negative ion fabms the compound showed an $[M-H]^-$ peak at *m/z* 655. In the eims spectrum the highest peak at *m/z* 477 was due to the removal of the terminal glucose unit, indicating the 6-deoxy-3-*O*-methyl-D-galactose is directly attached to the aglycone. There was also a strong peak at *m/z* 317 due to the aglycone without oxygen at C-3 and a base peak at *m/z* 299 $[317-H_2O]^+$.

The aglycone was obtained as a crystalline compound by acidic hydrolysis, and its structure was confirmed by spectroscopy. Because *C. tuberculata* belongs to the Asclepiadaceae, which are known to contain pregnane glycosides, and because the pregnane genins, boucerin and dihydroboucerin, have actually been isolated from the acid hydrolysate of the glycosides of this plant, it was expected that the aglycone could possibly be a pregnane type steroid. The ^{1}H- and ^{13}C-nmr spectra of **1** confirmed this assumption. However, the ^{13}C-nmr spectrum showed that an oxygen function at C-12 commonly found in pregnane aglycones of the Asclepiadaceae is missing (C-12, 39.44 ppm). The chemical shift of C-13 at 49.49 ppm with only one hydroxyl-bearing vicinal carbon atom (C-14, 84.76 ppm) and the chemical shift of C-11 at 21.63 ppm also support the absence of oxygen function in the neighboring C-12. This fact is further confirmed in the mass spectral data discussed above.

The ^{1}H-nmr of **1** in CD_3OD reveals methyl singlets at δ 0.83 (3H, H-19), 1.08 (3H, H-18), and 2.33 (3H, H-21). The last peak indicates the presence of a $COCH_3$ group in the compound. A doublet at δ 1.28 (3H, J = 6 Hz) is assigned to the secondary methyl group in the 6-deoxy sugar, and a singlet at δ 3.49 is assigned to the OMe group of the methylated sugar. A triplet at δ 2.90 (J = 5.6 Hz) can be assigned to H-17 vicinal to the ketonic group. This chemical shift and coupling are very close to those reported for H-17 in 13β,14-dihydroxy-14β-pregn-5-en-20-one (6). It is, therefore, concluded that the configuration of **1** at C-17 is the same as in this latter compound; i.e., the side chain at C-17 is β-oriented. The spectrum further shows two anomeric proton signals at δ 4.35 (d, J = 7.7 Hz) and 4.60 (J = 7.6 Hz). The latter is assigned to the anomeric proton of glucose, whereas the former is assigned to the H-1 signal of the

pling, hence β-glycosidic linkages. It also proves that the hydroxylic groups at the neighboring carbon atoms in the two sugars (C-2′ and C-2″) have the same equatorial configuration. It may be noted here that the Keller-Killiani test for 2-desoxy sugars gave negative results with **1**. This means that the 6-desoxy sugar possessed an OH group at C-2′.

Decoupling experiments, as well as COSY-45 experiments, show that the H-1′ doublet at δ 4.35 is coupled with a double doublet at δ 3.59 (J = 7.7, 10 Hz) due to H-2′. This means that $J_{2',3'}$ is equal to 10 Hz, and, therefore, H-3′ must also be axially oriented. The H-3′ signal becomes clear only in the ^{1}H-nmr spectrum of caratuberside A pentaacetate [3] where this signal is not shifted downfield because it bears a methoxy rather than a hydroxy group. It absorbs as a double doublet at δ 3.23 (J = 10, 2.5 Hz), which indicates that the methoxy group at C-3′ is equatorial and H-3′ is axial. The smaller $J_{3',4'}$ (2.5 Hz) also shows that H-4′ is equatorial. H-4′ absorbs as a broad doublet at δ 4.17; the $J_{4',5'}$ is very small and could not be calculated. H-5′ absorbs as a broad quartet at δ 3.65 (J = 6.3 Hz) which is coupled to the doublet at δ 1.28 as shown by the decoupling experiment or irradiation of the methyl doublet at 1.28 when this quartet is converted into a broad singlet.

The configuration of the methyl group (H-6′) was determined by nOe difference measurements of **3**. Irradiation of the H-5′ quartet at δ 3.49 resulted in 13.6% increase of the signal at δ 3.2 (H-3′) and 10.2% increase in the intensity of the doublet at δ 4.37 (H-1′). The intensity of the methyl doublet at δ 1.2 also increased by 19.3%. On the other hand, irradiation of the H-3′ double doublet at δ 3.2 led to 5.6% nOe of H-1′ at 4.37 but no increase in the intensity of methyl doublet at δ 1.2. This indicates that H-1′, H-3′, and H-5′ are axial, and 6-Me is equatorial. Thus, the methylated sugar was identified as 6-deoxy-3-*O*-methyl-D-galactose. The ^{13}C-nmr spectrum of **1** (Table 1) supports its proposed structure. It clearly shows the absence of olefinic carbon atoms, indicating that there is no carbon double bond in the compound. The assignments were aided by DEPT experiments as well as correlation with ^{13}C-nmr spectra of similar types of compounds (6).

Caratuberside B [2] was also obtained as a white crystalline compound, mp 182° (dec), which on acidic hydrolysis gave a pure genin as well as glucose and 6-deoxy-3-*O*-methyl-β-D-galactose. Caratuberside B does not show a carbonyl absorption in the ir spectrum. In the ^{13}C-nmr spectrum the peak assigned to the carbonyl carbon (C-20) of **1** is missing. Instead, a peak at 65.30 ppm is present indicating an additional hydroxyl-bearing carbon which, according to the DEPT spectrum, is secondary. These observations together with the $[M-H]^-$ peak at *m/z* 657 in the negative ion fab spectrum suggested that the carbonyl group in **1** has been reduced to a secondary alcohol in **2**. This is supported by the observation that the peak at 63.01 ppm (C-17) is shifted upfield to 57.08 ppm and the C-21 methyl peak from 32.37 ppm in **1** to 21.53 ppm in **2**. The correlation between **1** and **2** was confirmed by reduction of **1** to **2** with $NaBH_4$. Thus, the structure of **2** is proved to be 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-desoxygalactopyranosyl]-14,20-dihydroxy-14β-pregnane.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on Electrochemical Gallenkamp apparatus (capillary) and are uncorrected. Optical rotations were measured with a JASCO Dip-140 digital polarimeter. Tlc was carried out on Si gel PF 254 precoated glass plates (Merck 70–230 mesh ASTM) using the following solvent systems: (A) $CHCl_3$-MeOH-H_2O (7.5:2.5:0.2), (B) *n*-BuOH-HOAc-H_2O (12:3:5). Paper chromatography for sugars was run on Whatman paper No. 1 using solvent system (C) EtOAc-MeOH-H_2O-HOAc (65:15:15:20). A saturated solution of aniline phthalate in *n*-BuOH was used as a staining agent. Hplc analysis was performed with a LDC Constametric model III in-

TABLE 1. ^{13}C-nmr Data of Caracubenosides A [1] and B [2] in C_5D_5N (75.43 MHz).

Carbon	Compound	
	1	2
C-1	37.49	37.63
C-2	29.96	30.10
C-3	71.53	72.03
C-4	34.74	34.88
C-5	44.48	44.65
C-6	29.21	29.40
C-7	28.14	28.18
C-8	40.63	40.91
C-9	49.65	50.05
C-10	36.07	36.18
C-11	21.63	22.86
C-12	39.44	40.91
C-13	49.49	49.09
C-14	84.76	83.83
C-15	32.02	33.01
C-16	24.84	18.85
C-17	63.01	57.08
C-18	12.22	12.36
C-19	15.73	15.48
C-20	216.39	65.30
C-21	32.57	21.55
C-1'	102.41	102.51
C-2'	76.80	76.84
C-3'	85.50	85.58
C-4'	77.30	77.48
C-5'	70.48	70.55
C-6'	17.76	17.82
C-1"	105.51	105.58
C-2"	76.12	76.17
C-3"	78.34	78.41
C-4"	71.94	71.61
C-5"	78.51	78.59
C-6"	63.18	63.25
OMe	59.50	59.05

III machine with refractive index detector. Uv was recorded in MeOH on a Shimadzu uv-240 spectrophotometer. Ir spectra were recorded in KBr on Jasco A-302 infrared spectrophotometer. Electron impact mass spectra were determined on a Finnigan MAT 312 Varian MAT 112 double focusing mass spectrometer connected to a PDP 11/34 (DEC) computer system. Fast atomic bombardment mass spectral analysis was performed on the Varian MAT 312 fitted with a standard source and a high-field magnet. ^{1}H-nmr spectra were obtained from a Bruker AM-300 (300 MHz) spectrometer using both CD_3OD and C_5D_5N as solvents and TMS as an internal standard (s, singlet; d, doublet; q, quartet; m, multiplet). ^{1}H chemical shifts are reported from TMS and coupling constants are in Hz. ^{13}C-nmr spectra were measured in C_5D_5N and CD_3OD at 75.43 MHz with TMS as an internal standard using the Bruker AM-300 spectrometer. High-field ^{1}H-nmr spectroscopy was performed on a Bruker WM 400 instrument equipped with an Aspect 2000 computer. Two-dimensional COSY-45 experiments were performed at 400 MHz.

PLANT MATERIAL.—The whole plant of *C. tuberculata* (25 kg) was purchased from the vegetable market of Karachi, and identification was kindly carried out by Prof. Dr. S.I. Ali, Department of Botany, University of Karachi. A voucher specimen is deposited on a herbarium sheet in our department.

EXTRACTION AND SEPARATION.—The fresh material was chopped into small pieces and extracted with EtOH after percolation at room temperature for 15 days. The EtOH extract was evaporated under re-

partitioned between H_2O and *n*-BuOH. The *n*-BuOH extract (73 g) was redissolved in a small quantity of MeOH, then poured into cold Et_2O to precipitate the crude glycoside mixture. This was repeated several times, and the yellow precipitate was collected by filtration. The crude mixture (43 g) gave a positive test with Liebermann-Burchard, vanillin sulfate, and ceric sulfate reagents, whereas Keller-Kiliani and xanthydrol tests for 2-deoxy sugars were negative. The crude glycoside (36 g) was subjected to cc and eluted with $CHCl_3$-MeOH (9:1) which gave a mixture of two components. These two components were detected on tlc (PF 254) with ceric sulfate reagent after development with $CHCl_3$-MeOH-H_2O (7.5:2.5:0.5). The mixture of these two components was separated after repeated hplc on a reversed-phase Bondapak C-18 semipreparative column by using MeOH-H_2O (80:20) as a solvent with flow rate 5 ml/min, yielding caratuberside A (50 mg) and caratuberside B (25 mg).

CARATUBERSIDE A.—Caratuberside A [1] was obtained as a white, crystalline substance which was recrystallized from aqueous MeOH, mp 170–171°, $[\alpha]^{20}D$ +60° (c = 0.66, MeOH). Ir (KBr) ν max 3500 (OH), 2900 (CH_2), 1690 (C=O), 1100 (C-O) cm^{-1}; ^1H nmr (CD_3OD) δ 0.83 (3H, s, 19-Me), 1.08 (3H, s, 18-Me), 1.28 (3H, d, J = 6.0 Hz, 6-Me of methyldeoxygalactose), 2.23 (3H, s, 21-Me), 2.90 (1H, t, J = 5.6 Hz, H-17), 3.49 (3H, s, O-Me of methyldeoxygalactose), 3.5 (1H, m, H-3), 4.44 (1H, s, 14-OH), 4.35, 4.60 (two anomeric protons, J = 7.7 Hz and 7.6 Hz); ^{13}C-nmr see Table 1; fabms (negative ion mode) *m/z* $[M-H]^-$ 655 (100%); eims *m/z* (rel. int.) 477 (2), 345 (20), 317 (53), 299 (100), 281 (36), 219 (27), 161 (38).

ACID HYDROLYSIS OF CARATUBERSIDE A.—Caratuberside A (10 mg) was refluxed with 1% HCl (10 ml) in a boiling H_2O bath for 5 h. Thereafter, 10 ml H_2O was added and concentrated to 5 ml, and the reaction mixture was kept in a boiling H_2O bath for 30 min and extracted with $CHCl_3$. The organic extract so obtained was washed with H_2O and neutralized with 2 N Na_2CO_3, and the aqueous phase was again extracted with $CHCl_3$. On evaporation of $CHCl_3$, the precipitate was filtered off, and the filtrate was evaporated to dryness, then crystallized from aqueous MeOH to give the genin of caratuberside A (1.5 mg). The spectra of the isolated compound were compared with the spectra of caratuberside glycoside A and the spectral data of 13,14-dihydroxy-14-β-pregnane-5-ene-20-one (6). Colorless, sharp crystals ($CHCl_3$), mp 205–210°; ir ν max ($CDCl_3$) 3400–3500 (OH), 1690 (C=O) cm^{-1}; eims *m/z* 318 $[M]^+$, 301 $[M-H_2O]^+$, 296, 281, 271, 255, 228, 43 (base peak); ^1H nmr (300 MHz, $CDCl_3$) δ 1.00 (6H, s, 18-Me, 19-Me), 2.25 (3H, s, 21-Me), 2.95 (1H, t, 5.6 Hz, H-17), 3.47 (1H, m, H-3), 4.40 (1H, s, 14-OH); ^{13}C nmr (75.43 MHz, C_5D_5N) δ 37.49 (C-1), 29.96 (C-2), 17.49 (C-3), 34.71 (C-4), 44.49 (C-5), 29.20 (C-6), 28.15 (C-7), 40.39 (C-8), 49.69 (C-9), 36.04 (C-10), 21.62 (C-11), 39.44 (C-12), 49.46 (C-13), 83.79 (C-14), 32.00 (C-15), 25.80 (C-16), 62.89 (C-17), 12.22 (C-18), 15.72 (C-19), 218.39 (C-20), 32.29 (C-21).

The combined aqueous phase of the reaction mixture was concentrated under reduced pressure, tested for carbohydrates by tlc using EtOAc-MeOH-H_2O-HOAc (65:15:15:20) as a solvent system, and sprayed with freshly prepared aniline phthalate reagent. R_f values were calculated as 0.55 (glucose) and 0.75 (methyldeoxygalactose) (also confirmed by hplc).

ACETYLATION OF CARATUBERSIDE A.—Acetylation of caratuberside A (5 mg) was carried out in C_5H_5N (1 ml) and Ac_2O (2 ml), and the reaction mixture was left at room temperature overnight, then poured into ice H_2O and extracted with EtOAc. The EtOAc extract was concentrated in vacuum followed by Si gel cc (1% MeOH in $CHCl_3$) of the residue to give caratuberside A pentaacetate (2.32 mg). It forms colorless needles with $CHCl_3$; mp 190°; ir ($CDCl_3$) ν max 1725 (OAc), 1100 (C-O) cm^{-1}; eims *m/z* 523 $[M-OCOMe]^+$, 481, 439, 377, 177, 145, 74 (base peak); ^1H nmr (400 MHz, $CDCl_3$), δ 1.00 (6H, s, 18-Me and 19-Me), 1.99, 2.01, 2.057, 2.051, 2.10 (pentaacetate), 2.96 (1H, t, J = 5.6 Hz, H-17), 4.44 (1H, s, 14-OH).

REDUCTION OF CARATUBERSIDE A.—Compound 1 (5 mg) in MeOH (10 ml) was treated with an aqueous solution of $NaBH_4$ (15 mg), and the reaction mixture was stirred for 3 h. Dilute H_2SO_4 was then introduced into the reaction mixture. After neutralization and preparative layer chromatography [Si gel PF 254, $CHCl_3$-MeOH-H_2O (7.5:2.5:0.2)] of the reduction product, a pure compound (13.5 mg) was obtained which was found to be identical (^1H-nmr, ^{13}C-nmr, ir, ms, tlc) with caratuberside B.

CARATUBERSIDE B.—Colorless, shiny crystalline compound: mp 182–185° (dec) from aqueous MeOH; ir ν max (KBr) 3300–3400 (OH), 1100 (C-O) cm^{-1}; fabms negative ion *m/z* 657 $[M-H]^-$; eims *m/z* (rel: inc.) 343 (4), 317 (7.5), 301 (5.8), 299 (20), 283 (16), 257 (6), 55 (100); ^1H nmr (300 MHz, C_5D_5N) δ 0.70 (3H, s, 19-Me), 0.90 (3H, d, J = 9.0 Hz, 18-Me), 1.60 (3H, d, J = 6.3 Hz, 6-Me of methyldeoxygalactose), 2.20 (3H, s, 21-Me), 3.70 (3H, s, OMe of galactose), 4.34 and 4.60 (dd, J = 9.6 and 9.8 Hz, anomeric protons); ^{13}C-nmr see Table 1.

[illegible] B.—Caratuberside B (10 mg) was hydrolyzed as described

for caruxberside A. The identities of the genins and sugars were confirmed by chromatography and spectroscopy.

ACKNOWLEDGMENTS

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[illegible], Pakistan

Structures of Caratuberside E and F

GHAZALA H. RIZWANI[1], K. USMANGHANI[1], MANSOOR AHMAD[1] and ANWAR U. AHMED[2]

Ethanolic extract of whole plant of *Caralluma tuberculata* yielded two new pregnane glycoside caratuberside E and F, on repeated column chromatography. The Chemical structures were established on the basis of common and selective NMR spectroscopy. They were characterized as 3-O-[β-D-glucopyranosyl (1 → 6)-β-D glucopyranosyl(1 → 4)-β-D-(3-O-methyl-6-deoxygalactopyranosyl)]-14β-hydroxyl-pregn-20-one, and 3-O-[β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl (1 → 4)-β-D-(3-O-methyl-6-deoxygalactopyranosyl)-14β-20-dihydroxy-pregnane.

Die Struktur von Caratuberosid E und F

Mittels Säulenchromatographie wurden aus dem ethanolischen Extrakt aus *Caralluma tuberculata* die beiden neuen Pregnan-Glykoside Caratuberosid E und F isoliert. Die Strukturen wurden mittels NMR-Spektroskopie bestimmt. Es handelt sich um 3-O-[β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 4)-β-D-(3-O-methyl-6-deoxygalactopyranosyl]]-14β-hydroxyl-pregn-20-on und 3-O-[β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl(1 → 4)-β-D-(3-O-methyl-6-deoxy-galactropyranosyl)-14β-20-dihydroxy-pregnan.

1. Introduction

In an earlier investigation of constituents of *Caralluma tuberculata* (Asclepidaceae), Caratuberside A, B, C and D [1, 2] were reported. In this communication the isolation and structure of two new pregnane glycosides, Caratuberside E (1) and F (2) from the same source are described. The *ethanolic* extract, as previously reported [1] was repeatedly chromatographed over silica gel, flash and HPLC chromatography to give caratuberside E and F.

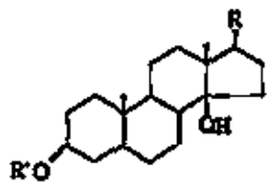
2. Investigations, results and discussion

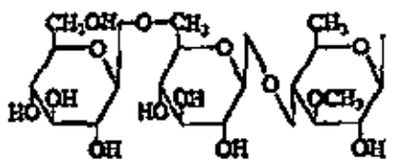
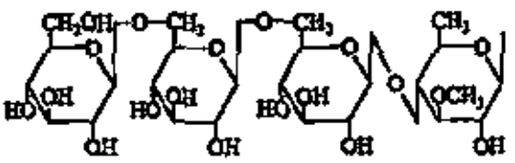
The crude mixture of glycosides of *Caralluma tuberculata* yielded two compounds 1 and 2 by silica gel open column, flash (resin) and HPLC (RP-18) CC.

Both compounds 1 and 2 are pregnane derivatives, while 1 possesses a carbonyl group at C-20, 2 bears a hydroxyl group at the same position. The IR spectrum showed carbonyl absorption at 1690 and 3300 cm^{-1}. The aglycone parts of 1 and 2 are similar to the already reported compounds caratuberside A and caratuberside B, respectively [1].

Compound 1 had a molecular weight of 818 which corresponded to a molecular formula of $C_{40}H_{66}O_{12}$. In FAB-MS (positive ion mode) it showed a molecular ion peak at m/z 819 [M + H, 20%] while in FAB-MS (negative ion mode) a peak at m/z 817 [M − H, 51%] was obtained. The fragmentation *pattern* was *deduced* from FI-MS *spectrum*, which displayed peaks at m/z 477 [M* − two hexose units], and 317 [M − three hexose units]. This compound was subjected to acid hydrolysis. It yielded glucose, 6-deoxy-3-O-methyl-galactose, genin and many other hydrolysed products formed due to the decomposition of genin and sugars. The hydrolysed products were compared with the hydrolysed products of caratuberside A and B [1] on silica gel and HPTLC plates in different solvent systems. The aglycone was found to be identical with caratuberside A.

The mass fragmentation pattern suggested that 1 consists of three sugar units. Further, the ^1H NMR spectrum displayed three anomeric proton signals at 4.38 (J = 7.6 Hz), 4.62 (J = 7.6 Hz) and 4.78 (J = 7.7 Hz) ppm. The later two signals were assigned to the anomeric protons of glucose, whereas the former was *assigned* to the H-1 signal of *methylated* deoxygalactose. In both cases the coupling constants indicate diaxial coupling hence β-glycosidic linkages. It was also proved that hydroxyl groups at the neighbouring carbon atom



	R	R'
1	$CH_3-\overset{O}{\overset{\|}{C}}-$	
2	$CH_3-CH(OH)-$	

[illegible] test for 2-deoxy sugars was negative as in the case of curatuberside A [1].

From the ^1H NMR spectrum the determination of linkage among the sugar moieties was carried out. The signals present at 3.82 and 3.60 (dd, J = 2.1, 6.6, 11.6, 11.6 Hz) ppm were assigned to C-6''', and the signals occuring downfield at 4.13 and 3.76 (dd, J = 2.5, 6.5, 11.6, 11.6 Hz) ppm indicated a substitution at C-6'' of the glucose unit.

The ^{13}C NMR signals of pregnane type derivatives were assigned using known chemical shift rules, such as OH substituent shift, acetylation shift, steric and β effects [3–8] and by chemical shift comparisons with previously reported data [1]. On the whole, the ^{13}C NMR spectrum showed 40 signals. From these, 21 signals were alloted to the pregnane skeleton having a carbonyl group at C-20, and 19 to three sugar moieties (one digitalose unit and two glucose units). The point of linkage between the aglycone, digitalose and glucose were confirmed by the occurrence of signals at 77.40, [illegible] (3-1'), 79.54, 104.26 (4' → 1'') 70.36, 105.64 (6'' → 1''') ppm. The signal at 219.68 ppm was assigned to the carbon of carbonyl group present in aglycone moiety.

The final confirmation of the proposed structure was carried out by COSY-45, NOSY, C–H correlation and decoupling experiments. Mild acid hydrolysis was made to determine and identify the terminal sugar. With the help of pyridine and acetic anhydride 1 was derivatised. This acetylated product was used in the determination of molecular weight, mass fragmentation, position of OH group and steric effects.

Compound 1 was characterized as 3-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl(1 → 4)-β-D(3-O-methyl-6-deoxy-galactopyranosyl)]-14β-hydroxy-pregn-20-one.

Compound 2 showed a similarity which previously reported compound curatuberside B [1], whereas it varied in the number of sugar moieties. The number and type of sugars were determined by MS, NMR, hydrolysis and comparison with authentic sample on TLC ad HPTLC in different solvent systems.

The molecular formula of 2 corresponded to $C_{46}H_{72}O_{21}$. The FAB-Ms (negative ion mode) furnished a molecular ion peak at [illegible]981 $[M-H]^-$, as well as other peaks at m/z 819 [M-one hexose unit], 657 [M-two hexose unit], 317 [M-four hexose units], and 339 [aglycone or genin].

The FAB-MS fragmentation pattern suggested that 2 consists of four sugar units. Furthermore, the ^1H NMR spectrum also showed four signals (doublets) at 4.35 (J = 7.6 Hz), 4.39 (J = 7.7 Hz), 4.59 (J = 7.7 Hz), 4.59 (J = 7.7 Hz) ppm. The chemical shifts for H-6'', H-6''' were observed at 4.05, 3.66 ppm (J = 11.96, 1.92, 188, 6.7, 11.96 Hz) and these were assigned to the protons (H-6''') of terminal glucose unit. The signal for the H-3 protons was found at 3.58 ppm.

The ^{13}C NMR spectrum showed a total of 46 carbon signals in its broad band scanning, and out of these, 21 signals were assigned to the aglycone of the pregnane skeleton having a hydroxyl group at C-20. The remaining 25 signals were designated to four sugar moieties (three units of glucose and one unit of digitalose or 6-deoxy-3-O-methyl galactose). Their configuration and linkage were deduced from ^{13}C-NMR, DEPT and COSY-45 experiments.

The ^{13}C NMR spectrum exhibited the signals at 78.05 and 102.82 ppm for C-3 and C-1' and 104.24 ppm for C-1'' of the first glucose attached to C-4' (78.08 ppm) of digitatose, 70.37 and 105.13 ppm for C-6'' and C-1''', 62.80 ppm for C-6'''. The signal present at 66.45 ppm was assigned to C-20. This value was identical with the reported values for pregnane derivatives [9, 10]. The spectrochemistry and configuration was deduced from the values of ^1H and ^{13}C NMR of the compound. The coupling constant of different signals of this compound were elucidated from J-resolved spectra.

Carbon atom	Genin	Curatuberside 1		Curatuberside 2	
		A		B	
1	37.00	37.49	38.71	37.63	38.38
2	28.91	29.96	29.98	30.10	28.66
3	72.40	76.53	77.40	77.03	78.05
4	35.62	34.74	35.34	34.88	35.33
5	43.40	44.48	45.70	44.65	45.71
6	28.13	29.21	29.98	29.40	30.00
7	27.10	28.14	28.65	28.18	28.66
8	39.25	40.63	41.38	40.91	41.38
9	47.65	49.55	51.08	50.05	51.05
10	35.42	36.07	36.96	36.18	36.98
11	20.00	21.63	19.04	22.86	22.04
12	38.50	39.44	40.83	40.91	40.83
13	48.30	49.65	49.71	49.09	48.14
14	82.52	84.76	85.73	83.83	85.69
15	33.00	32.02	33.15	32.01	33.14
16	24.40	24.48	22.10	18.85	30.44
17	61.48	63.01	63.74	57.08	57.45
18	12.00	12.22	12.61	12.26	12.61
19	14.99	15.73	15.34	15.48	15.34
20	218.39	216.39	219.68	65.30	66.43
21	31.07	32.37	30.71	21.53	22.10

Therefore, on these interpretation the isolated compound 2 was consequently elucidated as 3-O-[β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranosyl(1 → 6)-β-D-glucopyranosyl-(1 → 4)-β-D-(3-O-methyl-6-deoxygalactopyranosyl)]-14β, 20-dihydroxy-pregnane.

3. Experimental

The whole plant of *Caralluma tuberculata* (25 kg) was purchased from the vegetable market of Karachi, and identification was kindly carried out by Prof. Dr. S. I. Ali, Department of Botany, University of Karachi. A voucher specimen is deposited on a herbarium sheet in our department.

A fraction was subjected to RP-HPLC (flow rate 5 ml/min, MeOH/H_2O, 65:35), which yielded compound 1 (32 mg). Another fraction was subjected to flash CC (EtOAc/MeOH, 85:15). The obtained impure compound was further purified by RP-HPLC (flow rate 5 ml/min, MeOH/H_2O, 65:35), which finally yielded the pure compound 2 (65 mg).

Compound 1 was yielded as white amorphous powder having a m.p. of 142–149 °C, $[\alpha]_D^{25}$ −40° (C–H) 1680 (C=O), 1100 (C–C) cm^{-1}. The molecular formula

Table 2: ^{13}C-NMR Chemical shifts for sugar part of compound 1 and 2

Carbon atom	Curatuberside 1		Curatuberside 2	
	A		B	
1'	102.41	102.51	102.80	102.82
2'	76.80	76.84	75.81	75.85
3'	83.50	83.58	83.60	83.77
4'	77.30	77.48	79.54	78.06
5'	70.48	70.53	71.91	71.37
6'	17.76	17.82	17.59	17.60
OMe	59.50	59.05	58.60	58.62
1''	105.51	105.58	104.26	104.24
2''	76.12	76.17	75.15	75.18
3''	78.34	78.41	78.00	78.11
4''	71.94	71.61	71.67	71.67
5''	78.51	78.59	77.81	79.51
6''	63.18	63.25	70.36	70.39
1'''	–	–	105.64	105.13
2'''	–	–	75.11	74.80
3'''	–	–	78.07	77.47
4'''	–	–	71.36	71.67
5'''	–	–	77.40	77.47
6'''	–	–	62.76	70.37
1''''	–	–	–	105.13
2''''	–	–	–	75.85
3''''	–	–	–	78.05
4''''	–	–	–	71.94
5''''	–	–	–	78.11
6''''	–	–	–	62.80

($C_{46}H_{44}O_{18}$) was deduced from the FAB-MS spectrum. In FAB-MS (negative ion mode) the molecular ion peak appeared at m/z 867 (31%, [M − H]) while in FAB-MS (positive ion mode) a peak appeared at m/z 869 (20%, [M + H]). Furthermore, EI − MS showed important fragments at 477 (33%), 461 (85%), 345 (22%), 317 (61%), 299 (100%), 281 (40%), 255 (11%), 177 (18%). ^{1}H NMR (CD_3OD, 300 MHz, δ, ppm): 0.91 (3 H, s, C-19 H), 1.09 (3 H, δ, C-18 H, 1.27 (3 H, d, J = 6 Hz, C-6l + 1), 2.29 (3 H, s, C-21 H), 2.91 (1 H, t, J = 5.6 Hz, C-17 H), 3.52 (3 H, s, OCH_3), 3.35 (1 H, t, J = 5.6 Hz, C-17 H), 3.53 (3 H, s, OCH_3), 3.35 (1 Hm, C-3 H), 3.82 and 3.60 (2 H, dd, J = 2.1, 6.6, 11.6, 11.6 Hz, C-6'' H), 4.13 δ 3.76 (2 H, dd, J = 2.5, 6.5, 11.5 Hz, C-6'' H), 4.05 (1 H, s, C-140 H), 4.38, 4.63, 4.78 (1 H for each anomeric carbon, J = 7.6, 7.6 and 7.7 Hz, C-1' H, C-1'' H, C-1''' H), respectively.

Compound 2 was isolated as needle shape crystals from MeOH, m.p 189–190 °C (dec). $[\alpha]_D^{25}$ −40 (C = 0.01 MeOH). IR (KBr, ν, cm^{-1}): 3550 (OH), 2910 (C−H), 1080 (C−O−C). Molecular ion was m/z 982 which corresponds to a molecular formula of $C_{46}H_{54}O_{23}$. FAB-MS (negative ion mode) displayed peaks at m/z 981 (39.9%, [M − H]), 819 (13.2%, 657 (7%), 495 (6.3%), 334 (13.7%), 317 (23%), 281 (40%), 255 (100%), 334 (13.7%), 317 (23%), 281 (40%), 255 (100%), 227 (50%). ^{1}H NMR (CD_3OD, 400 MHz, δ, ppm): 0.84 (3 H, s, C-18 H), 1.20 (3 H, s, C-19 H, (3 H, d, J = 6.3 Hz, C-6', H), 1.28 (3 H, d, J = 3.2 Hz, C-21 H) 2.01 (1 H, m, −20 H), 3.25 (1 H, m, C-3 H), 3.35 (3 H, s, OCH_3), 3.57 and 3.56 (2 H, dd, J = 1.83, 6.2, 11.96, 11.96 Hz, C-6'' H), 9.06, 1.66 (4 H, m, J = 1.96, 1.92, 6.57, 6.58, (1.83, 11.84 Hz, C-6'' H, C-6''' H), 4.36, 4.39, 4.59, 4.49 (1 H for each anomeric carbon. d, J = 7.7, 7.7, 7.7, 7.7 Hz, C-1' H, C-1'' H, C-1''' H, C-1'''' H), respectively.

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Dr. K. Usmanghani
Department of Pharmacognosy
Faculty of Pharmacy
University of Karachi
Karachi-75270
Pakistan

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CARATUBERSIDE A2: A NEW PREGNANE FROM *CARALLUMA TUBERCULATA*

KEY WORDS: Caratuberside A2, *Caralluma tuberculata*, Asclepiadaceae, Pregnane.

Ghazala H. Rizwani *, Mansoor Ahmad[1], K. Uamanghani and Viqar Uddin Ahmad[2].

Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi-75270, Pakistan.

[1]Department of Pharmacognosy & Environmental Health Sciences, College of Pharmacy, The University of Rhode Island, Kingston, RI-02881, USA.

[2]HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

ABSTRACT:

A medicinal herb, *Caralluma tuberculata* (Asclepiadaceae), furnished a pregnane type compound, caratuberside A2. The sugar linkage was at C-14 which is a rare site of substitution. The structure was elucidated by spectroscopic means, including COSY-45, J-resolved, HMBC, HMQC, C-H correlation experiments and derivatization.

INTRODUCTION:

Our further studies on *Caralluma tuberculata*, family Asclepiadaceae, resulted in the isolation of a new pregnane, caratuberside A2. Previously reported compounds, caratuberside A and B[1], and this compound showed similarities in structure, but different behaviour on HPTLC and HPLC. This communication deals with the structural relationship among caratubergenin A1, caratuberside A & B and caratuberside A2.

RESULTS AND DISCUSSION:

Caralluma tuberculata is a medicinal herb of folkloric medicine[2]. From the chemistry of natural products point of view no work has been done on its

chemical constituents except that described in our previous report[1]. In continuation of our research work on *C. tuberculata* we are now reporting another pregnane glycoside which was not isolated previously from this plant. This compound had two basic differences in configuration when compared with caratuberside A[1], that is i) a double bond at C-5 and ii) attachment of sugars at C-14. The structure was elucidated with the help of spectroscopic techniques including DEPT, COSY-45, J-resolved, HMBC and HMQC.

This compound was isolated from the butanol fraction as a white amorphous powder through HPLC. Electron Ionization (EI) mass spectrum showed the molecular ion peak at m/z 654, corresponding to the molecular formula $C_{34}H_{54}O_{12}$, and the FAB mass spectrum confirmed the molecular weight i.e 654 with signals at m/z 653 $[M-H]^-$ and 745 [M+glycerol]$^-$. The IR spectrum exhibited the diagnostic peaks of hydroxyl, carbonyl groups and double bond at 3430, 1700 and 1615 cm^{-1},respectively. In comparison to our previously isolated compound, caratuberside A, there was no double bond peak in the IR spectrum. Further information for double bond was obtained from ^1H- and ^{13}C-NMR spectra. The ^1H-NMR spectrum showed a signal at 5.40 ppm (dd, J= 4.8, 1.2 Hz) which was assigned to H-6 on the basis of H-C correlation and HMBC spectra assignments (123.07 ppm). Another signal in ^{13}C-NMR spectrum at 140.76 ppm was assigned to C-5 due to the fact that no signal for proton of the C-5 was observed in the ^1H-NMR spectrum. In DEPT and HMQC experiments its signal appeared as a quaternary carbon. The effects of a double bond were observed on the neighbouring carbons, C-4 and C-6. The chemical shift for C-4 was found at 39.61 ppm (4.69 ppm downfield) due to a β–effect. Both β– and γ-effects were noted on C-8 (2.62 ppm upfield) due to the substitution at C-14 and a double between C-5 and C-6. The other signals of aglycone in ^1H- and ^{13}C-NMR were found to be identical with caratubergenin A1[1].

The linkage between the aglycone and sugar was also found unusual in that no evidence was detected for linkage between C-3 and sugar. In the ^1H-NMR spectrum the signal for the proton of C-3 appeared at 3.41 ppm as a multiplet. In our pregnane compounds there were only two positions of hydroxyl groups, i.e. C-3 and C-14. Whereas the signals for C-3 (71.47 ppm) were clear in ^{13}C-, C-H correlation, HMBC and HMQC there was now only the C-14 position left for the linkage. It was found there because C-14 showed its signal at 86.89 ppm in the ^{13}C-NMR spectrum, which was a clear indication of substitution at this position. The β–effects of this substitution were found on C-8, 13, 15 & 16. In case of C-15 and 16 signals were observed downfield (3.07 and 0.42 ppm) and upfield in C-8 and 13 (2.82 and 2.05 ppm).

The sugars were identified as 3-O-methyl fucose and glucose on the basis of acid hydrolysis and comparison on HPLC, TLC and HPTLC plates with authentic samples. The linkage between sugars were detected as 1→4 by the hydrolysis of the compound and spectroscopic data[1]. The sugar, glucose, was found as the terminal sugar and 3-O-methyl fucose as the bridge between glucose and aglycone. The evidences of C-H correlation, HMBC, HMQC and J-resolved spectra were in favour of our proposed structure. This linkage is also identical to caratuberside A[1]. Therefore, this compound was identified as 14-*O*-[β-d-glucopyranosyl(1→4)-(3-*O*-methylfucose)]-3β-hydroxyl-14β-pregn-20-one. The acetylation product and its spectroscopic data also confirmed this structure.

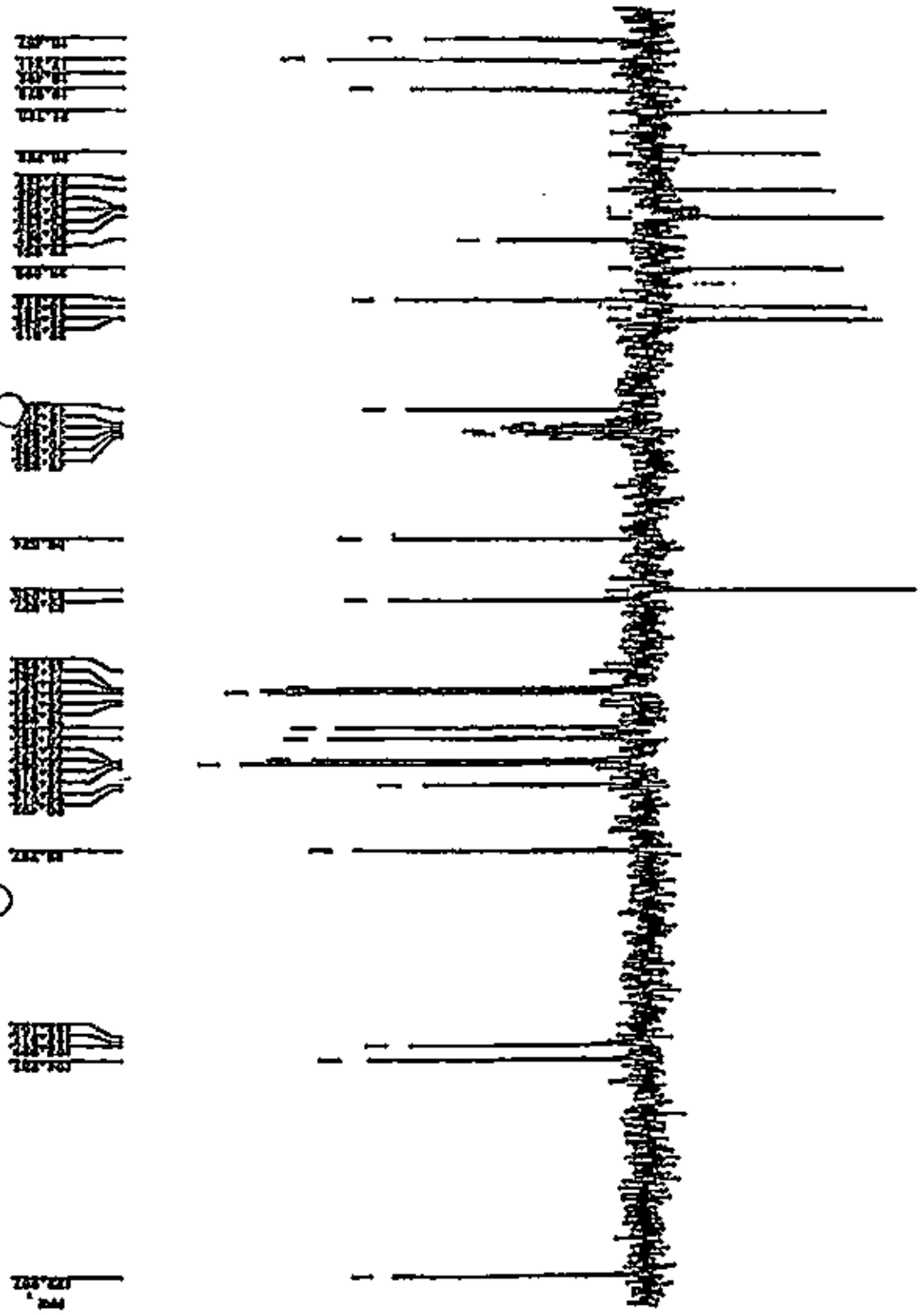


Fig. 1 A ^{13}C-NMR DEPT spectrum of caratuberside A2, measured in CD_3OD (75.42 MHz), shows CH_3 & CH signals up and CH_2 down.

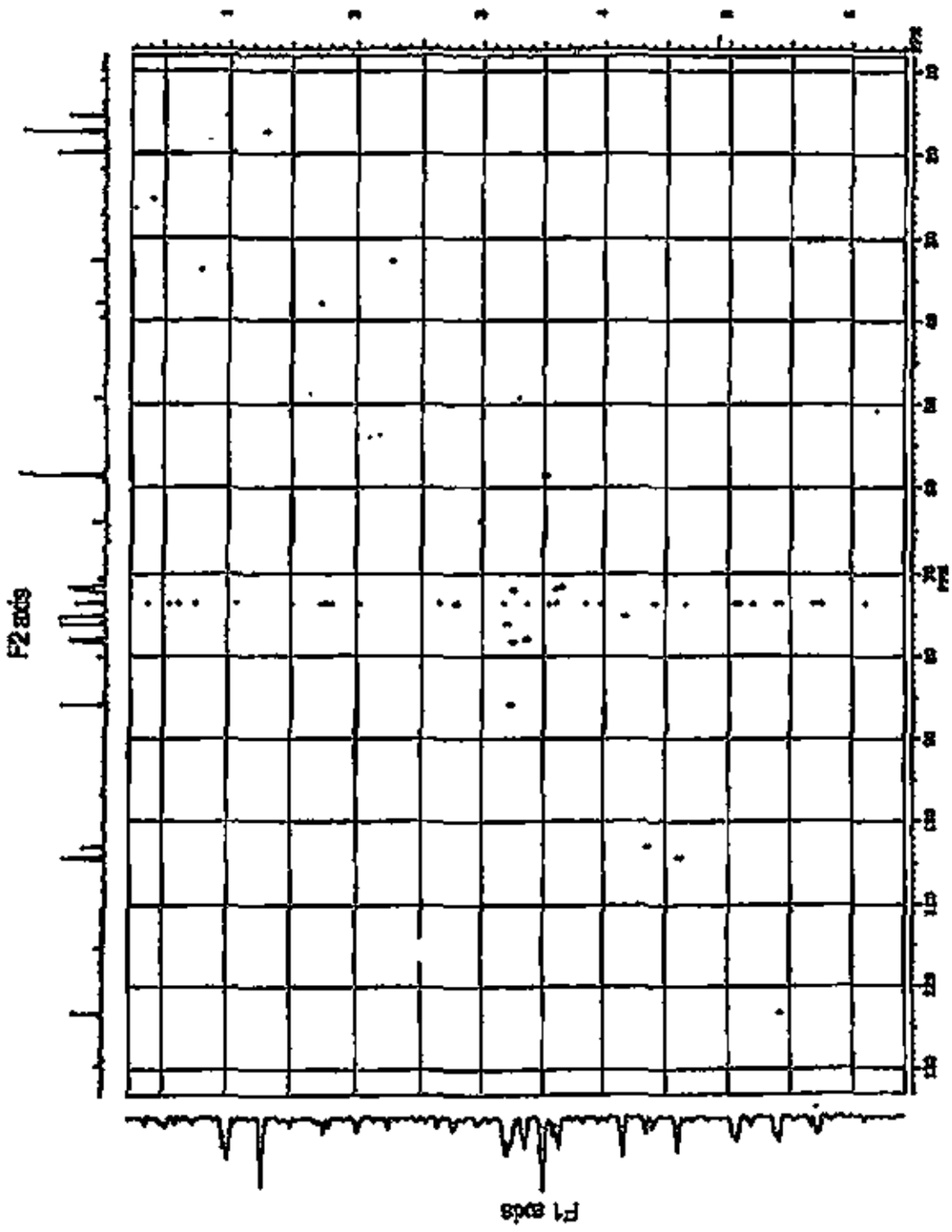


Fig. 2 Two-dimensional ^{1}H-^{13}C chemical shift correlation ship spectrum of caratuberside A2 in CD_3OD at 300/75.42 MHz presented a normal high resolution proton spectrum at F1 axis while 90°C projection to recover the protonated carbon spectrum along F2 axis.

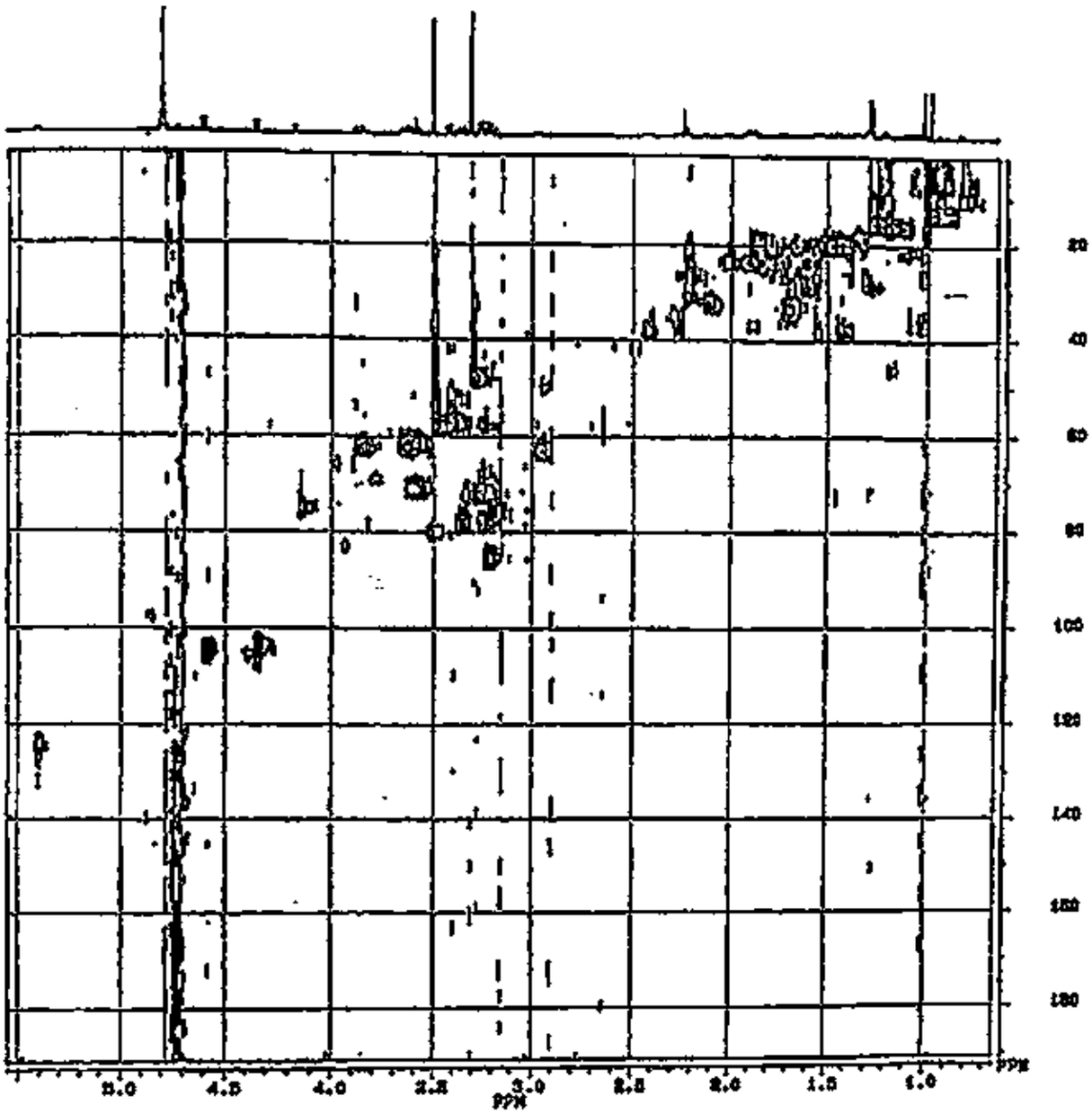
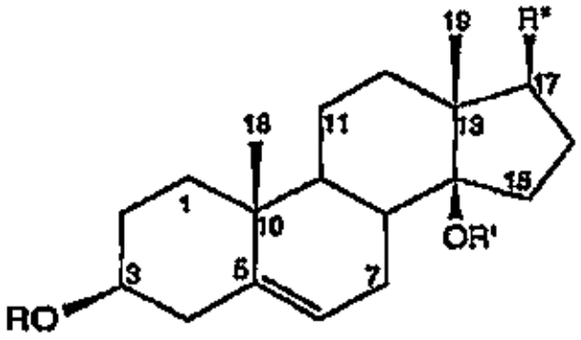


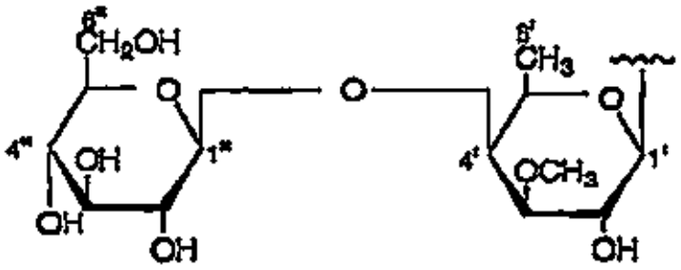
Fig.3 HMQC spectrum of caratuberside A2, measured in CD_3OD, shows a correlation among protons and carbons at high resolution.

EXPERIMENTAL:

The plant material was purchased from local vegetable market in Karachi and identification was kindly carried out by Prof. Dr. S.I. Ali, Department of Botany, University of Karachi. A voucher specimen of plant material was deposited in the Department of Pharmacognosy, University of Karachi. Extraction and fractionation procedure has already been described in our previous publication[1]. The butanol fraction was concentrated under reduced pressure and after the butanol was completely evaporated, it was dissolved in methanol. Later



Caratubergenin A1, R = R' = H, R" = CH_3-C=O
Caratuberside A, R = Glc.(1 → 4) -3-O-methyl fucose, R' = H, R" = CH_3-C=O
Caratuberside B, R = Glc.(1 → 4) -3-O-methyl fucose, R' = H, R" = CH_3-HCOH
Caratuberside A2, R = H, R' = Glc.(1 → 4) -3-O-methyl fucose, R" = CH_3-C=O



β-D-glucopyranosyl-(1→ 4)-3-O-methyl fucose

on ether was added for the formation of precipitate, and this precipitate contained glycosidic constituents, which were separated via Buchner funnel. This fraction was subjected to silica gel column chromatography eluted with ethyl acetate-methanol in increasing order of polarity. A fraction collected with eluent ethyl acetate-methanol (90:10) yielded two major fraction I & II. The fraction II was subjected to RP-HPLC (semi-preparative) with methanol-water (65:35), flow rate 4 ml/min., RI detector, and a pure compound was collected which yielded a white amorphous powder on lyophilization. It showed the following characteristic features: molecular weight 654; IR (KBr) 3430 (O-H), 2960 (C-H), 1700 (C=O), 1615 (C=C), 1040 (C-O-C) cm^{-1}: ^{1}H-NMR (300 MHz, CD_3OD) ppm; 0.98 (s, H-19), 1.02 (s, H-18), 1.28 (d, J=6.1 Hz, H-6'), 2.24 (s, H-21), 3.41 (m, H-3), 2.86 (dd, J=4.4, 8.8 Hz, H-17), 3.50 (s, OCH3), 3.18-3.70 (m, H-2',3',5',2",3",4",5"), 3.87 (dd, J= 11.6, 2.2 Hz, H-6"), 4.16 (d, J= 2.8 Hz, H-4'), 4.35 (d, J= 7.7 Hz, H-1'), 4.58 (d, J= 7.8 Hz, H-1"), 5.40 (dd, J= 4.9, 1.2 Hz, H-6): FAB-MS (-ve ion mode) at m/z 653 $[M-H]^-$, 745 [M+glycerol]$^-$, 421 [aglycone+glycerol-2H]$^-$,325 [glucose+3-O-methylfucose]$^-$: ^{13}C-NMR; see table 1.

Acid hydrolysis of caratuberside A2: 10 mg compound was used for acid hydrolysis as described in the literature[1,3,4]. Two different sugars were isolated and their type, structures & configurations were determined on the basis of comparison with standard sugars on TLC, HPTLC, HPLC and spectroscopic measurements. They were identified as glucose and 3-O-methyl fucose.

Table 1: ^{13}C-NMR chemical shifts of caratubergenin A1 (C_5D_5N), caratuberside A2 (OD_3OD), caratuberside A and caratuberside B (C_5D_5N).

C-atom	Caratubergenin	Caratuberosides		
	A1	A2	A	B
1	37.00	38.46, t	37.49	37.63
2	28.61	30.68, t	29.96	30.16
3	72.40	71.47, d	78.53	77.03
4	32.62	39.81, t	34.74	34.88
5	43.40	140.75, s	44.48	44.65
6	28.13	123.07, d	29.21	29.40
7	37.15	28.34, t	28.14	28.18
8	39.25	37.51, d	40.53	40.91
9	47.59	47.74, d	49.68	50.05
10	38.42	38.29, s	36.07	22.66
11	20.08	21.76, t	21.63	22.66
12	38.50	39.52, t	39.44	40.91
13	48.30	47.44, s	49.49	49.09
14	82.52	86.89, s	84.76	83.83
15	33.50	35.09, t	32.02	33.01
16	24.40	26.26, t	24.64	18.85
17	61.48	63.04, d	63.01	57.09
18	12.00	15.45, q	12.22	12.36
19	14.99	17.34, q	15.73	15.48
20	216.39	219.50, s	216.39	85.30, d
21	31.07	32.65, q	32.37	21.53
1'	-	102.89, d	102.41	102.51
2'	-	76.08, d	76.80	76.84
3'	-	88.79, d	88.50	88.58
4'	-	77.87, d	77.30	77.46
5'	-	71.67, d	70.48	70.65
6'	-	19.67, q	17.76	17.82
1"	-	104.28, d	106.69	106.58
2"	-	74.95, d	75.12	75.10
3"	-	80.42, d	78.84	78.41
4"	-	71.89, d	71.84	71.51
5"	-	78.91, d	78.51	78.59
6"	-	63.87, t	63.18	63.25
OCH_3	-	58.52, q	59.50	59.06

Acetylation of caratuberside A2: With the standard method of acetylation the compound caratuberside A2 was acetylated[1, 3,4]. The compound showed acetyl groups, five acetate groups on the sugar portion and one at the C-3 position. ^{1}H-NMR (300 MHz, $CDCl_3$) ppm: 0.84 (s, H-19), 1.12 (s, H-18), 2.16 (s, H-21), 2.20 (s, OAc), 4.65 (m, H-3), 5.41(dd, J= 5.0, 2.1 Hz, H-6). EI-MS at m/z 806 (M^+, 1.5%), 549 (0.5%, 3-O-methyl fucose+glucose acetate), 533 (0.6%, 3-O-methyl fucose+glucose acetate without oxygen) , 516 (6%, M-glucose acetate), 373 (32%, aglycone), 357 (10%, aglycone without oxygen).

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Further constituents from *Caralluma negevensis*

Ammar Bader[a], Alessandra Braca[b,*], Nunziatina De Tommasi[c], Ivano Morelli[b]

[a]*Faculty of Pharmacy, Al-Zaytoonah University of Jordan, PO Box 130, 11733 Amman, Jordan*
[b]*Dipartimento di Chimica Bioorganica e Biofarmacia, Università degli Studi di Pisa, Via Bonanno 33, I-56126 Pisa, Italy*
[c]*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Via Ponte Don Melillo, I-84084 Fisciano (SA), Italy*

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Dedicated to the memory of Professor Saverio Cristino

Abstract

Two new megastigmane glycosides (1 and 2) and two new flavone glycosides (3 and 4) were isolated from the methanol extract of the whole plant of *Caralluma negevensis* Zohary (Asclepiadaceae). The structures of the isolated compounds were characterized as (9*R*)-2β,9-dihydroxymegastigma-4,7-dien-3-one 9-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (1), 2β,9-dihydroxymegastigma-4-en-3-one 9-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (2), luteolin 3′-*O*-β-D-glucopyranoside-4′-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3), and luteolin 3′,4′-di-*O*-β-D-glucopyranoside (4). The structures of the isolated compounds were established on the basis of spectral evidence and chemical transformation.

Keywords: *Caralluma negevensis*; Asclepiadaceae; Megastigmane glycosides; Flavone glycosides

1. Introduction

The genus *Caralluma* belongs to the family Asclepiadaceae, which comprises some 200 genera and 2500 species. Plants belonging to this genus are rich in esterified polyhydroxypregnane glycosides, some of which showed antitumour activity and others were postulated as precursors of cardenolides (Deepak et al., 1989, 1997). The genus is also characterized by the presence of flavone glycosides (Ramesh et al., 1999; Rizwani et al., 1990). In the course of our screening for biologically active natural products, we have described 20 new pregnane glycosides and/or their esters from *Caralluma negevensis* Zohary (Braca et al., 2002), a succulent perennial herb occurring wild on the rocky desert of East Saharo-Arabian subregion (Feinbrun-Dothan, 1978). The plant is used by Bedouins to treat chronic lung diseases, such as tuberculosis and cancer. Further studies on the whole plant led to the isolation of two megastigmane glycosides and two flavonol glycosides. The presence of megastigmane glycosides in a plant of the genus *Caralluma* is now reported for the first time.

2. Results and discussion

The 1-butanol fraction of the methanol extract of the whole plant of *C. negevensis* was chromatographed over Sephadex LH-20 followed by DCCC and HPLC to afford four new compounds (1–4).

Compound 1 was isolated as an oil. Its molecular formula was derived as $C_{25}H_{40}O_{12}$ by ESI-MS, NMR and elemental analysis. The ^{1}H NMR spectrum of 1 showed signals assignable to a vinyl proton at δ 5.94 (*s*) and two mutually coupled vinyl protons at δ 5.78 (*dd*, J = 16.0 and 8.8 Hz) and δ 5.77 (*dd*, J = 16.0 and 6.5 Hz). Proton signals due to three methines (δ 4.39, 4.20, and 2.78), a vinyl methyl (δ 1.96 *d*, J = 1.5 Hz), a secondary methyl (δ 1.32, *d*, J = 6.5 Hz), and two tertiary methyl groups (δ 1.13, 0.94, each *s*) were observed in the aliphatic region. The presence of two sugar residues was suggested by two doublet signals [δ 4.36 (J = 8.0 Hz), and δ 4.74 (J = 1.8 Hz)] due to anomeric protons and a methyl signal [δ_H 1.29 (*d*, J = 6.5 Hz), δ_C 17.5]. The ^{13}C NMR spectrum of 1 showed 25 carbon signals, among

* Corresponding author. Tel.: +39-050-44734; fax: +39-050-43321.
E-mail address: braca@farm.unipi.it (A. Braca).

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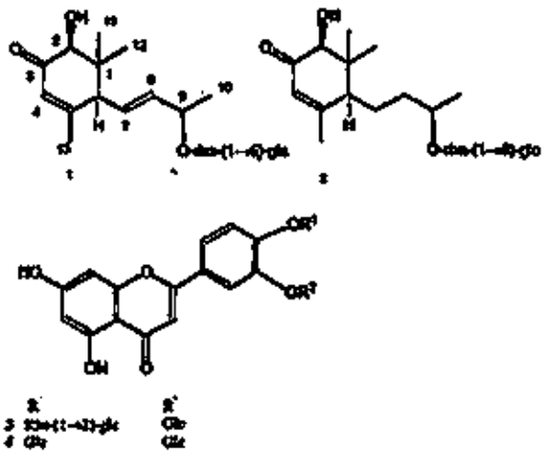

which 13 resonances were similar to those corresponding to a megastigmane (α-ionol) skeleton (De Tommasi et al., 1996; Otsuka et al., 1995), with an hydroxy group linked at C-2 (δ 77.3), confirmed by measurement of 2D NMR including DQF–COSY and HSQC spectral analyses. The position of hydroxy group at C-2 was also inferred from the presence of the singlet at δ 4.20, ascribable to a carbinyl proton and from key correlations observed in the HMBC experiment (H-2 and C-1, C-3, and C-12; H-4 and C-2, and C-6; H-6 and C-7, C-8, and C-11; H-8 and C-9). The remaining 12 carbon resonances were assigned to a β-D-glucopyranosyl and an α-L-rhamnopyranosyl moieties by means of 1D-TOCSY, DQF–COSY, and HSQC experiments. Acid hydrolysis of **1**, followed by TLC comparison with standards, yielded D-glucose and L-rhamnose as sugar moieties. The location of the sugar residues in **1** were established by the HMBC experiments. Hence, the anomeric proton signal (δ 4.36) of glucose, which was assigned from the DQF–COSY spectrum, was correlated through a three-bond coupling with C-9 (δ 76.0) of the aglycon. The other anomeric proton of rhamnose at δ 4.74 was also correlated with C-6′ of glucose at δ 68.0. So the disaccharide sugar chain was a rhamnopyranosyl-(1→6)-glucopyranosyl moiety linked at C-9 of the aglycon. In the 1D-ROESY experiment of **1**, correlation peaks between H-2 and H-6 were observed indicating the same α orientation of these protons. The absolute configuration at C-9 of the aglycon was assigned as *R* on the basis of a diagnostic chemical shift of the C-9 signal (76.0 ppm) in the ^{13}C-NMR spectrum (Pabst et al., 1992; Ito et al., 2001). Consequently, the structure of **1** was determined to be (9*R*)-2β,9-dihydroxymegastigma-4,7-dien-3-one 9-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

Compound **2** was obtained as an oil, and its ESI-MS showed a $[M+Na]^+$ ion peak at *m/z* 557, which was 2 mass units more than **1**. The molecular formula $C_{25}H_{42}O_{12}$ was confirmed by elemental analysis. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, both for the aglycon and the sugar moieties, except for the presence of two methylene groups (δ_H 1.62 and 1.60, δ_C 37.6 and 27.4) instead of one double bond. Upon acid hydrolysis, followed by TLC comparison with standards, compound **2** gave D-glucose and L-rhamnose. The DQF–COSY spectrum unambiguously revealed all the proton spin–spin connectivities and the correlations between protons and carbons were fully assigned from the HSQC experiment, allowing us to deduce the position of interglycosidic linkages. The sugar sequence was confirmed by HMBC cross peaks that showed correlations between H-1′–C-9 and H-1″–C-6′. The relative stereostructure of compound **2** was characterized by 1D-ROESY experiment: correlation peaks were observed between the signal at δ 4.18 (H-2) and 2.15 (H-6). The absolute configuration at C-9 of the aglycon was supposed to be *R* referring to the chemical shift of the C-9 signal in the ^{13}C NMR spectrum (75.3 ppm). On the basis of these data, the structure of **2** was assigned as 2β,9-dihydroxymegastigma-4-en-3-one 9-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

The molecular formulae $C_{33}H_{40}O_{20}$ for compound **3**, and $C_{27}H_{30}O_{16}$ for compound **4**, were determined by ESI-MS, ^{13}C and DEPT–^{13}C NMR analyses and were supported also by elemental analysis. Their ^{1}H and ^{13}C NMR spectra indicated that glycosides **3** and **4** had an identical aglycon portion but differed only in the saccharide chains. Their ^{1}H NMR spectra showed the presence of a flavone skeleton with two meta-doublet signals at δ 6.23 (J=1.3 Hz) and 6.52 (J=1.3 Hz) for compound **3** and δ 6.13 (J=1.5 Hz) and 6.46 (J=1.5 Hz) for compound **4**, respectively, one singlet at δ 6.71 and 6.80 ascribable to H-3, and an ABX system of ring B [δ 7.34 (*d*, J=8.0 Hz, H-5′), 7.70 (*dd*, J=8.0, 1.7 Hz, H-6′), 7.98 (*d*, J=1.7 Hz, H-2′) for **3** and δ 7.30 (*d*, J=8.0 Hz, H-5′), 7.67 (*dd*, J=8.0, 1.5 Hz, H-6′), 7.93 (*d*, J=1.5 Hz, H-2′) for **4**, respectively], permitting to identify the aglycon as luteolin (Agrawal, 1989). Their ^{1}H-NMR spectra indicated also signals for three and two sugar residues in compound **3** and **4**, respectively, easily clarified with the help of 1D-TOCSY and DQF–COSY experiments, that led also the assignation of the type of sugar and its anomeric configuration. Acid hydrolysis of both compounds, followed by TLC comparison with standards, yielded two β-D-glucose moieties and one α-L-rhamnose unit for compound **3** and two β-D-glucose residues for compound **4**, respectively. The exact disposition of the monosaccharide units and the position of the interglycosidic linkages in compound **3** and **4** were assigned in the following manner. HSQC permitted assignments of the interglycosidic linkages by comparison of the ^{13}C NMR shifts observed with those of the corresponding methyl pyranosides and taking into account the known effects of glycosidation (Breitmaier

and Voelter, 1987). The absence of any ^{13}C-NMR glycosidation shifts for one glucopyranosyl and the rhamnopyranosyl residues suggested that these sugars were terminal units in compound 3, while glycosidation shift for C-2 (~ +6 ppm) of the other glucopyranosyl unit indicated that it was substituted at C-2 position. The position of each sugar unit was achieved using HMBC experiment: diagnostic long range correlations were observed between H-1″glc and C-4′, H-1‴rha and C-2″glc, and H-1⁗glc and C-3′, allowing identification of compound 3 as luteolin 3′-*O*-β-D-glucopyranosido-4′-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside. Analogously the absence of any glycosidation shifts for the glucopyranosyl moieties in compound 4 and the HMBC correlation between H-1″glc and C-4′ and H-1⁗glc and C-3′, permitted to establish the structure for this compound as luteolin 3′,4′-di-*O*-β-D-glucopyranoside.

3. Experimental

3.1. General

Instrument to obtain physical and spectral data were the same as described by Braca et al. (2002). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ solution followed by heating. CC was performed over Sephadex LH-20 (Pharmacia); droplet countercurrent chromatography (DCCC) was performed on an apparatus manufactured by Ehrhi, equipped with 300 tubes; HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refrative index detector and with a Waters μ-Bondapak C_{18} column and Shimadzu injector. The other instruments used to obtain physical and spectral data were the same as described by Braca et al. (2002).

3.2. Plant material

The whole plant of *Carthamus tenuiflorus* Zohary was collected in Dabbet Hamoot, Qa' al Naqab, Jordan, in April, 1999 and identified by Professor Sawsan Al Oran, Department of Biology, University of Jordan, Amman, Jordan. A voucher specimen is deposited at the Orto Botanico, Università di Pisa, Pisa, Italy (No. 2000-0051/1).

3.3. Extraction and isolation

The 1-butanol fraction of the methanol extract prepared according the method reported previously (Braca et al., 2002), was chromatographed on Sephadex LH-20 with MeOH as eluent and collecting fractions of 8 ml that were pooled into 20 major fractions. Fraction 6 (760 mg) was submitted to DCCC [*n*-BuOH–Me_2CO–H_2O (33:10:50) descending mode, flow 10 ml/h], to yield two major fractions A (80 mg) and B (70 mg). Fraction A was finally purified by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 ml/min) with MeOH–H_2O 2:3 as eluent to yield pure compound 1 (5.0 mg, t_R = 17 min) and compound 2 (4.8 mg, t_R = 23 min). Fractions 12 (93 mg) and 16 (76 mg) from Sephadex LH-20 column were further purified on RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 ml/min) both with MeOH–H_2O 4.5:5.5 to obtain compound 3 (10.5 mg, t_R = 11 min) from fraction 12 and compound 4 (7.5 mg, t_R = 14 min) from fraction 16, respectively.

3.4. (9R)-2β,9-Dihydroxymegastigma-4,7-dien-3-one 9-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (1)

Oil; $[\alpha]_D^{25}$ = −18.2° (MeOH, *c* 0.1); UV (MeOH) λ_{max} (log ε) 232 sh (4.00) nm; ^{1}H and ^{13}C NMR (600 MHz, CD_3OD): see Table 1; ESI-MS: *m/z* 555 $[M+Na]^+$, 409 $[M+Na-146]^+$, 247 $[M+Na-146-162]^+$; elemental analysis (%): C 56.30, H 7.60, (calc. for $C_{25}H_{40}O_{12}$, C 56.38, H 7.57).

Table 1
^{1}H- and ^{13}C-NMR data of compounds 1 and 2 (CD_3OD, 600 and 150 MHz, respectively)[a,b]

No.		1		2	
		^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
	1		42.5		43.0
	2	4.20 *s*	77.3	4.18 *s*	77.0
	3		202.3		202.2
	4	5.94 *s*	124.0	5.85 *s*	123.8
	5		164.6		162.5
	6	2.28 *d* (8.8)	58.6	2.15 *dd* (9.0, 1.5)	54.0
	7	5.78 *dd* (16.0, 8.8)	128.4	1.62 *br m*	37.6
	8	5.77 *dd* (16.0, 6.5)	138.3	1.60 *br m*	27.4
	9	4.39 *q* (6.5)	76.0	3.79 *m*	75.3
	10	1.33 *d* (6.5)	21.0	1.18 *d* (6.5)	24.3
	11	0.94 *s*	21.0	0.88 *s*	21.7
	12	1.13 *s*	25.6	1.20 *s*	28.2
	13	1.96 *d* (1.5)	23.4	2.08 *d* (1.5)	24.0
glc	1′	4.36 *d* (8.0)	102.8	4.27 *d* (8.0)	102.3
	2′	3.18 *dd* (9.5, 8.0)	75.0	3.14 *dd* (9.0, 8.0)	75.0
	3′	3.36 *d* (9.5)	78.0	3.34 *d* (9.0)	77.9
	4′	3.28 *d* (9.5)	71.6	3.23 *d* (9.0)	71.4
	5′	3.38 *m*	78.8	3.33 *m*	76.0
	6′a	3.96 *dd* (12.0, 3.5)	68.0	3.94 *dd* (12.0, 3.0)	68.0
	6′b	3.69 *dd* (12.0, 5.0)		3.58 *dd* (12.0, 5.0)	
rha	1″	4.74 *d* (1.5)	102.8	4.73 *d* (1.8)	102.0
	2″	3.85 *dd* (2.5, 1.5)	72.1	3.80 *dd* (2.5, 1.8)	71.4
	3″	3.65 *dd* (9.0, 2.5)	72.3	3.62 *dd* (9.0, 2.5)	72.0
	4″	3.38 *t* (9.0)	73.9	3.35 *t* (9.0)	73.6
	5″	3.67 *m*	69.7	3.63 *m*	69.5
	6″	1.29 *d* (6.5)	17.9	1.26 *d* (6.0)	17.8

[a] Assignments were determined using the DQF-COSY, HSQC, and HMBC experiments.
[b] Multiplicity was determined by DEPT experiments and *J* values are given in parentheses.

3.5. 2β,9-Dihydroxymegastigma-4-en-3-one 9-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (2)

Oil; $[\alpha]_D^{25} = -14.0°$ (MeOH, c 0.1); UV (MeOH) λ_{max} (log ε) 235 sh (3.89) nm; ^1H and ^{13}C NMR (600 MHz, CD_3OD): see Table 1; ESI-MS: m/z 557 $[M+Na]^+$, 411 $[M+Na-146]^+$, 249 $[M+Na-146-162]^+$; elemental analysis (%): C 56.12, H 7.95, (calc. for $C_{25}H_{42}O_{12}$, C 56.17, H 7.92).

3.6. Luteolin 3′-O-β-glucopyranoside-4′-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (3)

Yellow amorphous powder, mp 215–220 °C dec; $[\alpha]_D^{25} = -25.7°$ (MeOH, c 0.1); UV (MeOH) λ_{max} (log ε) 267 sh (4.12), 319 (3.85) nm; ^1H and ^{13}C NMR (600 MHz, CD_3OD): see Table 2; ESI-MS: m/z 779 $[M+Na]^+$, 633 $[M+Na-146]^+$, 617 $[M+Na-162]^+$, 471 $[M+Na-46-162]^+$; elemental analysis (%): C 52.35, H 5.34, (calc. for $C_{33}H_{40}O_{20}$, C 52.38, H 5.33).

3.7. Luteolin 3′,4′-di-O-β-D-glucopyranoside (4)

Yellow amorphous powder, mp 200–205 °C; $[\alpha]_D^{25} = -34.5°$ (MeOH, c 0.1); UV (MeOH) λ_{max} (log ε) 263 sh (4.09), 317 (3.94) nm; ^1H and ^{13}C NMR (600 MHz, CD_3OD): see Table 2; ESI-MS: m/z 633 $[M+Na]^+$, 471 $[M+Na-162]^+$, 309 $[M+Na-162\times2]^+$; elemental analysis (%): C 53.07, H 4.96, (cal. for $C_{27}H_{30}O_{16}$, C 53.12, H 4.95).

3.8. Acid hydrolysis of 1–4

Each compound was heated with 1 ml of 1 M HCl, 1 ml of dioxane in a sealed tube at 90 °C for 4 h; then 5 ml of water were added and the aglycon was removed by extracting with $CHCl_3$ (3×10 ml). The aqueous layer was neutralized with Ag_2CO_3, filtered and evaporated to dryness. The sugar samples were identified by cellulose TLC (pyridine-AcOEt-AcOH-H_2O 36:36:7:21 as eluent) by comparison with authentic samples.

Table 2
^1H and ^{13}C NMR data of compounds 3 and 4 (CD_3OD, 600 and 150 MHz, respectively)[a,b]

	No.	3 ^1H-NMR	3 ^{13}C-NMR	4 ^1H-NMR	4 ^{13}C-NMR
	2		165.1		165.4
	3	6.71 s	103.2	6.60 s	103.5
	4		183.8		183.3
	5		163.2		163.0
	6	6.23 d (1.5)	100.1	6.13 d (1.5)	100.0
	7		165.6		165.0
	8	6.52 d (1.5)	95.1	6.46 d (1.5)	95.0
	9		158.4		159.6
	10		106.9		106.7
	1′		127.1		126.8
	2′	7.58 d (1.7)	115.1	7.83 d (1.7)	117.8
	3′		148.1		148.1
	4′		151.4		151.0
	5′	7.34 d (8.0)	118.1	7.30 d (8.0)	118.9
	6′	7.50 dd (8.0, 1.7)	123.3	7.63 dd (8.0, 1.7)	123.0
glc	1″	5.54 d (7.5)	100.9	5.12 d (7.0)	101.5
at C-3′	2″	3.73 dd (9.0, 7.5)	80.7	3.64 dd (9.0, 8.0)	74.5
	3″	3.63 d (9.0)	78.9	3.57 d (9.0)	78.5
	4″	3.46 d (9.0)	71.4	3.42 d (9.0)	71.1
	5″	3.47 m	78.1	3.55 m	77.9
	6″a	4.06 dd (11.5, 3.0)	62.4	3.95 dd (12.0, 1.5)	61.6
	6″b	3.86 dd (11.5, 3.0)		3.72 dd (12.0, 5.0)	
rha	1‴	5.28 d (1.5)	102.7		
	2‴	4.08 dd (2.0, 1.5)	72.1		
	3‴	3.70 dd (9.0, 2.0)	72.1		
	4‴	3.38 t (9.0)	74.0		
	5‴	3.57 m	70.5		
	6‴	1.25 d (6.0)	18.1		
glc	1⁗	5.00 d (7.5)	100.8	5.05 d (8.0)	103.0
at C-4′	2⁗	3.63 dd (9.5, 7.5)	78.8	3.64 dd (9.5, 8.0)	74.6
	3⁗	3.54 d (9.5)	78.5	3.57 d (9.5)	78.1
	4⁗	3.42 d (9.5)	71.1	3.40 d (9.5)	71.5
	5⁗	3.50 m	77.8	3.53 m	78.0
	6⁗a	3.97 dd (11.0, 2.0)	62.3	3.98 dd (12.0, 1.0)	62.5
	6⁗b	3.76 dd (11.0, 5.0)		3.79 dd (12.0, 5.0)	

[a] Assignments were determined using the DQF-COSY, HSQC, and HMBC experiments.
[b] Multiplicity was determined by DEPT experiments and J values are given in parenthesis.

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TETRAHEDRON

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New pregnane glycosides from *Caralluma negevensis*

Alessandra Braca,[a] Ammar Bader,[a] Ivano Morelli,[a] Roberto Scarpato,[b] Gino Turchi,[c] Cosimo Pizza[d] and Nunziatina De Tommasi[d,*]

[a]*Dipartimento di Chimica Bioorganica e Biofarmacia, Università di Pisa, Via Bonanno, 33, 56126 Pisa, Italy*
[b]*Dipartimento di Scienze dell'Uomo e dell'Ambiente, Università di Pisa, Via San Giuseppe 22, 56126 Pisa, Italy*
[c]*Istituto di Management e Differenziamento, Area della Ricerca, C.N.R., Via Moruzzi 1, 56126 Pisa, Italy*
[d]*Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA) Italy*

Dedicated to the memory of Professor Severo Catalano

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Abstract—Twenty new pregnane glycosides were isolated from the whole plant of *Caralluma negevensis*. Their structures were elucidated by extensive spectroscopic methods including 1D-(^{1}H, ^{13}C, ^{13}C DEPT, TOCSY, ROESY) and 2D-NMR experiments (DQF-COSY, HSQC, HMBC, HOHAHA) as well as ESI-MS analysis. Pregnane glycosides were tested for their cytotoxic and genotoxic activity. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The plants of the family Asclepiadaceae are known to contain cytotoxic and tumoricidal *CD-cis*-polycyclypregnane esters and glycosides.[1] *Caralluma negevensis* Zohary is a succulent perennial herb occurring wild on the rocky desert of East Saharo–Arabian subregion,[2] and it is used by Bedouins to treat chronic lung diseases, such as tuberculosis and cancer.

Previous phytochemical studies on plants of the genus *Caralluma* have reported the isolation of several pregnane glycosides and/or their esters,[3,4] some of which showed anti-tumor activity, and others were postulated as precursors of cardenolides.[5]

In the course of our screening for biologically active natural products, twenty new pregnane glycosides (compounds 1–20), along with two known pregnane glycosides russelioside B and russelioside C,[4] were isolated from the whole plant of *C. negevensis*. The structures of these compounds are based on the known polycyclypregnane skeleton of boucerin,[1,6] calogenin,[6,7] or 5α-dihydrocalogenin,[8] as well as acetyl, tigloyl, and *o*-hydroxybenzoyl ester moieties linked at C-12 and/or at C-20 of the aglycons. In addition all compounds possess an oligosaccharide portion linked at C-3 and/or C-20 to the aglycon consisting of one to six sugar unit, well known to occur in Asclepiadaceae plants.

This paper deals with the isolation of these pregnane glycosides and their structural elucidation by means of one- and two-dimensional NMR techniques (^{1}H, ^{13}C, ^{13}C DEPT, 1D-TOCSY, 1D-ROESY, DQF-COSY, HSQC, HMBC, HOHAHA), ESI-MS, and with evaluation of cytotoxic[9] and genotoxic[10,11] activity on some of them selected on the basis of their structural differences.

2. Results and discussion

The chloroform and methanol extracts of the whole plant of *C. negevensis* were processed as described in the Section 3 to afford twenty new pregnane glycosides (1–20) together with the known derivatives russelioside B and russelioside C.

The compounds can be sorted into three series by the nature of the pregnane aglycons: compounds 1–16 are characterized by the boucerin aglycon, compounds 17–19 have the calogenin structure, and compound 20 presents the 5α-dihydrocalogenin skeleton. ^{1}H and ^{13}C NMR assignments for all compounds 1–20 (Section 3 and Tables 1–4) were based on the analysis of NMR spectral data (^{1}H, ^{13}C, DEPT, DQF-COSY, 1D-TOCSY, HOHAHA, HSQC, HMBC).

Compounds 1–16. ESI-MS and NMR data of compounds 1–16 indicated that they were derivatives of the *CD-cis*-polycyclypregnane boucerin by comparison with aglycon data previously reported in the literature.[1,6] In addition to the pregnane moiety, the ^{1}H and ^{13}C NMR spectra of compounds 1–3 and 5–16 showed signals due to tigloyl and/or acetyl and/or *o*-hydroxybenzoyl groups. The ester

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* Corresponding author. Tel: +39-81-962643; fax: +39-89-962222; e-mail: demtommasi@unisa.it

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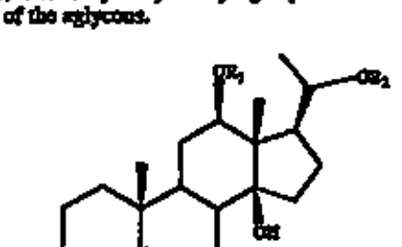
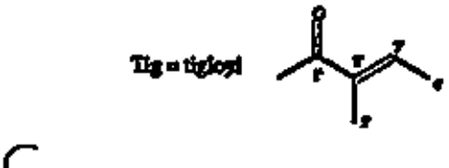
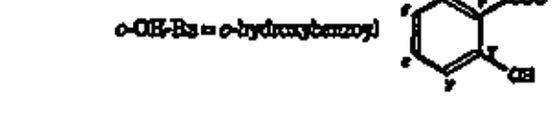
linkages were located at C-12 and/or C-20 on the basis of the chemical shifts of the double doublet of H-12 (δ~4.60 when esterified, δ~3.63 when free) and the quartet of H-20 (δ~4.95 when esterified, δ~3.80 when free) (see Experimental Section). This was confirmed by the results of HMBC experiments which showed clear long-range correlation peaks between the carbonyl carbon of the tigloyl, acetyl, and *o*-hydroxybenzoyl groups and H-12 and/or H-20 of the aglycone.



Compounds	R	R_1	R_2
1	E	H	Tig
2	E	Tig	Tig
3	F	Tig	Ac
4	G	H	H
5	G	*o*-OH-Bz	H
6	G	Tig	Ac
7	G	Tig	Tig
8	G	Tig	H
9	W	Tig	H
10	W	Tig	Tig
11	W	*o*-OH-Bz	H
12	W	Ac	H
13	I	Tig	Tig
14	M	H	Tig
15	M	Tig	Tig
16	L	Tig	Ac





Ac = acetyl

Compounds 1 and 2 (chain E). Compounds 1 and 2 had molecular formulae $C_{35}H_{56}O_{10}$ and $C_{40}H_{60}O_{12}$, respectively, deduced from ESI-MS and NMR analysis. An examination of their NMR spectra revealed signals of protons and carbons attributed to aglycon 20-*O*-tigloylbocconin for compound 1 and 12β, 20-*O*-ditigloylbocconin for compound 2, respectively. In addition to the aglycon signals, ^{13}C NMR spectra of both compounds exhibited 27 signals ascribable to the saccharide portion made up of three 3-*O*-methyl-2,6-dideoxyhexopyranosyl and one hexopyranosyl units. A detailed comparison of their sugar region NMR spectra showed that the saccharide chain was identical in the two compounds. Their ^{1}H NMR spectra also supported the above results by the presence of four anomeric protons at δ 4.48, 4.63, 4.83, and 4.89, three doublet methyls at δ 1.21, 1.23, 1.40, and three *O*-methoxyl at δ 3.47, 3.48, 3.50. All proton signals due to the four sugar units (Table 2) were assigned by a DQF-COSY experiment together with HOHAHA and 1D-TOCSY experiments which allowed the sequential assignments of hydrogens from H-1 to H-6 within each sugar unit starting from the anomeric and methyl signals. The β-linkages of the four sugar moieties were shown by the large (J=9.0 Hz) coupling constants of the anomeric proton signals as well as by the presences of C-2, C-3, and C-5 characteristic of β forms.[13,14] The assignments of all protonated carbons were accomplished by interpretation of the HSQC NMR spectrum and allowed therefore the identification of the sugars as a terminal β-D-glucopyranosyl as well as a β-D-oleandropyranosyl and two β-D-cymaropyranosyl inner units. Each sugar unit was glycosylated at C-4 as shown by the glycosidation shifts observed for C-4_{Glc}, C-$4_{\text{Cym II}}$, C-$4_{\text{Cym I}}$, and C-4_{Ole} (Table 2). Direct evidence for the sugar sequence and their linkage sites was derived from the results of HMBC experiment which showed unequivocal correlation peaks between H-$1_{\text{Cym I}}$–C-3, H-$1_{\text{Cym II}}$–C-$4_{\text{Cym I}}$, H-1_{Ole}–C-$4_{\text{Cym II}}$, H-1_{Glc}–C-4_{Ole}. It is also interesting to note that C-1 of D-cymarose characteristically resonates upfield (97.0 ppm) when linked at C-3 of the aglycon in contrast with the resonance at 101.0 ppm when it is linked to the hydroxyl group of a sugar unit.[15] On the basis of this NMR evidence, the structure of the sugar chain of compounds 1 and 2 was determined to be β-D-glucopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

Compound 3 (chain F). The aglycon moiety of compound 3 was determined to be 12β-*O*-tigloyl-20-*O*-acetylbocconin by spectroscopic evidence (Section 3 and Table 1), while the sugar chain attached to the C-3 position was a tetrasaccharide, as revealed by four anomeric signals (^{1}H NMR δ 4.47, 4.64, 4.81, and 4.87; ^{13}C NMR δ 96.9, 101.2, 102.6, and 104.1) in its spectra. The proton coupling network of each sugar residue was derived from a combination of 1D- and 2D-NMR experiments, which indicated that a β-D-quinovopyranose unit was present instead of the β-D-oleandropyranose unit observed in the saccharide chain E (Section 3). In fact, the 1D-TOCSY subspectrum obtained when irradiating the anomeric proton signal at δ 4.47 (1H, d, J=7.5 Hz) showed a set of coupled proton signals at δ 3.14, 3.54, 3.47, 3.43, 1.45 assigned from H-1 to H_3-6 of a β-D-quinovopyranose unit. To establish the nature of the sugar sequence required analysis of the HMBC spectrum which showed correlation peaks between H-1 of quinovose and C-4 of the second unit of cymarose and between H-1 of glucose and C-4 of quinovose. Thus, the structure of compound 3 was established as 12β-*O*-tigloyl-20-*O*-acetylbocconin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-quinovopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

Table 1. ^{13}C NMR data for aglycon moieties of compounds 1–3, 5, 8, 12, 17, 18, 20 (500 MHz, CD_3OD)

Position	1	2	3	5	8	12	17	18	20
1	38.2 (t)	38.3	38.2	38.3	38.3	38.3	38.3 (t)	38.5	38.2 (t)
2	30.5 (t)	30.4	30.5	30.4	30.6	30.4	30.6 (t)	30.6	30.1 (t)
3	80.4 (d)	79.0	80.0	78.0	79.2	79.0	79.9 (d)	80.0	79.3 (d)
4	39.5 (t)	39.7	39.5	39.8	39.6	39.8	39.5 (t)	39.5	39.3 (t)
5	141.0 (s)	141.0	140.5	141.0	141.0	141.0	141.0 (s)	141.0	45.6 (d)
6	123.2 (d)	123.0	122.8	123.2	123.1	123.2	123.8 (d)	123.8	22.7 (t)
7	28.5 (t)	27.9	28.3	28.0	27.9	28.0	28.1 (t)	28.1	30.6 (t)
8	32.9 (d)	31.8	32.4	32.2	32.4	32.2	31.8 (d)	31.8	41.3 (d)
9	42.0 (d)	44.6	44.5	44.7	44.6	44.7	42.7 (d)	42.7	50.6 (d)
10	38.1 (s)	38.0	38.0	37.8	37.6	37.6	37.8 (s)	37.8	37.0 (s)
11	26.5 (t)	27.1	26.5	26.7	26.8	26.7	21.1 (t)	21.1	21.2 (t)
12	71.4 (d)	78.2	78.8	78.5	79.0	78.8	41.3 (t)	41.3	41.9 (t)
13	52.6 (s)	53.6	53.2	53.6	53.6	53.3	47.0 (s)	47.0	47.5 (s)
14	84.7 (s)	82.4	87.0	87.3	87.5	87.3	86.0 (s)	85.0	86.1 (s)
15	32.8 (t)	32.8	32.8	33.6	33.3	33.4	33.8 (t)	33.8	33.1 (t)
16	25.4 (t)	25.0	25.3	24.0	26.5	25.0	20.3 (t)	20.5	21.5 (t)
17	52.0 (d)	50.9	52.0	53.3	53.4	53.3	53.5 (d)	57.9	57.7 (d)
18	9.8 (q)	9.8	10.2	11.0	10.0	11.0	15.0 (q)	15.0	12.3 (q)
19	19.7 (q)	19.8	19.8	19.6	19.6	19.8	19.6 (q)	19.6	15.3 (q)
20	78.8 (d)	74.8	75.2	77.3	77.7	77.3	75.5 (d)	79.0	78.8 (d)
21	19.6 (q)	19.3	19.7	23.0	22.8	23.0	19.5 (q)	17.3	16.9 (q)
Tig at C-12									
1′		169.5	169.0 (s)		169.9 (s)				
2′		130.1	130.0 (s)		130.9 (s)				
3′		138.2	138.8 (d)		139.0 (d)				
4′		14.3	14.0 (q)		14.0 (q)				
5′		12.0	12.3 (q)		12.0 (q)				
Tig at C-20									
1′	168.8 (s)	168.9							
2′	130.1 (s)	128.9							
3′	138.6 (d)	138.3							
4′	14.3 (q)	14.4							
5′	12.0 (q)	12.0							
Ac									
CO			178.0 (s)			178.0	178.0		
CH_3			21.3 (q)			21.2	21.0		
p-OH-Bz									
1′				112.0 (s)					
2′				161.3 (s)					
3′				119.4 (d)					
4′				120.7 (d)					
5′				131.2 (d)					
6′				118.3 (d)					
COO				173.0 (s)					

Compounds 4–8 (chain G). Compounds 4–8 had molecular formulae $C_{40}H_{68}O_{19}$, $C_{53}H_{86}O_{21}$, $C_{51}H_{84}O_{21}$, $C_{38}H_{64}O_{18}$, and $C_{49}H_{82}O_{20}$, respectively, as deduced from ESI-MS and NMR spectra. Their aglycons were identified as boucerin,[14] 12β-*O*-*o*-hydroxybenzoylboucerin, 12β-*O*-tigloyl-20-*O*-acetylboucerin, 12β,20-*O*-ditigloylboucerin, and 12β-*O*-tigloylboucerin, respectively, by NMR data (Table 1). A detailed comparison of their NMR spectra showed that the sugar chain was the same. Also in this case, the proton coupling network within each sugar residue was determined using a combination of NMR experiments, which led to the identification of two β-D-cymatopyranose, one 6-deoxy-3-*O*-methyl-β-D-allopyranose, and one β-D-glucopyranose. Once again direct evidence of the sugar sequence and the linkage site was derived from HSQC and HMBC experiments. The absence of any ^{13}C glycosidation shift for the glucopyranose residue suggested that this sugar was the terminal unit, while the glycosidation shift on $C\text{-}4_{cym\,II}$, $C\text{-}4_{cym\,I}$, or $C\text{-}4_{all}$ indicated the sequence of the saccharidic chain. Also in this case one D-cymatose unit was linked to C-3 of the aglycon as shown by HMBC experiment; key correlations were observed between $H\text{-}1_{cym\,I}$–C-3, $H\text{-}1_{cym\,II}$–$C\text{-}4_{cym\,I}$, and $H\text{-}1_{all}$–$C\text{-}4_{cym\,II}$. Thus the structure of chain G was established as β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-cymatopyranosyl-(1→4)-β-D-cymatopyranoside.

Compounds 9–12 (chain W). Compounds 9–12 had molecular formulae $C_{51}H_{86}O_{20}$, $C_{58}H_{92}O_{21}$, $C_{53}H_{88}O_{20}$, and $C_{50}H_{84}O_{20}$, respectively, as deduced from MS and NMR analyses. The NMR spectra revealed that the aglycon moieties of 9–12 were 12β-*O*-tigloylboucerin, 12β,20-*O*-ditigloylboucerin, 12β-*O*-*o*-hydroxybenzoylboucerin, 12β-*O*-acetylboucerin, respectively. ^1H and ^{13}C NMR spectra revealed that 9–12 had an identical trisaccharidic sugar chain made up of two D-cymatose, one D-thevetose, and one D-glucose units. By means of NMR spectral data each carbon and hydrogen signal was assigned as shown in Table 2. Consequently it was possible to identify a terminal β-D-glucopyranose as well as two inner β-D-cymatopyranose, and a β-D-thevetopyranose, each glycosylated at C-4 as inferred by HSQC and HMBC

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Table 2. ^1H and ^{13}C NMR data for sugar moieties of compounds 1, 2, 4–8, 13 (600 MHz, CD_3OD)

Position	1, 2 chain E		4–8 chain G		13 chain I	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
D-Cym I						
1	4.89 dd (9.5, 2.0)	97.0	4.89 dd (9.5, 2.0)	97.2	4.87 dd (9.5, 2.0)	97.6
2a	1.54 br dd (16.0, 12.0)	36.5	1.56 br dd (16.0, 12.0)	36.5	1.61 br dd (16.0, 12.0)	36.5
2b	2.09 br dd (16.0, 3.0)		2.09 br dd (16.0, 3.0)		2.14 br dd (16.0, 3.0)	
3	3.87 q (3.0)	72.5	3.87 q (3.0)	72.0	3.86 q (3.0)	72.7
4	3.30 dd (9.5, 3.0)	83.8	3.33 dd (9.5, 3.0)	83.4	3.27 dd (9.5, 3.0)	84.0
5	3.90 dq (9.5, 6.0)	69.8	3.83 dq (9.5, 6.0)	69.8	3.83 dq (9.5, 6.0)	69.7
6	1.21 d (6.0)	18.4	1.21 d (6.0)	18.5	1.21 d (6.0)	18.4
-OMe	3.48 s	58.4	3.46 s	58.5	3.45 s	58.5
D-Cym II						
1	4.83 dd (9.5, 2.0)	101.0	4.83 dd (9.5, 2.0))	101.4		
2a	1.62 br dd (16.0, 12.0)	36.4	1.60 br dd (16.0, 12.0)	36.3		
2b	2.13 br dd (16.0, 3.0)		2.15 br dd (16.0, 3.0)			
3	3.85 q (3.0)	72.6	3.85 q (3.0)	72.4		
4	3.26 dd (9.5, 3.0)	84.0	3.26 dd (9.5, 3.0)	84.0		
5	3.84 dq (9.5, 6.0)	69.9	3.82dq (9.5, 6.0)	70.0		
6	1.23 d (6.0)	18.0	1.22 d (6.0)	18.3		
-OMe	3.50 s	58.2	3.45 s	58.6		
D-Glc						
1	4.63 dd (8.5, 1.5)	102.7			4.63 dd (8.5, 1.5)	103.0
2a	1.48 m	32.6			1.46 m	33.3
2b	2.25 m				2.34 m	
3	3.43 m	82.4			3.50 m	79.2
4	3.25 t (9.5)	84.0			3.22 t (9.5)	84.0
5	3.44 dq (9.5, 6.0)	72.0			3.45 dq (9.5, 6.0)	72.6
6	1.49 d (6.0)	19.0			1.40 d (6.0)	19.5
-OMe	3.47 s	58.4			3.46 s	59.0
-OMe D-Allo						
1			4.61 d (8.0)	101.5	4.38 d (8.0)	102.3
2			3.58 dd (8.0, 2.5)	72.3	3.40 dd (8.0, 2.5)	72.6
3			3.58 dd (3.5, 2.5)	83.1	3.97 dd (3.5, 2.5)	83.2
4			3.35 (9.0, 3.5)	84.0	3.35 (9.0, 3.5)	84.2
5			3.39 m	72.0	3.37 m	73.0
6			1.28 d (6.0)	18.5	1.28 d (6.0)	18.4
-OMe			3.62 s	61.5	3.62 s	61.7
D-Glc						
1	4.63 d (8.0)	104.5	4.37 d (8.0)	106.0	4.39 d (7.8)	106.6
2	3.22 dd (9.5, 8.0)	75.6	3.21 dd (9.5, 8.0)	75.5	3.20 dd (9.5, 7.8)	75.6
3	3.37 d (9.5)	78.2	3.37 d (9.5)	77.8	3.38 d (9.5)	78.0
4	3.27 d (9.5)	71.7	3.26 d (9.5)	71.8	3.25 d (9.5)	71.8
5	3.28 m	78.3	3.22 m	78.0	3.29 m	78.3
6a	3.67 dd (12.0, 5.0)	63.0	3.68 dd (12.0, 5.0)	62.8	3.68 dd (12.0, 5.0)	62.9
6b	3.89 dd (12.0, 3.5)		3.94 dd (12.0, 3.0)		3.91 dd (12.0, 3.0)	

J values are in parentheses and reported in Hz; chemical shifts are given in ppm.

spectral data. The position of each sugar unit was confirmed by 1D-ROESY experiments that showed a cross peak between the signal at δ 4.89 (H-$1_{cym\,I}$) and the signal at δ 3.53 (H-3), and other key correlation peaks between the signal at δ 4.83 (H-$1_{cym\,II}$) and the signal at δ 3.30 (H-$4_{cym\,I}$), δ 4.37 (H-1_{Glc}) and 3.31 (H-$4_{cym\,II}$), δ 4.45 (H-1_{glc}) and 3.40 (H-4_{Glc}). NMR spectra also revealed that chain W is related to chain G by replacement of the unit of 6-deoxy-3-*O*-methyl-β-D-allopyranose with a β-D-thevetopyranose. On the basis of the above data, the structure of the tetrasaccharide of compounds 9–12 was deduced as β-D-glucopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

Compound 13 (chain I). The structure 12β,20-*O*-diglycylbouceorin 3-*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside was assigned to compound 13 on the basis of the following evidence. Compounds 13 and 7 were shown to be isomers with identical molecular formulae ($C_{73}H_{92}O_{22}$); the structural differences, as shown by their NMR spectra, involved a different sugar in the tetrasaccharide moiety. In particular chain I was related to chain G by replacement of the second unit of cymarose with an oleandrose, as deduced from results of DQF-COSY, 1D-TOCSY, and HSQC experiments (Table 2). Unambiguous determination of the interglycosidic linkages and sugar sequence was obtained from HMBC experiment. Key cross peaks due to long-range correlation were observed between H-1 of oleandrose and C-4 of cymarose, H-1 of 6-deoxy-3-*O*-methyl-allose and C-4 of oleandrose, and H-1 of glucose and C-4 of 6-deoxy-3-*O*-methyl-allose.

Compounds 14 and 15 (chain M). Compounds 14 ($C_{68}H_{98}O_{28}$) and 15 ($C_{71}H_{104}O_{29}$) were 20-*O*-diglycylbouceorin and 12β,20-*O*-diglycylbouceorin derivatives, respectively. ^1H and ^{13}C NMR data suggested that the sugar chain of compounds 14 and 15 consists of six

Table 3. ^1H and ^{13}C NMR data for sugar moieties of compounds 9–12, 14–16 (600 MHz, CD_3OD)

Position	9–12 chain W		16 chain L		14, 15 chain M	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
D-Cym I						
1	4.89 dd (9.5, 2.0)	97.0	4.89 dd (9.5, 2.0)	97.0	4.89	97.0
2a	1.56 br dd (16.0, 12.0)	36.2	1.57 br dd (16.0, 12.0)	36.2	1.57	36.2
2b	2.09 br dd (16.0, 3.5)		2.08 br dd (16.0, 3.5)		2.08	
3	3.87 q (3.5)	78.5	3.87 q (3.5)	78.6	3.87	78.6
4	3.30 dd (9.5, 3.5)	83.8	3.29 dd (9.5, 3.5)	83.8	3.26	83.8
5	3.50 dq (9.5, 6.0)	69.8	3.86 dq (9.5, 6.0)	70.0	3.83	70.0
6	1.22 d (6.0)	18.4	1.21 d (6.0)	18.4	1.21 d	18.4
-OMe	3.46 s	58.4	3.45 s	58.5	3.45 s	58.5
D-Cym II						
1	4.83 dd (9.2, 2.0)	101.9	4.83 dd (9.2, 2.0)	102.1	4.83	102.3
2a	1.63 br dd (16.0, 12.0)	36.4	1.63 br dd (16.0, 12.0)	36.6	1.63	36.6
2b	2.17 br dd (16.0, 3.5)		2.17 br dd (16.0, 3.5)		2.17	
3	3.85 q (3.0)	78.6	3.87 q (3.0)	78.8	3.83	78.8
4	3.33 dd (9.5, 3.0)	84.2	3.35 dd (9.5, 3.0)	84.0	3.28	83.8
5	3.86 dq (9.5, 6.0)	69.9	3.87 dq (9.5, 6.0)	69.9	3.83	69.8
6	1.32 d (6.0)	18.4	1.31 d (6.0)	18.2	1.31	18.2
-OMe	3.45 s	58.5	3.45 s	58.6	3.45	58.6
D-Tho						
1	4.37 d (8.5)	106.0	4.36 d (8.5)	106.0	4.38	106.6
2	3.32 t (8.7)	74.9	3.32 t (9.0)	74.9	3.32	74.8
3	3.21 t (8.7)	86.1	3.21 t (9.0)	86.0	3.21	86.0
4	3.40 t (8.7)	82.4	3.38 t (9.0)	82.7	3.38	82.8
5	3.49 m	72.4	3.49 m	72.4	3.49	72.4
6	1.40 d (6.0)	18.4	1.40 d (6.0)	18.6	1.40	18.6
-OMe	3.63 s	61.0	3.66 s	61.1	3.66	61.1
-OMe D-Allo						
1			4.60 d (8.0)	103.8	4.60	103.8
2			3.40 dd (8.0, 2.5)	72.3	3.40	72.3
3			3.99 dd (3.5, 2.5)	83.2	3.99	83.2
4			3.37 dd (9.0, 3.5)	83.8	3.37	83.6
5			3.40 m	72.4	3.40	72.4
6			1.31 d (6.0)	18.4	1.31	18.4
-OMe			3.63 s	61.9	3.63	61.9
D-Glc I						
1	4.45 d (8.0)	102.9	4.46 d (7.8)	106.1	4.32	106.2
2	3.20 dd (9.5, 8.0)	75.7	3.21 dd (9.5, 7.8)	75.6	3.20	75.6
3	3.38 d (9.5)	78.0	3.37 d (9.5)	77.9	3.39	84.0
4	3.24 d (9.5)	71.8	3.24 d (9.5)	71.8	3.25	71.9
5	3.29 m	78.3	3.28 m	78.2	3.23	77.8
6a	3.83 dd (12.0, 3.0)	63.0	3.83 dd (12.0, 3.0)	63.1	3.83	62.9
6b	3.50 dd (12.0, 3.5)		3.52 dd (12.0, 3.5)		3.50	
D-Glc II						
1					4.46 d (8.0)	104.1
2					3.21 dd (9.5, 8.0)	75.6
3					3.37 d (9.5)	77.8
4					3.24 d (9.5)	71.6
5					3.28 m	78.1
6a					3.83 dd (12.0, 3.0)	63.0
6b					3.72 dd (12.0, 3.0)	

J values are in parentheses and reported in Hz; chemical shifts are given in ppm.

residues: six anomeric proton signals that resonated at δ 4.36, 4.38, 4.46, 4.60, 4.83, and 4.89, and correlated in the HSQC spectrum to carbons at 105.0, 106.2, 104.1, 103.8, 100.3, and 97.0 ppm, respectively, were easily identified. By a combination of 1D- and 2D-NMR experiments, 1D-TOCSY subspectrum of six monosaccharide units could be interpreted and, at the same time, the type of sugar and its configuration assigned (Table 3). The β-configuration at the anomeric carbons of the six sugars was confirmed by the *J* values of their anomeric signals (Table 3).[12,13] An HSQC experiment correlated all proton resonances with those of the corresponding carbons and allowed to deduce the position of interglycosidic linkages. The sugar sequence was confirmed by HMBC cross peaks that showed correlations between H-$1_{Glc\ II}$–C-$3_{Glc\ I}$, H-$1_{Glc\ I}$–C-4_{Dc}, H-1_{Dc}–C-4_{Allo}, and H-1_{Allo}–C-$4_{Cym\ II}$. Finally confirmation of the sugar sequence was derived from the chemical shift of C-$1_{Cym\ I}$ (97.0 ppm). Therefore the sugar chain of compounds 14 and 15 was established to be β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside.

Compound 16 (chain L). Compound 16 possessed a pentasaccharide sugar chain attached to C-3 of the aglycon that was identified as 12β-*O*-tigloyl-20-*O*-acetyldeacetin. The

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Table 4. ^1H and ^{13}C NMR data for sugar moieties of compounds 17–20 (600 MHz, CD_3OD)

Position	17, 20 chain B		18, 19 chain C		18 chain D	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
–Oble D-Fuc						
1	4.38 d (8.0)	103.0	4.39 d (8.0)	103.0		
2	3.61 dd (9.5, 8.0)	71.6	3.63 dd (9.5, 8.0)	71.7		
3	3.27 dd (9.5, 4.0)	83.9	3.26 dd (9.5, 4.0)	83.8		
4	4.20 dd (4.0, 2.5)	74.8	4.20 dd (4.0, 2.5)	74.7		
5	3.67 m	71.0	3.68 m	71.4		
6	1.32 d (6.0)	17.3	1.30 d (6.0)	17.3		
–OMe	3.53 s	58.0	3.54 s	58.3		
D-Glc I						
1	4.41 d (8.0)	104.3	4.43 d (7.8)	104.4		
2	3.17 dd (9.5, 8.0)	75.2	3.23 dd (9.5, 7.8)	76.0		
3	3.39 d (9.5)	78.9	3.39 d (9.5)	77.7		
4	3.27 d (9.5)	71.0	3.37 d (9.5)	71.2		
5	3.23 m	78.2	3.49 m	76.5		
6a	3.70 dd (12.0, 5.0)	62.5	3.82 dd (12.0, 5.0)	70.0		
6b	3.89 dd (12.0, 2.5)		4.19 dd (12.0, 2.0)			
D-Glc II						
1			4.64 d (8.0)	104.2		
2			3.24 dd (9.5, 8.0)	75.3		
3			3.37 d (9.5)	78.2		
4			3.29 d (9.5)	71.6		
5			3.30 m	78.1		
6a			3.69 dd (12.0, 5.0)	62.4		
6b			3.90 dd (12.0, 2.5)			
D-Glc III						
1					4.41 d (8.0)	104.5
2					3.23 dd (9.5, 8.0)	75.5
3					3.37 d (9.5)	78.0
4					3.38 d (9.5)	71.1
5					3.23 m	78.2
6a					3.61 dd (12.0, 5.0)	62.4
6b					3.93 dd (12.0, 2.5)	
D-Glc IV						
1					4.63 d (7.8)	104.2
2					3.22 dd (9.5, 7.8)	76.0
3					3.40 d (9.5)	78.8
4					3.37 d (9.5)	71.1
5					3.48 m	77.3
6a					3.81 dd (12.0, 5.0)	62.5
6b					3.98 dd (12.0, 3.0)	
L-Rha						
1					4.74 d (1.8)	102.0
2					3.83 dd (3.5, 1.8)	72.6
3					3.66 dd (9.0, 3.5)	72.5
4					3.38 t (9.0)	74.0
5					3.67 m	69.8
6					1.29 d (6.5)	18.0

J values are in parentheses and reported in Hz; chemical shifts are given in ppm.

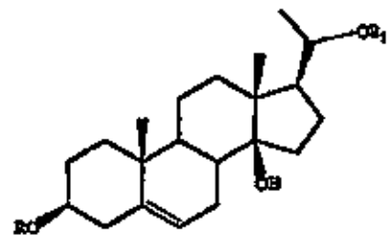
ESI-MS spectrum of compound 16 (molecular formula $C_{62}H_{100}O_{32}$) showed the $[M+Na]^+$ ion at *m/z* 1267 and a fragmentation pattern similar to that of 15. The ^{13}C and ^{13}C DEPT NMR spectra of 16 showed 62 signals of which 34 were assigned to the saccharidic portion. Analysis of sugar chain NMR data of compound 16 and comparison with those of 15 showed that 16 differed from 15 only in the absence of the terminal glucopyranosyl unit (Table 3). Therefore, the structure 12β-*O*-tigloyl-20-*O*-acetylbonoorin 3-*O*-β-D-glucopyranosyl-(1→4)-6-deoxy-3-*O*-methyl-β-D-allopyranosyl-(1→4)-β-D-thevetopyranosyl-(1→4)-β-D-cymaropyranosyl-(1→4)-β-D-cymaropyranoside was assigned to compound 16.

Compound 17 (chain B). Compound 17 was assigned $C_{70}H_{112}O_{33}$ molecular formula, as shown by its ESI-MS data (*m/z* 721 $[M+Na]^+$) in combination with the ^{13}C NMR spectrum. Compound 17 showed two anomeric protons and carbons at δ_H 4.38 and 4.41 and δ_C 103.0 and 104.3 (Table 4). The structure of the sugar unit was determined by NMR spectral data as 3-*O*-methyl-β-D-fucose attached through a 1→4 linkage to β-D-glucose and confirmed by comparison with literature values.[4] The genin was identified as calogenin from a careful analysis of its NMR spectra and by comparison with literature data.[4,5] Attachment of the glycoside chain at C-3 was indicated by the significant downfield shift observed for this carbon in compound 17 relative to the corresponding signal in calogenin and was subsequently confirmed from a long-range correlation between C-3 (79.9 ppm) and $H\text{-}1_{Fuc}$ (δ 4.38) in the HMBC spectrum. 1D-ROESY experiment showed a correlation peak between $H\text{-}1_{Glc}$ and $H\text{-}4_{Fuc}$ that

established the position of each sugar unit. The ester was determined to be an acetyl group as shown by NMR data (δ_H 1.93; δ_C 21.0, 178.0). The ester linkage was located at C-20 on the basis of the chemical shift of the double doublet signal (δ 4.42) ascribable to H-20 of esterified calogenin and confirmed by HMBC peak between H-20 and signal at 178.0 ppm. From the foregoing evidence, compound 17 was deduced as 20-*O*-acetylcalogenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside or 20-*O*-acetyl musalioside C.

Compound 18 (chains C and D). Compound 18 ($C_{72}H_{116}O_{28}$) was identified as a further calogenin derivative possessing two sugar moieties at C-3 and C-20. The ^{13}C and ^{13}C DEPT NMR spectra of 18 showed 58 signals, of which 21 were assigned to the steroid moiety and 37 to the saccharide portion. In compound 18, C-20 appeared at 79.0 ppm in the ^{13}C NMR and H-20 at δ 4.03 in the ^1H NMR spectra, indicating that the hydroxyl group at C-20 was glycosylated. Attachment of the glycosidic chain at C-3 was confirmed by comparison with compound 17 spectral data. The identity and sequence of sugar units was deduced by a combination of 1D and 2D NMR techniques. The results of 1D-TOCSY and DQF-COSY experiments allowed the sequential assignments of all the proton resonances to the individual monosaccharide as reported in Table 4. Thus, the chemical shifts of the sugar resonance were attributable to the β-D-3-*O*-methylfucopyranosyde ($\delta_{H\text{-}1_{Me}}$=4.39), β-D-glucopyranoside ($\delta_{H\text{-}1_{Glc\ I}}$=4.43), β-D-glucopyranoside ($\delta_{H\text{-}1_{Glc\ II}}$=4.64), β-D-glucopyranoside ($\delta_{H\text{-}1_{Glc\ III}}$=4.41), β-D-glucopyranoside ($\delta_{H\text{-}1_{Glc\ IV}}$=4.63), and α-L-rhamnopyranoside ($\delta_{H\text{-}1_{Rha}}$=4.74). HSQC experiments led to the correlation of all the proton resonances with those of each corresponding carbon. The absence of any glycosidation shift for one β-D-glucopyranose and α-L-rhamnopyranose moieties suggested that these sugars were terminal units. Glycosidation shifts were observed for C-4_{Me} (74.7 ppm), C-$6_{Glc\ I}$ (70.0 ppm), C-$6_{Glc\ III}$ (68.4 ppm), and C-$6_{Glc\ IV}$ (63.5 ppm). Finally the position of the sugar residues in 18 was defined by the HMBC experiment. A cross peak due to long-range correlation between C-3 (80.0 ppm) of the aglycon and H-1_{Me} indicated that 3-*O*-methylfucose was the residue linked to C-3; a cross peak between C-4_{Me} and H-$1_{Glc\ I}$ indicated that glucose I was the second unit; a cross peak between C-$6_{Glc\ I}$ and H-$1_{Glc\ II}$ indicated that glucose II was the third unit of the trisaccharide chain at C-3. Similarly, the sequence of the trisaccharide chain at C-20 was indicated by the cross peaks between C-20 and H-$1_{Glc\ III}$, and between C-$6_{Glc\ III}$ and H-$1_{Glc\ IV}$ and between C-$6_{Glc\ IV}$ and H-1_{Rha}. On the basis of all this evidence compound 18 was identified as calogenin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside-20-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6) β-D-glucopyranoside.

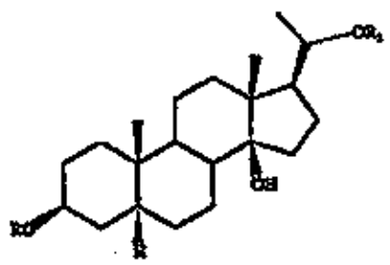
Compound 19 (chains C and A). Compound 19 had molecular formula $C_{66}H_{106}O_{27}$ as obtained from NMR and ESI-MS spectral data. Its NMR spectra showed, together with signals of the aglycon calogenin, 25 signals ascribable to sugar units. ESI-MS of compound 19 showed the $[M+Na]^+$ ion at *m/z* 1003 with prominent fragments at *m/z* 841 $[(M+Na)-162]^+$ (cleavage of one hexose unit) and *m/z* 519 $[(M+Na)-(162+160+162)]^+$ due to the subsequent loss of two hexose units. Analysis of the NMR data of compound 19 and comparison with those of 18 showed they possessed the same sugar chain at C-3 (Table 4), while saccharidic moiety at C-20 was the point of difference. The sugar chain at C-20 of compound 19 was established to be one β-D-glucopyranose (Experimental section). Therefore, the structure of calogenin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside-20-*O*-β-D-glucopyranoside was assigned to 19.



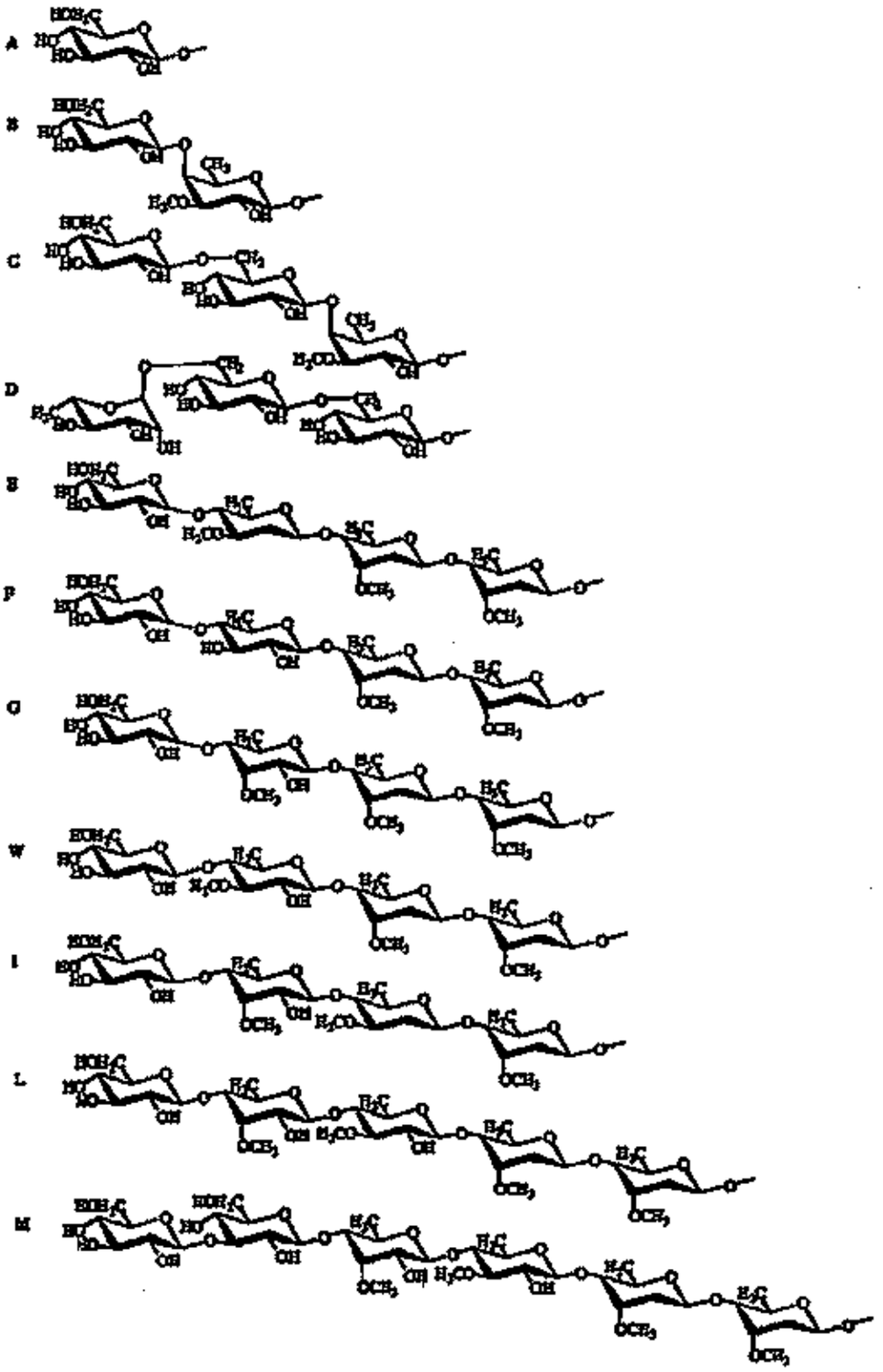
Compounds	R	R_1
17	B	Ac
18	C	D
19	C	A

Compound 20 (chains B and A). Finally, compound 20 had molecular formula $C_{60}H_{96}O_{27}$ (^{13}C NMR data and ESI-MS, *m/z* 843 $[M+Na]^+$) that presented only two mass unit of difference if compared with musalioside B.[4] This evidence, together with NMR analysis, suggested the presence of the 5α-dihydrocalogenin aglycon[4] with the same sugar moiety of musalioside B. The comparison of the ^1H and ^{13}C NMR spectra of 20 with those of musalioside B showed the lack of the double bond between C-5 and C-6 and the presence of one methyne and one methylene at δ 1.11 and 1.09, 2.05, respectively, which correlated in the HSQC spectrum with signals at 45.6 and 28.7 ppm (Table 1). Thus the structure of compound 20 was established as 5α-dihydrocalogenin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-3-*O*-methylfucopyranoside-20-*O*-β-D-glucopyranoside or dihydromusalioside B.

The known compounds were identified as musalioside B and musalioside C by comparison with literature NMR data.[4]



Compound	R	R_1
20	B	A



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2.1. Cytotoxic and genotoxic activity

Some pregnane glycosides from *C. negevensis*, selected on the basis of their structural differences were submitted to cytotoxic and genotoxic activity evaluation studies. The cytotoxic test[9] assesses the ability of a molecule to arrest or inhibit tumor cell growth, while the genotoxic assay evaluates the induction of micronuclei (small chromatine mass resembling the main nucleus and easily observed in the cytoplasm of cells undergoing at least one cell division). This methodology has been previously used to preserve biological activity of newly characterized chemicals of plant origin.[10,11] None of the tested compounds inhibited tumor cell (LoVo and HT29) growth up to 25 μg/mL, except for compound 7 that is cytotoxic for both cell lines. This effect seems to be the consequence of a general toxicity rather than of a specific activity against the DNA as there is no difference in the pattern of toxicity between LoVo and HT29 cell lines. Compound 7 slightly increased micronuclei (MN) frequency only at 150 μg/mL.[10,11] These results indicated that the tested compounds did not show effective anticancer activity against the two cell lines used and were not genotoxic in the in vitro human lymphocyte micronucleus assay, especially when compared to other related molecules which are active at concentrations at least 50-fold lower.

3. Experimental

3.1. General

Melting points were determined on a Koller apparatus. Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin–Elmer-Lambda 11 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^1H and 150.86 MHz for ^{13}C, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in δ (ppm) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 (for CD_3OD). ^{13}C DEPT, 1D-TOCSY, 1D-ROESY, ^1H–^1H DQF-COSY, HOHAHA, ^1H–^{13}C HSQC, and HMBC experiments were carried out using the conventional pulse sequences as described in the literature.[12] ESI-MS (positive mode) were obtained from a Finnigan LC-Q Deca instrument from Termoquest (San Jose, CA, USA) equipped with Excalibur software. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ solution followed by heating. Column chromatography was performed over Si gel (40–63 μm, Merck) and Sephadex LH-20 (Pharmacia); droplet countercurrent chromatography (DCCC) was performed on an apparatus manufactured by Büchi, equipped with 300 tubes; HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column and Shimadzu injector.

3.2. Plant material

The whole plant of *C. negevensis* Zohary was collected in Dabbet Hamoot, Qa' al Nejeb, Jordan, in April, 1999 and identified by Professor Sawsan Al Oran, Department of Biology, University of Jordan, Amman, Jordan. A voucher specimen is deposited at the Orto Botanico, Università di Pisa, Pisa, Italy (No. 2000-0051/1).

3.3. Extraction and isolation

The dried whole plant (80 g) of *C. negevensis* was defatted with *n*-hexane and then extracted successively at room temperature with $CHCl_3$ and MeOH to give 4.9 and 9.5 g of residues, respectively. The chloroform extract was dissolved in a mixture MeOH–H_2O 8:2 and part of the soluble portion (1.1 g) was submitted to flash column chromatography over Si gel with $CHCl_3$ containing an increasing amount of MeOH (99:1, 98:2, 9:1, 8:2, 1:1, V/V) to give six main fractions. Fractions 4 (190 mg), 5 (180 mg), and 6 (45 mg) were further purified on RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 4:1 for fraction 4, MeOH–H_2O 75:25 for fraction 5, and MeOH–H_2O 7:3 for fraction 6, to yield compounds 16 (2.0 mg, t_R=17 min), 1 (2.5 mg, t_R=22 min), 3 (3.5 mg, t_R=25 min), and 2 (6.3 mg, t_R=45 min) from fraction 4, compounds 4 (2.3 mg, t_R=10 min), 5 (2.7 mg, t_R=30 min), 9 (3.8 mg, t_R=35 min), 6 (6.5 mg, t_R=45 min), 14 (3.8 mg, t_R=50 min), 13 (4.5 mg, t_R=82 min), 10 (3.0 mg, t_R=90 min), and 7 (6.5 mg, t_R=98 min) from fraction 5; russelioside C (3.5 mg, t_R=22 min), compounds 11 (2.0 mg, t_R=56 min), 12 (3.3 mg, t_R=65 min), and 8 (2.4 mg, t_R=70 min) from fraction 6, respectively. The methanolic residue was partitioned between *n*-BuOH–H_2O; the *n*-BuOH fraction (4.0 g) was chromatographed on Sephadex LH-20 with MeOH as eluent, collecting 20 major fractions. Fraction 3 was purified by DCCC ($CHCl_3$–MeOH–H_2O–*n*-PrOH (9:12:8:1), descending mode, flow 10 mL/h) to give three fractions: A (100 mg), B (40 mg), and C (60 mg). Fraction C was submitted to final separation by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 83:17 as eluent to obtain pure compound 15 (3.0 mg, t_R=25 min). Fraction 5 was submitted to DCCC (*n*-BuOH–Me_2CO–H_2O (33:10:50) descending mode, flow 10 mL/h), to yield pure compound 19 (4.0 mg) together with two main fractions A' (80 mg) and B' (50 mg). Fraction B' was purified by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 1:1 as eluent to yield pure compound 17 (6.2 mg, t_R=35 min). Fraction 6 was submitted to DCCC (*n*-BuOH–Me_2CO–H_2O (33:10:50) descending mode, flow 10 mL/h), to yield pure compound 18 (6.5 mg) together with one fraction B" (70 mg) that was finally purified by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 3:2 as eluent to yield pure russelioside B (50 mg, t_R=21 min) and compound 20 (5.8 mg, t_R=27 min).

3.3.1. Compound 1. Amorphous powder, mp 135–138°C; $[\alpha]_D^{25}$=+10.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 218 sh (2.56) nm; ^1H NMR of the aglycone: δ 1.05 (3H, s, Me-19), 1.06 (3H, s, Me-18), 1.29 (3H, d, *J*=6.5 Hz, Me-21), 1.87 (3H, d, *J*=6.5 Hz, H-4'), 1.89 (1H, m, H-7a), 1.94 (3H, s, H-5'), 2.05 (1H, m, H-17), 2.20 (1H, dd, *J*=10.2, 2.5 Hz, H-4a), 2.27 (1H, m, H-7b), 2.38 (1H, br

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t, J=9.5 Hz, H-4b), 3.45 (1H, m, H-3), 3.66 (1H, dd, J=12.0, 4.0 Hz, H-12), 4.62 (1H, m, H-20), 5.48 (1H, br d, J=4.0 Hz, H-6), 6.96 (1H, br q, J=6.5 Hz, H-3′); ^{13}C NMR data of the aglycon: see Table 1, ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1049 $[M+Na]^+$; anal. C 61.90% H 8.46%, O 29.64%, calcd for $C_{53}H_{86}O_{23}$, C 61.95%, H 8.44%, O 29.60%.

3.3.2. Compound 2. White crystalline powder, mp 133–135°C; $[\alpha]_D^{23}$=+19.7 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 215 sh (3.31), 272 (2.90) nm; ^{1}H NMR of the aglycon: δ 1.01 (3H, s, Me-18), 1.03 (3H, s, Me-19), 1.15 (3H, d, J=6.5 Hz, Me-21), 1.82 (3H, d, J=6.5 Hz, H-4′ at C-12), 1.83 (3H, s, H-5′ at C-20), 1.84 (1H, m, H-7a), 1.88 (3H, d, J=6.5 Hz, H-4′ at C-20), 1.90 (3H, s, H-5′ at C-12), 2.06 (1H, m, H-17), 2.16 (1H, dd, J=10.0, 2.5 Hz, H-4a), 2.22 (1H, m, H-7b), 2.36 (1H, br t, J=9.5 Hz, H-4b), 3.51 (1H, m, H-3), 4.72 (1H, dd, J=12.0, 4.3 Hz, H-12), 5.11 (1H, m, H-20), 5.47 (1H, br d, J=4.0 Hz, H-6), 6.79 (1H, br q, J=6.5 Hz, H-3′ at C-12), 6.93 (1H, br q, J=6.5 Hz, H-3′ at C-20); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1131 $[M+Na]^+$; anal. C 62.75%, H 8.37%, O 28.88%, calcd for $C_{55}H_{88}O_{24}$, C 62.78%, H 8.36%, O 28.86%.

3.3.3. Compound 3. Amorphous powder, mp 130°C; $[\alpha]_D^{23}$=+19.0 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 219 sh (4.12) nm; ^{1}H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.08 (3H, s, Me-18), 1.14 (3H, d, J=6.5 Hz, Me-21), 1.82 (3H, d, J=6.5 Hz, H-4′), 1.90 (1H, m, H-7a), 1.91 (3H, s, H-5′), 2.00 (3H, s, COMe), 2.08 (1H, m, H-17), 2.18 (1H, dd, J=10.0, 2.5 Hz, H-4a), 2.26 (1H, m, H-7b), 2.33 (1H, br t, J=9.0 Hz, H-4b), 3.52 (1H, m, H-3), 4.64 (1H, dd, J=12.0, 4.0 Hz, H-12), 4.95 (1H, m, H-20), 5.47 (1H, br d, J=4.3 Hz, H-6), 7.05 (1H, br q, J=6.5 Hz, H-3′); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H NMR of the sugar moiety: δ 1.20 (3H, d, J=6.4 Hz, H-6$_{gal}$), 1.25 (3H, d, J=6.0 Hz, H-6$_{rha}$), 1.45 (3H, d, J=6.0 Hz, H-6$_{glc}$), 1.53 (1H, br dd, J=16.0, 12.0 Hz, H-2b$_{rha}$), 1.63 (1H, br dd, J=16.0, 12.0 Hz, H-2b$_{rha}$), 2.09 (1H, br dd, J=16.0, 3.0 Hz, H-2a$_{rha}$), 2.15 (1H, br dd, J=16.0, 3.0 Hz, H-2a$_{rha}$), 3.14 (1H, dd, J=10.0, 7.0 Hz, H-2$_{glc}$), 3.20 (1H, dd, J=9.5, 7.5 Hz, H-2$_{gal}$), 3.23 (1H, d, J=9.5 Hz, H-4$_{glc}$), 3.25 (1H, dd, J=9.5, 3.0 Hz, H-4$_{rha}$), 3.26 (1H, m, H-5$_{gal}$), 3.30 (1H, dd, J=9.5, 3.0 Hz, H-4$_{rha}$), 3.37 (1H, d, J=9.5 Hz, H-3$_{glc}$), 3.45 (1H, m, H-5$_{glc}$), 3.47 (1H, t, J=10.0 Hz, H-4$_{glc}$), 3.51 (3H, s, OMe$_{rha}$), 3.53 (3H, s, OMe$_{rha}$), 3.54 (1H, t, J=10.0 Hz, H-3$_{glc}$), 3.64 (1H, dd, J=12.0, 3.5 Hz, H-6b$_{glc}$), 3.82 (1H, dq, J=9.5, 6.0 Hz, H-5$_{rha}$), 3.85 (1H, dq, J=9.5, 6.4 Hz, H-5$_{rha}$), 3.87 (1H, q, J=3.0 Hz, H-3$_{rha}$), 3.88 (1H, q, J=3.0 Hz, H-3$_{rha}$), 3.94 (1H, dd, J=12.0, 5.0 Hz, H-6a$_{glc}$), 4.47 (1H, d, J=7.5 Hz, H-1$_{glc}$), 4.64 (1H, d, J=7.5 Hz, H-1$_{glc}$), 4.81 (1H, dd, J=9.0, 2.0 Hz, H-1$_{rha}$), 4.87 (1H, dd, J=9.5, 2.0 Hz, H-1$_{rha}$); ^{13}C NMR of the sugar moiety: δ 18.0 (q, C-6$_{rha}$), 18.5 (q, C-6$_{rha}$), 18.6 (q, C-6$_{gal}$), 36.5 (t, C-2$_{rha}$), 36.8 (t, C-2$_{rha}$), 58.0 (q, OMe$_{rha}$), 58.1 (q, OMe$_{rha}$), 62.9 (t, C-6$_{glc}$), 69.7 (d, C-5$_{rha}$), 69.9 (d, C-5$_{rha}$), 71.8 (d, C-4$_{glc}$), 72.6 (d, C-5$_{glc}$), 74.4 (d, C-2$_{glc}$), 75.3 (d, C-2$_{gal}$), 78.1 (d, C-5$_{glc}$), 78.0 (d, C-3$_{rha}$), 78.2 (d, C-3$_{glc}$), 78.6 (d, C-3$_{rha}$), 78.7 (d, C-3$_{rha}$), 83.6 (d, C-4$_{glc}$), 83.7 (d, C-4$_{rha}$), 84.0 (d, C-4$_{rha}$), 96.9 (d, C-1$_{rha}$), 101.2 (d, C-1$_{rha}$), 102.6 (d, C-1$_{glc}$), 104.1 (d, C-1$_{glc}$); ESI-MS m/z: 1093 $[M+Na]^+$; anal. C 60.49%, H 8.11%, O 31.40%, calcd for $C_{54}H_{86}O_{22}$, C 60.54%, H 8.09%, O 31.36%.

3.3.4. Compound 4. Amorphous powder, mp 165–170°C; $[\alpha]_D^{23}$=+29.0 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 225 sh (2.24), 280 (3.38) nm; NMR data for the aglycon moiety are superimposable on those for hoeceda;[15] ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 983 $[M+Na]^+$; anal. C 59.90%, H 8.42%, O 31.68%, calcd for $C_{48}H_{80}O_{20}$, C 59.98%, H 8.39%, O 31.63%.

3.3.5. Compound 5. Amorphous powder, mp 118–120°C; $[\alpha]_D^{23}$=+20.3 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 222 sh (4.20), 250 sh (3.76), 278 (3.05) nm; ^{1}H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.23 (3H, d, J=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 2.19 (1H, dd, J=9.5, 2.5 Hz, H-4a), 2.25 (1H, m, H-7b), 2.40 (1H, br t, J=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, J=12.0, 4.0 Hz, H-12), 5.48 (1H, br d, J=4.5 Hz, H-6), 6.60 (1H, t, J=8.0 Hz, H-4′), 7.20 (1H, t, J=8.0 Hz, H-5′), 7.77 (1H, d, J=8.0 Hz, H-3′), 7.91 (1H, d, J=8.0 Hz, H-6′); ^{13}C NMR data of the aglycon: see Table 1, ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1103 $[M+Na]^+$; anal. C 61.06%, H 7.84%, O 31.10%, calcd for $C_{53}H_{84}O_{22}$, C 61.10%, H 7.83%, O 31.07%.

3.3.6. Compound 6. Amorphous powder, mp 133–139°C; $[\alpha]_D^{23}$=+35.6 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 220 sh (3.86), 282 (4.76) nm; NMR data for the aglycon moiety are identical to those for compound 3; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1107 $[M+Na]^+$; anal. C 60.80%, H 8.20%, O 31.00%, calcd for $C_{52}H_{88}O_{22}$, C 60.87%, H 8.17%, O 30.96%.

3.3.7. Compound 7. Amorphous powder, mp 152–157°C; $[\alpha]_D^{23}$=+34.7 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 280 (3.99) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1147 $[M+Na]^+$, 1047, 947, 439; anal. C 61.83%, H 8.27%, O 29.85%, calcd for $C_{56}H_{92}O_{23}$, C 61.90%, H 8.24%, O 29.86%.

3.3.8. Compound 8. Amorphous powder, mp 133–135°C; $[\alpha]_D^{23}$=+25.0 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 220 sh (2.40) nm; ^{1}H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.23 (3H, d, J=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.84 (3H, d, J=6.5 Hz, H-4′), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 1.89 (3H, s, H-5′), 2.19 (1H, dd, J=9.5, 2.0 Hz, H-4a), 2.26 (1H, m, H-7b), 2.40 (1H, br t, J=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, J=12.0, 4.0 Hz, H-12), 5.48 (1H, br d, J=4.0 Hz, H-6), 6.98 (1H, br q, J=6.5 Hz, H-3′); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS m/z: 1105 $[M+Na]^+$, 1025; anal. C 61.04%, H 8.30%, O 30.66%, calcd for $C_{53}H_{86}O_{22}$, C 61.02%, H 8.31%, O 30.67%.

3.3.9. Compound 9. Amorphous powder, mp 120–123°C; $[\alpha]_D^{23}$=+4.0 (c 0.4, MeOH); UV (MeOH) λ_{max} (log ε): 220

sh (2.29) nm; NMR data for the aglycon moiety are identical to those for compound 8; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1065 $[M+Na]^+$, 965; anal. C 60.98%, H 8.33%, O 30.69%, calcd for $C_{50}H_{82}O_{20}$, C 61.02%, H 8.31%, O 30.67%.

3.3.10. Compound 10. Amorphous powder, mp 152°C; $[\alpha]_D^{25}$=+16.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 217 sh (3.50), 278 (3.88) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1147 $[M+Na]^+$, 1047, 585; anal. C 61.81%, H 8.27%, O 29.92%, calcd for $C_{56}H_{92}O_{24}$, C 61.90%, H 8.24%, O 29.85%.

3.3.11. Compound 11. Amorphous powder, mp 160°C; $[\alpha]_D^{25}$=+16.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 220 sh (3.85), 247 sh (2.92), 280 (3.68) nm; NMR data for the aglycon moiety are identical to those for compound 6; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1103 $[M+Na]^+$; anal. C 61.07%, H 7.85%, O 31.08%, calcd for $C_{53}H_{84}O_{22}$, C 61.10%, H 7.83%, O 31.07%.

3.3.12. Compound 12. Amorphous powder, mp 140–142°C; $[\alpha]_D^{25}$=+12.0 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log *e*): 225 (3.90) nm; ^{1}H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.23 (3H, d, *J*=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 2.05 (3H, s, COMe), 2.19 (1H, dd, *J*=10.0, 2.5 Hz, H-4a), 2.26 (1H, m, H-7b), 2.40 (1H, br t, *J*=10.0 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, *J*=12.0, 4.2 Hz, H-12), 5.48 (1H, br d, *J*=4.5 Hz, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1025 $[M+Na]^+$, 965; anal. C 59.79%, H 8.26%, O 31.95%, calcd for $C_{50}H_{82}O_{20}$, C 59.86%, H 8.24%, O 31.90%.

3.3.13. Compound 13. White crystalline powder, mp 140–145°C; $[\alpha]_D^{25}$=+49.0 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log *e*): 218 sh (3.21), 276 (3.50) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1147 $[M+Na]^+$; anal. C 61.91%, H 8.19%, O 29.90%, calcd for $C_{56}H_{92}O_{24}$, C 61.90%, H 8.24%, O 29.86%.

3.3.14. Compound 14. Amorphous powder, mp 125–128°C; $[\alpha]_D^{25}$=+19.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 221 sh (4.15), 279 (4.30) nm; NMR data of the aglycon moiety are identical to those for compound 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1357 $[M+Na]^+$, 1257; anal. C 58.00%, H 7.98%, O 34.02%, calcd for $C_{62}H_{102}O_{26}$, C 58.05%, H 7.97%, O 33.98%.

3.3.15. Compound 15. Amorphous powder, mp 165–170°C; $[\alpha]_D^{25}$ +34.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 218 sh (4.42) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1469 $[M+Na]^+$; anal. C 58.84%, H 7.96%, O 33.20%, calcd for $C_{71}H_{114}O_{30}$, C 58.91%, H 7.94%, O 33.16%.

3.3.16. Compound 16. Amorphous powder, 185–190°C dec; $[\alpha]_D^{25}$=+24.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 220 sh (2.45), 275 (4.21) nm; NMR data for the aglycon moiety are identical to those for compound 3; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1267 $[M+Na]^+$, 1207; anal. C 59.71%, H 8.10%, O 32.19%, calcd for $C_{59}H_{96}O_{24}$, C 59.79%, H 8.09%, O 32.12%.

3.3.17. Compound 17. Amorphous powder, mp 174°C; $[\alpha]_D^{25}$=-22 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 265 (4.25) nm; ^{1}H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.14 (3H, s, Me-18), 1.32 (3H, d, *J*=6.4 Hz, Me-21), 1.70 (1H, m, H-17), 1.90 (1H, m, H-7a), 1.93 (3H, s, COMe), 2.25 (1H, m, H-7b), 2.31 (1H, dd, *J*=10.0, 2.5 Hz, H-4a), 2.47 (1H, br t, *J*=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 4.42 (1H, m, H-20), 5.45 (1H, m, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 4; ESI-MS *m/z*: 721 $[M+Na]^+$, 661; anal. C 61.82%, H 8.36%, O 29.80%, calcd for $C_{36}H_{58}O_{10}$, C 61.87%, H 8.37%, O 29.76%.

3.3.18. Compound 18. Amorphous powder, mp 142°C; $[\alpha]_D^{25}$=-4.0 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log *e*): 277 (4.37), 320 sh (3.97) nm; ^{1}H NMR of the aglycon: δ 1.08 (3H, s, Me-19), 1.15 (3H, s, Me-18), 1.31 (3H, d, *J*=6.4 Hz, Me-21), 1.70 (1H, m, H-17), 1.90 (1H, m, H-7a), 2.25 (1H, m, H-7b), 2.31 (1H, dd, *J*=9.5, 2.5 Hz, H-4a), 2.47 (1H, br t, *J*=9.5 Hz, H-4b), 3.55 (1H, m, H-3), 4.03 (1H, m, H-20), 5.45 (1H, br d, *J*=4.0 Hz, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 4; ESI-MS *m/z*: 1311 $[M+Na]^+$; anal. C 54.00%, H 7.52%, O 38.48%, calcd for $C_{62}H_{96}O_{28}$, C 54.03%, H 7.50%, O 38.47%.

3.3.19. Compound 19. Amorphous powder, mp 195°C; $[\alpha]_D^{25}$=-11.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 220 sh (4.13), 283 (3.78) nm; NMR data for the aglycon moiety are superimposable on those of compound 18; ^{1}H NMR of the sugar moiety: chain A δ 3.16 (1H, dd, *J*=9.5, 7.8 Hz, H-2$_{glc}$ m), 3.28 (1H, d, *J*=9.5 Hz, H-4$_{glc}$ m), 3.30 (1H, m, H-5$_{glc}$ m), 3.38 (1H, d, *J*=9.5 Hz, H-3$_{glc}$ m), 3.68 (1H, dd, *J*=12.0, 5.0 Hz, H-6a$_{glc}$ m), 3.83 (1H, dd, *J*=12.0, 3.0 Hz, H-6b$_{glc}$ m), 4.40 (1H, d, *J*=7.8 Hz, H-1$_{glc}$ m); chain C: see Table 4; ^{13}C NMR of the sugar moiety: chain A δ 62.4 (C-6$_{glc}$ m), 71.7 (C-4$_{glc}$ m), 75.3 (C-2$_{glc}$ m), 77.6 (C-3$_{glc}$ m), 78.2 (C-5$_{glc}$ m), 104.5 (C-1$_{glc}$ m); chain C: see Table 4; ESI-MS *m/z*: 1003 $[M+Na]^+$, 841, 519; anal. C 56.28%, H 7.82%, O 35.90%, calcd for $C_{48}H_{76}O_{20}$, C 56.31%, H 7.81%, O 35.88%.

3.3.20. Compound 20. Amorphous powder, 270–275°C dec; $[\alpha]_D^{25}$=-6.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *e*): 225 (4.55), 238 sh (3.78) nm; ^{1}H NMR of the aglycon: δ 0.86 (3H, s, Me-18), 1.10 (3H, s, Me-19), 1.30 (3H, d, *J*=6.4 Hz, Me-21), 1.65 (1H, m, H-17), 3.67 (1H, m, H-3), 4.03 (1H, m, H-20); ^{13}C NMR data of the aglycon: see Table 1; for ^{1}H and ^{13}C NMR data of the sugar moiety see compound 19 (chain A) and Table 4 (chain B); ESI-MS *m/z*: 843 $[M+Na]^+$; anal. C 58.45%, H 8.37%, O 33.18%, calcd for $C_{42}H_{68}O_{16}$, C 58.32%, H 8.35%, O 33.13%.

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3.4. Cytotoxic and genotoxic activity

3.4.1. In vitro cytotoxicity test for anticancer activity in tumor cell lines. Cell lines: HT29, colorectal human cancer cell line, DNA mismatch repair proficient, established from non polyposic sporadic tumor; LoVo, colorectal human cancer cell line, DNA mismatch repair deficient, established from non polyposic hereditary tumor. The two cancer cell lines were treated with 10, 25, 75, and 100 μg/mL from 24 h to 48 h. All tested compounds were dissolved in DMSO not exceeding 1% final concentration. Negative controls were set up according to the maximum amount of solvent used to prepare each chemical. The ability of single cells to proliferate and form colony during 48 h grown in culture medium was measured. The results are expressed, in percent, as relative cloning efficiency that is the ratio of the number of cell grown after chemical treatment with respect to untreated cell growth.[9]

3.4.2. In vitro genotoxicity test in human somatic cells. Phytohemagglutinin-stimulated peripheral blood lymphocytes cultured up to 72 h were used. The addition of cytochalasin B to cultures 44 h before cell harvest blocked the cytodieresis of dividing lymphocytes which assumed a characteristic binucleated shape (a cell with two main nuclei) at the following interphase. Only in these cells was observed the presence of micronuclei. Lymphocyte cultures were treated with 0.1, 1.0, 5.0 50, 100, and 150 μg/mL from 24 to 72 h. All tested compounds were dissolved in DMSO not exceeding 1% final concentration. Negative controls were set up according to the maximum amount of solvent used to prepare each chemical. The frequency of micronuclei (MN) is expressed as the number of micronucleated lymphocytes (containing one or more MN) on 1000 cells scored.[9,11]

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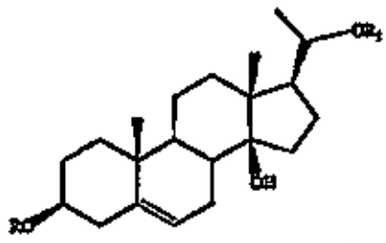
established the position of each sugar unit. The ester was determined to be an acetyl group as shown by NMR data (δ_H 1.93; δ_C 21.0, 178.0). The ester linkage was located at C-20 on the basis of the chemical shift of the double doublet signal (δ 4.42) ascribable to H-20 of esterified calogenin and confirmed by HMBC peak between H-20 and signal at 178.0 ppm. From the foregoing evidence, compound 17 was deduced as 20-*O*-acetylcalogenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside or 20-*O*-acetyl russelioside C.

Compound 18 (chains C and D). Compound 18 ($C_{70}H_{116}O_{31}$) was identified as a further calogenin derivative possessing two sugar moieties at C-3 and C-20. The ^{13}C and ^{13}C DEPT NMR spectra of 18 showed 58 signals, of which 21 were assigned to the steroid moiety and 37 to the saccharide portion. In compound 18, C-20 appeared at 79.0 ppm in the ^{13}C NMR and H-20 at δ 4.03 in the ^1H NMR spectra, indicating that the hydroxyl group at C-20 was glycosylated. Attachment of the glycosidic chain at C-3 was confirmed by comparison with compound 17 spectral data. The identity and sequence of sugar units was deduced by a combination of 1D and 2D NMR techniques. The results of 1D-TOCSY and DQF-COSY experiments allowed the sequential assignments of all the proton resonances to the individual monosaccharide as reported in Table 4. Thus, the chemical shifts of the sugar resonance were attributable to the β-D-3-*O*-methylfucopyranosyde ($\delta_{H\text{-}1_{gle\ I}}$=4.39), β-D-glucopyranoside ($\delta_{H\text{-}1_{glc\ II}}$=4.43), β-D-glucopyranoside ($\delta_{H\text{-}1_{glc\ III}}$=4.64), β-D-glucopyranoside ($\delta_{H\text{-}1_{glc\ III}}$=4.41), β-D-glucopyranoside ($\delta_{H\text{-}1_{glc\ IV}}$=4.63), and α-L-rhamnopyranoside ($\delta_{H\text{-}1_{rha}}$=4.74). HSQC experiment led to the correlation of all the proton resonances with those of each corresponding carbon. The absence of any glycosidation shift for one β-D-glucopyranose and α-L-rhamnopyranose moieties suggested that these sugars were terminal units. Glycosidation shifts were observed for $C\text{-}4_{rha}$ (74.7 ppm), $C\text{-}6_{glc\ I}$ (70.0 ppm), $C\text{-}6_{glc\ III}$ (68.4 ppm), and $C\text{-}6_{glc\ IV}$ (68.5 ppm). Finally the position of the sugar residues in 18 was defined by the HMBC experiment. A cross peak due to long-range correlation between C-3 (80.0 ppm) of the aglycon and $H\text{-}1_{gle}$ indicated that 3-*O*-methylfucose was the residue linked to C-3; a cross peak between $C\text{-}4_{gle}$ and $H\text{-}1_{glc\ I}$ indicated that glucose I was the second unit; a cross peak between $C\text{-}6_{glc\ I}$ and $H\text{-}1_{glc\ II}$ indicated that glucose II was the third unit of the trisaccharide chain at C-3. Similarly, the sequence of the trisaccharide chain at C-20 was indicated by the cross peaks between C-20 and $H\text{-}1_{glc\ III}$, and between $C\text{-}6_{glc\ III}$ and $H\text{-}1_{glc\ IV}$ and between $C\text{-}6_{glc\ IV}$ and $H\text{-}1_{rha}$. On the basis of all this evidence compound 18 was identified as calogenin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside-20-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6) β-D-glucopyranoside.

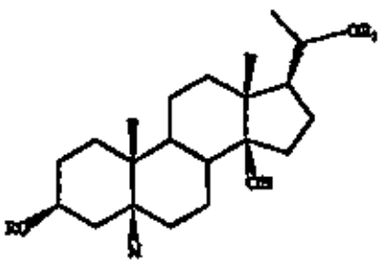
Compound 19 (chains C and A). Compound 19 had molecular formula $C_{58}H_{96}O_{22}$ as obtained from NMR and ESI-MS spectral data. Its NMR spectra showed, together with signals of the aglycon calogenin, 25 signals ascribable to sugar units. ESI-MS of compound 19 showed the $[M+Na]^+$ ion at *m/z* 1003 with prominent fragments at *m/z* 841 $[(M+Na)-162]^+$ (cleavage of one hexose unit) and *m/z* 519 $[(M+Na)-(162+160+162)]^+$ due to the subsequent loss of two hexose units. Analysis of the NMR data of compound 19 and comparison with those of 18 showed they possessed the same sugar chain at C-3 (Table 4), while saccharidic moiety at C-20 was the point of difference. The sugar chain at C-20 of compound 19 was established to be one β-D-glucopyranose (Experimental section). Therefore, the structure of calogenin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside-20-*O*-β-D-glucopyranoside was assigned to 19.



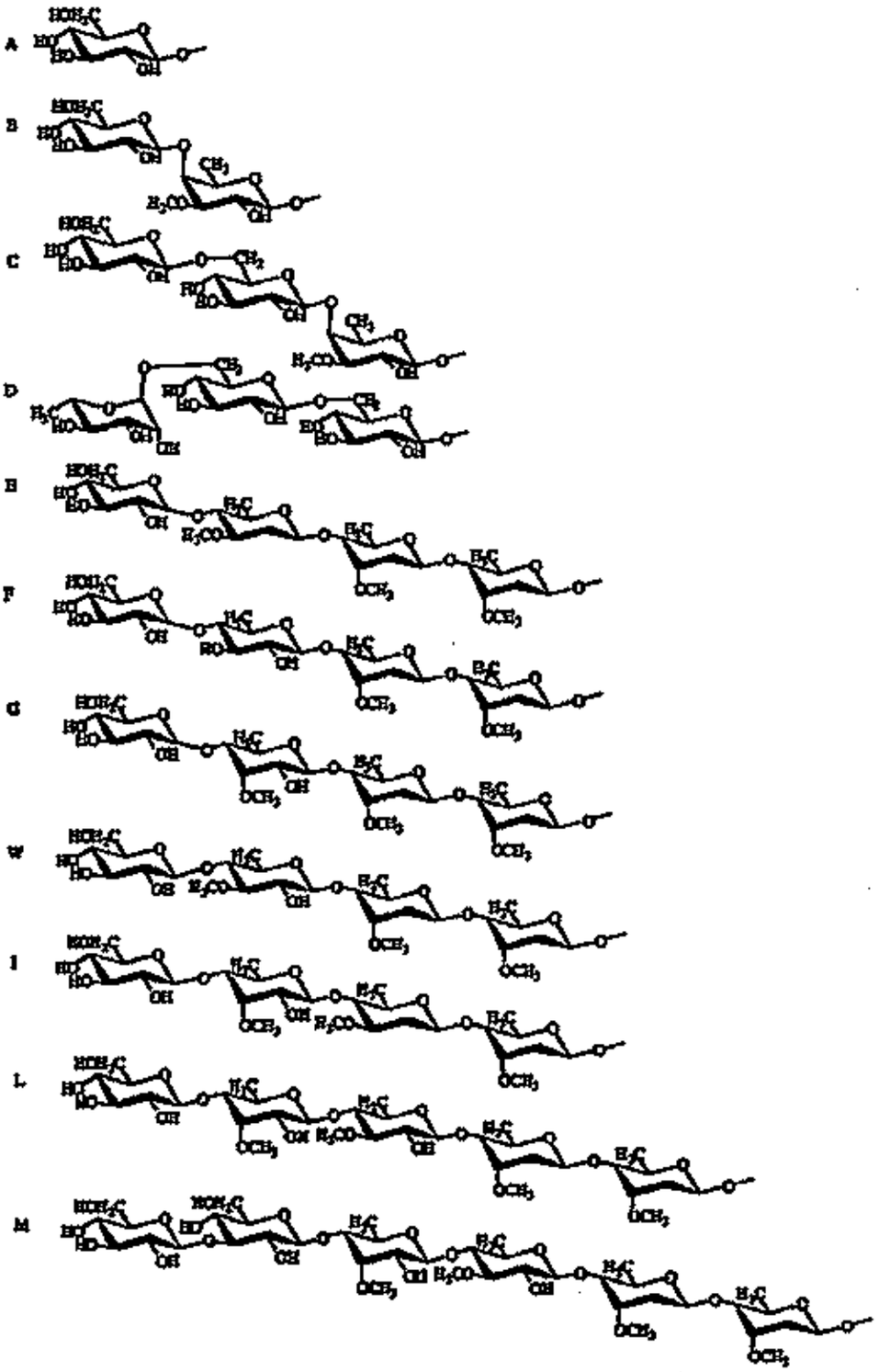
Compounds	R	R_1
17	B	Ac
18	C	D
19	C	A

Compound 20 (chains B and A). Finally, compound 20 had molecular formula $C_{50}H_{86}O_{17}$ (^{13}C NMR data and ESI-MS, *m/z* 843 $[M+Na]^+$) that presented only two mass unit of difference if compared with russelioside B.[4] This evidence, together with NMR analysis, suggested the presence of the 5α-dihydrocalogenin aglycon[5] with the same sugar moiety of russelioside B. The comparison of the ^1H and ^{13}C NMR spectra of 20 with those of russelioside B showed the lack of the double bond between C-5 and C-6 and the presence of one methyne and one methylene at δ 1.11 and 1.09, 2.05, respectively, which correlated in the HSQC spectrum with signals at 45.6 and 28.7 ppm (Table 1). Thus the structure of compound 20 was established as 5α-dihydrocalogenin 3-*O*-β-D-glucopyranosyl-(1→4)-β-D-3-*O*-methylfucopyranoside-20-*O*-β-D-glucopyranoside or dihydrorussellioside B.

The known compounds were identified as russelioside B and russelioside C by comparison with literature NMR data.[4]



Compound	R	R_1
20	B	A



64
390
391

2.1. Cytotoxic and genotoxic activity

Some pregnane glycosides from *C. negrevensis*, selected on the basis of their structural differences were submitted to cytotoxic and genotoxic activity evaluation studies. The cytotoxic test[9] assesses the ability of a molecule to arrest or inhibit tumor cell growth, while the genotoxic assay evaluates the induction of micronuclei (small chromatine mass resembling the main nucleus and easily observed in the cytoplasm of cells undergoing at least one cell division). This methodology has been previously used to pursuance biological activity of newly characterized chemicals of plant origin.[10,11] None of the tested compounds inhibited tumor cell (LoVo and HT29) growth up to 25 μg/mL, except for compound 7 that is cytotoxic for both cell lines. This effect seems to be the consequence of a general toxicity rather than of a specific activity against the DNA as there is no difference in the pattern of toxicity between LoVo and HT29 cell lines. Compound 7 slightly increased micronuclei (MN) frequency only at 150 μg/mL.[10,11] These results indicated that the tested compounds did not show effective anticancer activity against the two cell lines used and were not genotoxic in the in vitro human lymphocyte micronucleus assay, especially when compared to other related molecules which are active at concentrations at least 50-fold lower.

3. Experimental

3.1. General

Melting points were determined on a Kofler apparatus. Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were recorded on a Perkin–Elmer-Lambda 11 spectrophotometer. A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^1H and 150.86 MHz for ^{13}C, using the UXNMR software package, was used for NMR experiments; chemical shifts are expressed in δ (ppm) referring to the solvent peaks δ_H 3.34 and δ_C 49.0 for CD_3OD. ^{13}C DEPT, 1D-TOCSY, 1D-ROESY, ^1H–^1H DQF-COSY, HOHAHA, ^1H–^{13}C HSQC, and HMBC experiments were carried out using the conventional pulse sequences as described in the literature.[12] ESI-MS (positive mode) were obtained from a Finnigan LC-Q Deca instrument from Termoquest (San Jose, CA, USA) equipped with Excalibur software. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ solution followed by heating. Column chromatography was performed over Si gel (40–63 μm, Merck) and Sephadex LH-20 (Pharmacia); droplet counter-current chromatography (DCCC) was performed on an apparatus manufactured by Büchi, equipped with 300 tubes; HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column and Shimadzu injector.

3.2. Plant material

The whole plant of *C. negrevensis* Zohary was collected in Dabbet Hmoot, Qa' al Naqab, Jordan, in April, 1999 and identified by Professor Sawsan Al Oran, Department of Biology, University of Jordan, Amman, Jordan. A voucher specimen is deposited at the Orto Botanico, Universita di Pisa, Pisa, Italy (No. 2000-0051/1).

3.3. Extraction and isolation

The dried whole plant (80 g) of *C. negrevensis* was defatted with *n*-hexane and then extracted successively at room temperature with $CHCl_3$ and MeOH to give 4.9 and 9.5 g of residues, respectively. The chloroform extract was dissolved in a mixture MeOH–H_2O 8:2 and part of the soluble portion (1.1 g) was submitted to flash column chromatography over Si gel with $CHCl_3$ containing an increasing amount of MeOH (99:1, 98:2, 9:1, 8:2, 1:1, V/V) to give six main fractions. Fractions 4 (190 mg), 5 (180 mg), and 6 (45 mg) were further purified on RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 4:1 for fraction 4, MeOH–H_2O 75:25 for fraction 5, and MeOH–H_2O 7:3 for fraction 6, to yield compounds 16 (2.0 mg, t_R=17 min), 1 (2.5 mg, t_R=22 min), 3 (5.5 mg, t_R=25 min), and 2 (6.3 mg, t_R=45 min) from fraction 4, compounds 4 (2.3 mg, t_R=10 min), 5 (2.7 mg, t_R=30 min), 9 (5.8 mg, t_R=35 min), 6 (6.5 mg, t_R=45 min), 14 (3.8 mg, t_R=50 min), 13 (4.5 mg, t_R=82 min), 10 (3.0 mg, t_R=90 min), and 7 (6.5 mg, t_R=98 min) from fraction 5; nussellioside C (3.5 mg, t_R=22 min), compounds 11 (2.0 mg, t_R=56 min), 12 (5.3 mg, t_R=65 min), and 8 (2.4 mg, t_R=70 min) from fraction 6, respectively. The methanolic residue was partitioned between *n*-BuOH–H_2O: the *n*-BuOH fraction (4.0 g) was chromatographed on Sephadex LH-20 with MeOH as eluent, collecting 20 major fractions. Fraction 3 was purified by DCCC ($CHCl_3$–MeOH–H_2O–*n*-PrOH (9:12:8:1), descending mode, flow 10 mL/h) to give three fractions: A (100 mg), B (40 mg), and C (60 mg). Fraction C was submitted to final separation by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 83:17 as eluent to obtain pure compound 15 (5.0 mg, t_R=25 min). Fraction 5 was submitted to DCCC (*n*-BuOH–Me_2CO–H_2O (33:10:50) descending mode, flow 10 mL/h), to yield pure compound 19 (4.0 mg) together with two main fractions A' (80 mg) and B' (50 mg). Fraction B' was purified by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 1:1 as eluent to yield pure compound 17 (6.2 mg, t_R=35 min). Fraction 6 was submitted to DCCC (*n*-BuOH–Me_2CO–H_2O (33:10:50) descending mode, flow 10 mL/h), to yield pure compound 18 (6.5 mg) together with one fraction B'' (40 mg) that was finally purified by RP-HPLC on a C-18 μ-Bondapak column, (30 cm×7.8 mm, flow rate 2.0 mL/min) with MeOH–H_2O 3:2 as eluent to yield pure nussellioside B (50 mg, t_R=21 min) and compound 20 (3.8 mg, t_R=27 min).

3.3.1. Compound 1. Amorphous powder, mp 135–138°C; $[\alpha]_D^{25}$=+10.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 218 sh (2.56) nm; ^1H NMR of the aglycon: δ 1.05 (3H, s, Me-19), 1.06 (3H, s, Me-18), 1.29 (3H, d, *J*=6.5 Hz, Me-21), 1.87 (3H, d, *J*=6.5 Hz, H-4'), 1.89 (1H, m, H-7a), 1.94 (2H, s, H-5'), 2.05 (1H, m, H-17), 2.20 (1H, dd, *J*=10.2, 2.5 Hz, H-4a), 2.27 (1H, m, H-7b), 2.38 (1H, br

t, J=9.5 Hz, H-4b), 3.45 (1H, m, H-3), 3.66 (1H, dd, J=12.0, 4.0 Hz, H-12), 4.62 (1H, m, H-20), 5.43 (1H, br d, J=4.0 Hz, H-6), 6.96 (1H, br q, J=6.5 Hz, H-3'); ^{13}C NMR data of the aglycone: see Table 1, ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1049 $[M+Na]^+$; anal. C 61.90% H 8.46%, O 29.64%, calcd for $C_{53}H_{86}O_{19}$, C 61.95%, H 8.44%, O 29.60%.

3.3.2. Compound 2. White crystalline powder, mp 133–135°C; $[\alpha]_D^{25}$=+19.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 215 sh (3.31), 272 (2.90) nm; ^{1}H NMR of the aglycone: δ 1.01 (3H, s, Me-18), 1.03 (3H, s, Me-19), 1.15 (3H, d, J=6.5 Hz, Me-21), 1.82 (3H, d, J=6.5 Hz, H-4' at C-12), 1.83 (3H, s, H-5' at C-20), 1.84 (1H, m, H-7a), 1.88 (3H, d, J=6.5 Hz, H-4' at C-20), 1.90 (3H, s, H-5' at C-12), 2.06 (1H, m, H-17), 2.16 (1H, dd, J=10.0, 2.5 Hz, H-4a), 2.22 (1H, m, H-7b), 2.36 (1H, br t, J=9.5 Hz, H-4b), 3.51 (1H, m, H-3), 4.72 (1H, dd, J=12.0, 4.3 Hz, H-12), 5.11 (1H, m, H-20), 5.47 (1H, br d, J=4.0 Hz, H-6), 6.79 (1H, br q, J=6.5 Hz, H-3' at C-12), 6.93 (1H, br q, J=6.5 Hz, H-3' at C-20); ^{13}C NMR data of the aglycone: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1131 $[M+Na]^+$; anal. C 62.75%, H 8.37%, O 28.88%, calcd for $C_{59}H_{96}O_{20}$, C 62.78%, H 8.36%, O 28.86%.

3.3.3. Compound 3. Amorphous powder, mp 130°C; $[\alpha]_D^{25}$=+19.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 219 sh (4.12) nm; ^{1}H NMR of the aglycone: δ 1.05 (3H, s, Me-19), 1.08 (3H, s, Me-18), 1.14 (3H, d, J=6.5 Hz, Me-21), 1.82 (3H, d, J=6.5 Hz, H-4'), 1.90 (1H, m, H-7a), 1.91 (3H, s, H-5'), 2.00 (3H, s, COMe), 2.08 (1H, m, H-17), 2.13 (1H, dd, J=10.0, 2.5 Hz, H-4a), 2.26 (1H, m, H-7b), 2.38 (1H, br t, J=9.0 Hz, H-4b), 3.52 (1H, m, H-3), 4.64 (1H, dd, J=12.0, 4.0 Hz, H-12), 4.95 (1H, m, H-20), 5.47 (1H, br d, J=4.3 Hz, H-6), 7.05 (1H, br q, J=6.5 Hz, H-3'); ^{13}C NMR data of the aglycone: see Table 1; ^{1}H NMR of the sugar moiety: δ 1.20 (3H, d, J=6.4 Hz, H-6$_{gua}$), 1.25 (3H, d, J=6.0 Hz, H-6$_{cym}$), 1.45 (3H, d, J=6.0 Hz, H-6$_{gal}$), 1.53 (1H, br dd, J=16.0, 12.0 Hz, H-2b$_{cym}$), 1.63 (1H, br dd, J=16.0, 12.0 Hz, H-2b$_{gua}$), 2.09 (1H, br dd, J=16.0, 3.0 Hz, H-2a$_{gua}$), 2.15 (1H, br dd, J=16.0, 3.0 Hz, H-2a$_{cym}$), 3.14 (1H, dd, J=10.0, 7.0 Hz, H-2$_{gal}$), 3.20 (1H, dd, J=9.5, 7.5 Hz, H-2$_{gle}$), 3.23 (1H, d, J=9.5 Hz, H-4$_{gle}$), 3.25 (1H, dd, J=9.5, 3.0 Hz, H-4$_{cym}$), 3.26 (1H, m, H-5$_{gle}$), 3.30 (1H, dd, J=9.5, 3.0 Hz, H-4$_{cym}$), 3.37 (1H, d, J=9.5 Hz, H-3$_{gle}$), 3.45 (1H, m, H-5$_{gal}$), 3.47 (1H, t, J=10.0 Hz, H-4$_{gal}$), 3.51 (3H, s, OMe$_{cym}$), 3.53 (3H, s, OMe$_{cym}$), 3.54 (1H, t, J=10.0 Hz, H-3$_{gal}$), 3.64 (1H, dd, J=12.0, 3.5 Hz, H-6b$_{gle}$), 3.82 (1H, dq, J=9.5, 6.0 Hz, H-5$_{cym}$), 3.85 (1H, dq, J=9.5, 6.4 Hz, H-5$_{cym}$), 3.87 (1H, q, J=3.0 Hz, H-5$_{cym}$), 3.88 (1H, q, J=3.0 Hz, H-3$_{cym}$), 3.94 (1H, dd, J=12.0, 3.0 Hz, H-6a$_{gle}$), 4.47 (1H, d, J=7.5 Hz, H-1$_{gal}$), 4.64 (1H, d, J=7.5 Hz, H-1$_{gle}$), 4.81 (1H, dd, J=9.0, 2.0 Hz, H-1$_{cym}$), 4.87 (1H, dd, J=9.5, 2.0 Hz, H-1$_{cym}$); ^{13}C NMR of the sugar moiety: δ 18.0 (q, C-6$_{cym}$), 18.5 (q, C-6$_{cym}$), 18.6 (q, C-6$_{gal}$), 36.5 (t, C-2$_{cym}$), 36.8 (t, C-2$_{cym}$), 58.0 (q, OMe$_{cym}$), 58.1 (q, OMe$_{cym}$), 62.9 (t, C-6$_{gal}$), 69.7 (d, C-5$_{cym}$), 69.9 (d, C-5$_{cym}$), 71.8 (d, C-4$_{gal}$), 72.6 (d, C-5$_{gal}$), 74.4 (d, C-2$_{gal}$), 75.3 (d, C-2$_{gle}$), 78.1 (d, C-5$_{gle}$), 78.0 (d, C-3$_{gal}$), 78.2 (d, C-3$_{gle}$), 78.6 (d, C-3$_{cym}$), 78.7 (d, C-3$_{cym}$), 83.6 (d, C-4$_{gal}$), 83.7 (d, C-4$_{cym}$), 84.0 (d, C-4$_{cym}$), 96.9 (d, C-1$_{cym}$), 101.2 (d, C-1$_{cym}$), 102.6 (d, C-1$_{gle}$), 104.1 (d, C-1$_{gal}$); ESI-MS *m/z*: 1093 $[M+Na]^+$; anal. C 60.49%, H 8.11%, O 31.40%, calcd for $C_{54}H_{86}O_{21}$, C 60.54%, H 8.09%, O 31.36%.

3.3.4. Compound 4. Amorphous powder, mp 165–170°C; $[\alpha]_D^{25}$=+29.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 225 sh (2.24), 280 (3.38) nm; NMR data for the aglycon moiety are superimposable on those for boucerin;[15] ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 983 $[M+Na]^+$; anal. C 59.90%, H 8.42%, O 31.68%, calcd for $C_{48}H_{80}O_{19}$, C 59.98%, H 8.39%, O 31.63%.

3.3.5. Compound 5. Amorphous powder, mp 118–120°C; $[\alpha]_D^{25}$=+20.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 222 sh (4.20), 250 sh (3.76), 278 (3.05) nm; ^{1}H NMR of the aglycone: δ 1.05 (3H, s, Me-19), 1.23 (3H, d, J=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 2.19 (1H, dd, J=9.5, 2.5 Hz, H-4a), 2.26 (1H, m, H-7b), 2.40 (1H, br t, J=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, J=12.0, 4.0 Hz, H-12), 5.48 (1H, br d, J=4.5 Hz, H-6), 6.60 (1H, t, J=8.0 Hz, H-4'), 7.20 (1H, t, J=8.0 Hz, H-5'), 7.77 (1H, d, J=8.0 Hz, H-3'), 7.91 (1H, d, J=8.0 Hz, H-6'); ^{13}C NMR data of the aglycone: see Table 1, ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1103 $[M+Na]^+$; anal. C 61.06%, H 7.84%, O 31.10%, calcd for $C_{53}H_{84}O_{21}$, C 61.10%, H 7.83%, O 31.07%.

3.3.6. Compound 6. Amorphous powder, mp 133–139°C; $[\alpha]_D^{25}$=+33.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 220 sh (3.86), 282 (4.76) nm; NMR data for the aglycon moiety are identical to those for compound 3; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1107 $[M+Na]^+$; anal. C 60.80%, H 8.20%, O 31.00%, calcd for $C_{52}H_{84}O_{21}$, C 60.87%, H 8.17%, O 30.96%.

3.3.7. Compound 7. Amorphous powder, mp 152–157°C; $[\alpha]_D^{25}$=+34.7 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 280 (3.99) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1147 $[M+Na]^+$, 1047, 947, 489; anal. C 61.88%, H 8.27%, O 29.85%, calcd for $C_{59}H_{92}O_{21}$, C 61.90%, H 8.24%, O 29.86%.

3.3.8. Compound 8. Amorphous powder, mp 133–135°C; $[\alpha]_D^{25}$=+25.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε): 220 sh (2.40) nm; ^{1}H NMR of the aglycone: δ 1.05 (3H, s, Me-19), 1.23 (3H, d, J=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.84 (3H, d, J=6.5 Hz, H-4'), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 1.89 (3H, s, H-5'), 2.19 (1H, dd, J=9.5, 2.0 Hz, H-4a), 2.26 (1H, m, H-7b), 2.40 (1H, br t, J=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, J=12.0, 4.0 Hz, H-12), 5.48 (1H, br d, J=4.0 Hz, H-6), 6.98 (1H, br q, J=6.5 Hz, H-3'); ^{13}C NMR data of the aglycone: see Table 1; ^{1}H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1105 $[M+Na]^+$, 1005; anal. C 61.04%, H 8.30%, O 30.66%, calcd for $C_{53}H_{86}O_{20}$, C 61.02%, H 8.31%, O 30.67%.

3.3.9. Compound 9. Amorphous powder, mp 120–123°C; $[\alpha]_D^{25}$=+4.0 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε): 220

sh (2.29) nm; NMR data for the aglycon moiety are identical to those for compound 8; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1065 $[M+Na]^+$, 965; anal. C 60.98%, H 8.33%, O 30.69%, calcd for $C_{51}H_{86}O_{20}$, C 61.02%, H 8.31%, O 30.67%.

3.3.10. Compound 10. Amorphous powder, mp 157°C; $[\alpha]_D^{25}$=+16.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 217 sh (3.50), 278 (3.83) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1147 $[M+Na]^+$, 1047, 585; anal. C 61.81%, H 8.27%, O 29.92%, calcd for $C_{56}H_{92}O_{23}$, C 61.90%, H 8.24%, O 29.86%.

3.3.11. Compound 11. Amorphous powder, mp 160°C; $[\alpha]_D^{25}$=+16.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 220 sh (3.85), 247 sh (2.92), 280 (3.68) nm; NMR data for the aglycon moiety are identical to those for compound 8; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1103 $[M+Na]^+$; anal. C 61.07%, H 7.85%, O 31.08%, calcd for $C_{53}H_{84}O_{21}$, C 61.10%, H 7.83%, O 31.07%.

3.3.12. Compound 12. Amorphous powder, mp 140–142°C; $[\alpha]_D^{25}$=+12.0 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log *ε*): 225 (3.90) nm; ^1H NMR of the aglycon: *δ* 1.05 (3H, s, Me-19), 1.23 (3H, d, *J*=6.5 Hz, Me-21), 1.29 (3H, s, Me-18), 1.85 (1H, m, H-17), 1.88 (1H, m, H-7a), 2.05 (3H, s, COMe), 2.19 (1H, dd, *J*=10.0, 2.5 Hz, H-4a), 2.26 (1H, m, H-7b), 2.40 (1H, br t, *J*=10.0 Hz, H-4b), 3.53 (1H, m, H-3), 3.83 (1H, m, H-20), 4.63 (1H, dd, *J*=12.0, 4.2 Hz, H-12), 5.48 (1H, br d, *J*=4.5 Hz, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1025 $[M+Na]^+$, 965; anal. C 59.79%, H 8.26%, O 31.95%, calcd for $C_{50}H_{82}O_{20}$, C 59.86%, H 8.24%, O 31.90%.

3.3.13. Compound 13. White crystalline powder, mp 140–145°C; $[\alpha]_D^{25}$=+49.0 (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log *ε*): 218 sh (3.21), 276 (3.30) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^1H and ^{13}C NMR data of the sugar moiety: see Table 2; ESI-MS *m/z*: 1147 $[M+Na]^+$; anal. C 61.91%, H 8.19%, O 29.90%, calcd for $C_{56}H_{92}O_{23}$, C 61.90%, H 8.24%, O 29.86%.

3.3.14. Compound 14. Amorphous powder, mp 125–128°C; $[\alpha]_D^{25}$=+19.3 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 221 sh (4.15), 279 (4.30) nm; NMR data of the aglycon moiety are identical to those for compound 1; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1387 $[M+Na]^+$, 1287; anal. C 58.00%, H 7.93%, O 34.02%, calcd for $C_{62}H_{100}O_{28}$, C 58.05%, H 7.97%, O 33.98%.

3.3.15. Compound 15. Amorphous powder, mp 165–170°C; $[\alpha]_D^{25}$ +34.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 218 sh (4.42) nm; NMR data for the aglycon moiety are superimposable on those of compound 2; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1469 $[M+Na]^+$; anal. C 58.84%, H 7.96%, O 33.20%, calcd for $C_{71}H_{114}O_{29}$, C 58.91%, H 7.94%, O 33.16%.

3.3.16. Compound 16. Amorphous powder, 185–190°C dec; $[\alpha]_D^{25}$=+24.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 220 sh (2.45), 275 (4.21) nm; NMR data for the aglycon moiety are identical to those for compound 3; ^1H and ^{13}C NMR data of the sugar moiety: see Table 3; ESI-MS *m/z*: 1267 $[M+Na]^+$, 1207; anal. C 59.71%, H 8.10%, O 32.19%, calcd for $C_{59}H_{96}O_{25}$, C 59.79%, H 8.09%, O 32.12%.

3.3.17. Compound 17. Amorphous powder, mp 174°C; $[\alpha]_D^{25}$=-22 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 265 (4.25) nm; ^1H NMR of the aglycon: *δ* 1.06 (3H, s, Me-19), 1.14 (3H, s, Me-18), 1.32 (3H, d, *J*=6.4 Hz, Me-21), 1.70 (1H, m, H-17), 1.90 (1H, m, H-7a), 1.93 (3H, s, COMe), 2.25 (1H, m, H-7b), 2.31 (1H, dd, *J*=10.0, 2.5 Hz, H-4a), 2.47 (1H, br t, *J*=9.5 Hz, H-4b), 3.53 (1H, m, H-5), 4.42 (1H, m, H-20), 5.45 (1H, m, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^1H and ^{13}C NMR data of the sugar moiety: see Table 4; ESI-MS *m/z*: 721 $[M+Na]^+$, 661; anal. C 61.82%, H 8.38%, O 29.80%, calcd for $C_{36}H_{58}O_{12}$, C 61.87%, H 8.37%, O 29.76%.

3.3.18. Compound 18. Amorphous powder, mp 142°C; $[\alpha]_D^{25}$=-4.0 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log *ε*): 277 (4.57), 320 sh (3.37) nm; ^1H NMR of the aglycon: *δ* 1.08 (3H, s, Me-19), 1.15 (3H, s, Me-18), 1.31 (3H, d, *J*=6.4 Hz, Me-21), 1.70 (1H, m, H-17), 1.90 (1H, m, H-7a), 2.25 (1H, m, H-7b), 2.31 (1H, dd, *J*=9.5, 2.5 Hz, H-4a), 2.47 (1H, br t, *J*=9.5 Hz, H-4b), 3.53 (1H, m, H-3), 4.03 (1H, m, H-20), 5.45 (1H, br d, *J*=4.0 Hz, H-6); ^{13}C NMR data of the aglycon: see Table 1; ^1H and ^{13}C NMR data of the sugar moiety: see Table 4; ESI-MS *m/z*: 1311 $[M+Na]^+$; anal. C 54.00%, H 7.52%, O 38.48%, calcd for $C_{62}H_{96}O_{24}$, C 54.03%, H 7.50%, O 38.47%.

3.3.19. Compound 19. Amorphous powder, mp 195°C; $[\alpha]_D^{25}$=-11.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 220 sh (4.13), 283 (3.78) nm; NMR data for the aglycon moiety are superimposable on those of compound 18; ^1H NMR of the sugar moiety: chain A *δ* 3.16 (1H, dd, *J*=9.5, 7.8 Hz, H-2_{gl} m), 3.28 (1H, d, *J*=9.5 Hz, H-4_{gl} m), 3.30 (1H, m, H-5_{gl} m), 3.38 (1H, d, *J*=9.5 Hz, H-3_{gl} m), 3.68 (1H, dd, *J*=12.0, 5.0 Hz, H-$6a_{gl}$ m), 3.83 (1H, dd, *J*=12.0, 3.0 Hz, H-$6b_{gl}$ m), 4.40 (1H, d, *J*=7.8 Hz, H-1_{gl} m); chain C: see Table 4; ^{13}C NMR of the sugar moiety: chain A *δ* 62.4 (C-6_{gl} m), 71.7 (C-4_{gl} m), 75.3 (C-2_{gl} m), 77.6 (C-3_{gl} m), 78.2 (C-5_{gl} m), 104.5 (C-1_{gl} m); chain C: see Table 4; ESI-MS *m/z*: 1003 $[M+Na]^+$, 841, 519; anal. C 56.28%, H 7.82%, O 35.90%, calcd for $C_{48}H_{76}O_{20}$, C 56.31%, H 7.81%, O 35.88%.

3.3.20. Compound 20. Amorphous powder, 270–275°C dec; $[\alpha]_D^{25}$=-6.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log *ε*): 225 (4.53), 238 sh (3.78) nm; ^1H NMR of the aglycon: *δ* 0.86 (3H, s, Me-18), 1.10 (3H, s, Me-19), 1.30 (3H, d, *J*=6.4 Hz, Me-21), 1.55 (1H, m, H-17), 3.67 (1H, m, H-3), 4.03 (1H, m, H-20); ^{13}C NMR data of the aglycon: see Table 1; for ^1H and ^{13}C NMR data of the sugar moiety see compound 19 (chain A) and Table 4 (chain B); ESI-MS *m/z*: 843 $[M+Na]^+$; anal. C 58.45%, H 8.37%, O 33.18%, calcd for $C_{42}H_{68}O_{16}$, C 58.52%, H 8.35%, O 33.13%.

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3.4. Cytotoxic and genotoxic activity

3.4.1. In vitro cytotoxicity test for anticancer activity in tumor cell lines. Cell lines: HT29, colorectal human cancer cell line, DNA mismatch repair proficient, established from non polyposis sporadic tumor; LoVo, colorectal human cancer cell line, DNA mismatch repair deficient, established from non polyposis hereditary tumor. The two cancer cell lines were treated with 10, 25, 75, and 100 μg/mL from 24 h to 48 h. All tested compounds were dissolved in DMSO not exceeding 1% final concentration. Negative controls were set up according to the maximum amount of solvent used to prepare each chemical. The ability of single cells to proliferate and form colony during 48 h grown in culture medium was measured. The results are expressed, in percent, as relative cloning efficiency that is the ratio of the number of cell grown after chemical treatment with respect to untreated cell growth.[9]

3.4.2. In vitro genotoxicity test in human somatic cells. Phytohemagglutinin-stimulated peripheral blood lymphocytes cultured up to 72 h were used. The addition of cytochalasin B to cultures 44 h before cell harvest blocked the cytodieresis of dividing lymphocytes which assumed a characteristic binucleated shape (a cell with two main nuclei) at the following interphase. Only in these cells was observed the presence of micronuclei. Lymphocyte cultures were treated with 0.1, 1.0, 5.0 50, 100, and 150 μg/mL from 24 to 72 h. All tested compounds were dissolved in DMSO not exceeding 1% final concentration. Negative controls were set up according to the maximum amount of solvent used to prepare each chemical. The frequency of micronuclei (MN) is expressed as the number of micronucleated lymphocytes (containing one or more MN) on 1000 cells scored.[9,11]

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ACYLATED C-21 STEROIDAL BISDESMOSIDIC GLYCOSIDES FROM *CARALUMA UMBELLATA*

SHENG-XIANG QIU, LONG-ZE LIN, GEOFFREY A. CORDELL,* MULLAGHI RAJESH,† BOREALA RAVI KUMAR,† MARUPAKA RADHAKRISHNA,‡ GOTTUMUKKALA KRISHNA MOHAN,† BOMMINENI MADHAVA REDDY,§ YEPURI NAGESWARA RAO,‡ BAJJURI SRINIVAS,† NARISETTY SUNIL THOMAS† and ACHANTA VENKATA NARASIMHA APPA RAO†

Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The University of Illinois at Chicago, 833 South Wood Street, Chicago, IL 60612, U.S.A.; †University College of Pharmaceutical Sciences, Kakatiya University, Warangal-506 009 A. P. India; ‡Government Polytechnic for Women, Warangal, A. P. India; §Al-Ameen College of Pharmacy, Bangalore, India

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Key Word Index—*Caraluma umbellata*; Asclepiadaceae; C-21 steroidal glycosides; Bisdesmosidic glycosides, Caralumagenin; Carumbelloside III; Carumbelloside IV; Carumbelloside V; Structure elucidation; NMR assignments; Two-dimensional NMR techniques.

Abstract—From the whole plant of *Caraluma umbellata*, three new C-21 steroidal glycosides, named as carumbellosides III–V, were isolated and their structures elucidated by extensive spectroscopic experiments, devoid of any derivatisation, as caralumagenin 3-*O*-β-D-glucopyranosyl(1→4)-β-D-digitalopyranoside-20-*O*-β-D-glucopyranoside, caralumagenin 3-*O*-β-D-glucopyranosyl(1→4)-β-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-β-D-glucopyranoside and caralumagenin 3-*O*-[6-*O*-benzoyl-β-D-glucopyranosyl(1→4)]-β-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-β-D-glucopyranoside. The determination of the absolute configuration of the aglycone as (20*R*), the conformations of the sugars and the unambiguous assignments of their NMR spectroscopic signals were achieved by a combination of 2D-NMR techniques. The isolates were devoid of significant cytotoxity in the UIC human cancer cell panel. © 1997 Elsevier Science Ltd

INTRODUCTION

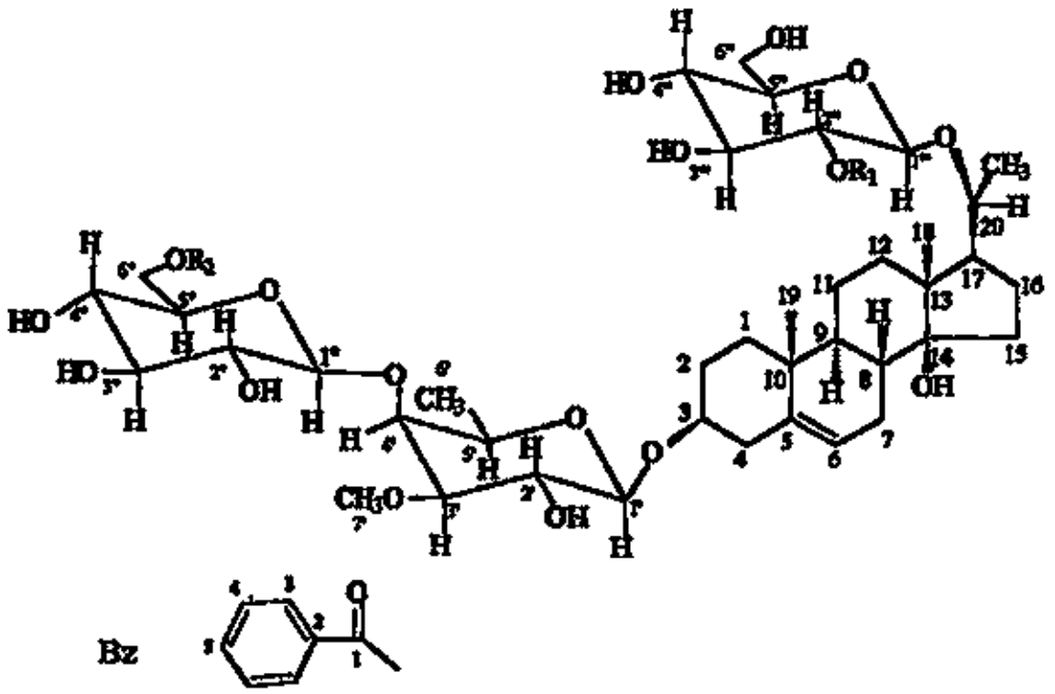
Caraluma umbellata Haw. (syn. *Boucerosia umbellata* W. and A.) (Asclepiadaceae) is a succulent, perennial herb growing wild in Tirupathi and surrounding places of Andhra Pradesh, India. Previously, we reported on the isolation and identification of two new pregnane glycosides, carumbellosides I and II, from this plant [1]. In this report, we present the isolation, characterization and complete NMR assignments of three further new C-21 steroidal glycosides carumbellosides III (1), IV (2) and V (3) from this plant.

RESULT AND DISCUSSION

Carumbelloside III (1) gave a positive Liebermann–Burchard reaction, as a steroidal glycoside, and exhibited a molecular formula $C_{59}H_{94}O_{27}$ (MW = 818) based on its ^{13}C NMR (DEPT) spectrum and FAB-MS. In the latter, a quasi-molecular ion was observed at *m/z* 817 $[M-H]^-$ in the negative ion FAB-mass spectrum and a sodiated molecular ion at *m/z* 841 $[M+Na]^+$ in the positive ion FAB-mass spectrum.

Compound 1 displayed no absorption in the UV spectrum indicating the absence of any conjugated double bonds. The IR spectrum revealed no other significant absorptions except for a strong hydroxyl group band at 3500 cm^{-1} and glycosidic bonds (C—O) at 1110 cm^{-1}. The ^1H NMR spectrum of 1 displayed three doublet anomeric proton signals δ 5.09, 4.82 and 4.73 with coupling constants of 8.0 Hz, diagnostic of an axial orientation for all three sugar moieties. This is in good agreement with the negative FAB-mass spectral fragmentation pattern of 1, which showed *m/z* 817 $[M-H]^-$, 655 $[M-H-162]^-$ and 493 $[M-H-162-162]^-$, suggesting the presence of two hexose units. The third sugar could be inferred as 6-deoxy-3-*O*-methyl-hexose based on the observed predominant fragmentation at *m/z* 479, which was probably generated from the quasi-molecular ion at *m/z* 819, corresponding to $[M+H]^+$ from the positive mode FAB-mass spectrum, by the loss of a hexose unit and a

*Author to whom correspondence should be addressed.
†Present address: Dr Reddy's Research Foundation, Bolaram Road, Miyapur, Hyderabad, India.



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Carumbelloside III (1) $R_1 = R_2 = H$
Carumbelloside IV (2) $R_1 = Bz$, $R_2 = H$
Carumbelloside V (3) $R_1 = R_2 = Bz$

molecule of water. Furthermore, the combination fragmentation patterns of positive and negative FAB-mass spectra suggest that 1 is a bidesmosidic glycoside, i.e. with one hexose attached directly to the aglycone, and another hexose linked to the aglycone-bound deoxyhexose. The NMR spectral outline of the aglycone derived by subtraction of the sugar chain from that of the glycoside, taking into account the 'glycoside shift' effect, is analogous to those of calogenin, isolated by Khare *et al.* from *Periploca calophylla* [2] and *Hemidesmus indicus* [3], with the exception of the configuration at the stereogenic centers C-17 and C-20, since these centres in calogenin remained to be assigned stereochemically.

In classical approaches, the structure of glycosides are traced by analysis of the fragments obtained by chemical and/or enzymic degradation, and can be associated with disadvantages such as artifact formation and tedious workup of various derivatization reactions [4]. Moreover, these degradative methods consume precious sample which is frequently difficult to isolate and could be preserved for biological evaluation. In the current study, an effort was made to characterise the intact glycoside structure primarily based on NMR spectroscopic techniques. The structure characterization and stereochemical assignment of the glycosides were achieved through the unambiguous rationalization of the ^1H and ^{13}C NMR resonances using a combination of DQF-COSY, HOHAHA, HETCOR, HMBC and ROESY techniques. Proton connectivities were determined by DQF-COSY and HOHAHA experiments and the signals of all carbons with directly attached protons were assigned using the HETCOR spectrum. The ROESY spectrum was employed to assign the stereochemistry of the aglycone along with the configurations and conformations of the sugars and the sugar-chain sequences and its attachment to the aglycone. Finally, the HMBC spectrum was used to assign the quaternary carbons and to verify the correctness of the connectivities established by the interpretation of the other spectra with consequent confirmation of the oligosaccharide chain linkage and sequences.

The ^{13}C DEPT experiment of 1 revealed a total of 40 carbon resonances, of which five corresponded to methyl carbons, ten to methylene carbons, twenty-one to methine carbons, and four to non-proton bearing carbons, corresponding to a C-21 steroid triglycoside. Two olefinic carbons at δ 122.67 and 139.63 and three anomeric carbons resonating at δ 102.57, 103.29 and 104.86 could be recognized readily. The ^1H NMR spectrum displayed two distinct chemical shift regions as a set of oxygenated methine and methylene protons (olefinic proton included) in the range δ 3.50–5.50, and the aliphatic methylene and methine protons in the range δ 0.80–2.70 arising mainly from the aglycone portion. Except for the well-resolved signals of the methyl groups and some of the non-equivalent methylene protons, most of the signals at high field were severely overlapped and could only be distinguished in the HETCOR spectrum in a 'fuzzy logic' fashion. However, it was still possible to assign

the corresponding ^{13}C resonances unambiguously through the investigation of the DQF-COSY, HOHAHA and HMBC spectra.

The proton singlet at δ 0.83, assignable to the methyl CH_3-19, showed a cross-peak with the quaternary carbon signals at δ 37.54 and δ 139.63, a methylene carbon at δ 37.35 and a methine carbon at δ 46.39 in the HMBC spectrum, resulting in their assignment to C-10, the olefinic carbon C-5, and C-1 and C-9, respectively. Accordingly, the proton singlet at δ 1.33 should be assigned to the CH_3-18, since it correlated with the quaternary carbons C-13 at δ 47.40 and C-14 at δ 83.90, the methylene carbon at δ 40.62 and a methine carbon at δ 57.20 ppm, leaving the latter to be assigned to C-12 and C-17, respectively. One of the two doublet methyl signals at δ 1.37 (*d*, J = 6.0 Hz) could be assigned to CH_3-21 based on a correlation with the C-17 carbon signal in the HMBC spectrum.

Following the interpretation of the coupling pattern using DQF-COSY of the HOHAHA coupling fragments C_1-C_2-C_3-C_4, C_5-C_6-C_7-C_8-C_9-C_{11}-C_{12} and C_{15}-C_{16}-C_{17}, H_2-2, H-3, H_2-4, H_2-7, H-8, H-9, H_2-11, H_2-12, H_2-15, H_2-16, H-17 were identified, resulting in the ^{13}C assignments of C-2, C-3, C-4, C-7, C-8, C-9, C-11, C-15 and C-16 by means of the HETCOR experiment. The stereochemical assignments of the methine and non-equivalent methylene protons, except for the almost coincident H_2-15, were achieved with the aid of the ROESY spectrum, in a similar way as with gymnemanargenin [5] and marsdenacigenins A and B [6]. The H_2-19 showed a strong correlation contour with the C-1 and C-4 methylene proton signals at δ 1.72 and δ 2.33, which should be assigned to H-1β and H-4β, leaving their partners at δ 1.05 and δ 2.64 to be assigned to H-1α and H-4α, respectively. The β-configuration of H-8 was also evident from its strong ROE correlation contour with H_2-19.

It is a well-established feature of pregnane steroids with a $\Delta^{5,6}$-double bond that H-6 and H-7β are spatially close and afford an easily observed ROE cross-peak [5], which enables δ 2.59 and 2.02 to be assigned to H-7β and H-7α, respectively. The α-orientation of H-9 was confirmed by the ROE correlation contour between H-7α and H-9, and in addition, H_2-11, H_2-12 and H_2-16 could also be differentiated by relative configuration analogies accordingly.

The H-17 signal appeared as a doublet of doublets (δ 1.55, *dd*, J = 11.5, 6.5 Hz), therefore, the configuration of the side-chain was deduced to be β-oriented (i.e. H-17α) by coupling pattern analysis [7]. The absolute configuration of C-20, which is left unassigned in most published papers on pregnane steroids bearing a hydroxyl group at the C-20 positions [8, 9], was finally determined to be *R*- by the ROESY spectrum and molecular modelling (see below). Thus, the aglycone of **1** was deduced to be 3β,14β,20(*R*)-trihydroxy-pregnane and was designated cestrumargenin to avoid confusion with the C-17 and C-20 stereochemically undefined pregnane calogenin [2, 3].

Proton-3 of the aglycone showed a strong ROE correlation contour with a doublet anomeric proton H-1′ at δ 4.73 (*d*, J = 8.0 Hz), which in turn was coupled with a double doublet signal H-2′ at δ 4.37 (*dd*, J = 9.5, 8.0 Hz) and was relay coupled with a double doublet signal H-3′ at δ 3.52 (*dd*, J = 9.5, 0.5 Hz) in the HOHAHA (45 ms) spectrum and an overlapping signal H-4′ at δ 4.07 in the HOHAHA (55 ms) spectrum. The quadruplet signal at δ 3.69, coupling with a methyl signal at δ 1.52 (*d*, J = 6.5 Hz), with no observed cross-peak with H-4′ due to a small coupling constant, could be attributed to H-5′ based on its ROE effects on both H-1′ and H-3′ in the ROESY spectrum. From the ongoing evidence, the inner sugar of the oligosaccharide chain could be established as β-D-digitalopyranose in a $^4C_1$ conformation.

The doublet proton signal at δ 5.09 (*d*, J = 8.0 Hz) was readily assigned to that of the terminal sugar H-1″ from its strong ROE cross contour with H-4′, implying attachment to the C-4′ position of the inner sugar digitalose. Protons-2″ (δ 3.92, *t*, J = 8.5 Hz) and H-3″ (δ 4.18, *t*, J = 8.5 Hz) are well-resolved and were assigned by the DQF-COSY spectrum; consequently, the ^{13}C assignments of C-1″, C-2″ and C-3″ are straightforward. Protons-4″ and H-5″ could not be well-defined due to the limits of resolution. However, the ^{13}C assignments of C-4″, C-5″ and C-6″ (Table 2) could be made after careful interpretation of DQF-COSY, HOHAHA, HETCOR and HMBC spectra and were found to be almost superimposable with those of methyl β-D-glucopyranoside [10]. Thus, the disaccharide unit should be β-D-glucopyranosyl-β-D-digitalopyranose.

The third sugar was deduced to be β-D-glucose and its ^{13}C assignments (Table 2) were accomplished in a similar way as mentioned above. The notable ROE cross-peaks (Fig. 1) between H-1‴ at δ 4.82 (*d*, J = 8.0 Hz) with the doublet signal of H-21 at δ 1.37 (*d*, J = 6.0 Hz) and the singlet signal of H_3-18 at δ 1.33 (*s*) allowed the conclusion that it was attached to the aglycone directly at the C-20 hydroxyl group. This could be further confirmed by key observations of correlation contours between H-1′ and C-3, H-1″ and C-4′, as well as H-1‴ and C-20, in the HMBC spectrum.

The ROE results were also applicable for the determination of the absolute configuration of C-20. The individual structures of the 20(*R*)- and 20(*S*)- isomers were generated by the molecular modelling program PCMODEL V 5.0 for Windows, using the MMX force field calculations for energy minimization. The calculated distances between H-1‴ and H_3-18, H_3-21 and H-20 were 2.32, 2.19 and 2.45 Å, respectively, for the C-20(*R*) isomer, consistent with the strong ROE correlations observed between each of these pairs; while for the C-20(*S*)- isomer, the calculated distance between H_3-21 and H-1‴ was 4.53 Å, which was considered to be too far away to account for the observed ROE correlation.

Table 1. ^{13}C NMR data of Caruntelloside III, IV and V (1-3)

	1	2	3
Aglycone			
1	37.35 (t)	37.23 (t)	37.22 (t)
2	30.13 (t)	30.15 (t)	30.17 (t)
3	77.90 (d)	77.86 (d)	77.89 (d)
4	39.04 (t)	39.01 (t)	39.03 (t)
5	139.63 (s)	139.20 (s)	139.19 (s)
6	122.67 (d)	122.76 (d)	122.75 (d)
7	27.81 (t)	27.65 (t)	27.66 (t)
8	37.30 (d)	35.98 (d)	35.97 (d)
9	46.39 (d)	43.99 (d)	45.97 (d)
10	37.54 (s)	37.50 (s)	37.51 (s)
11	19.72 (t)	20.11 (t)	20.25 (t)
12	40.62 (t)	40.15 (t)	40.14 (t)
13	47.40 (s)	47.00 (s)	46.99 (s)
14	83.90 (s)	83.22 (s)	83.25 (s)
15	31.40 (t)	31.09 (t)	31.09 (t)
16	33.30 (t)	32.72 (t)	32.67 (t)
17	57.20 (d)	56.65 (d)	56.64 (d)
18	15.19 (q)	15.15 (q)	15.17 (q)
19	19.48 (q)	19.28 (q)	19.28 (q)
20	77.92 (d)	79.24 (d)	79.59 (d)
21	21.61 (q)	21.75 (q)	21.76 (q)
Digitalose			
1'	102.57 (d)	102.53 (d)	102.55 (d)
2'	71.33 (d)	71.33 (d)	71.33 (d)
3'	85.37 (d)	85.43 (d)	85.44 (d)
4'	76.16 (d)	76.04 (d)	76.06 (d)
5'	70.39 (d)	70.41 (d)	70.42 (d)
6'	17.67 (q)	17.69 (q)	17.70 (q)
$-OCH_3$	58.85 (q)	58.86 (q)	58.90 (q)
Glucose			
1''	105.29 (d)	105.36 (d)	105.37 (d)
2''	76.01 (d)	76.20 (d)	76.26 (d)
3''	78.22 (d)	78.27 (d)	78.29 (d)
4''	71.36 (d)	71.78 (d)	71.79 (d)
5''	78.96 (d)	78.56 (d)	75.38 (d)
6''	62.99 (t)	62.44 (t)	63.08 (t)
Glucose			
1'''	104.86 (d)	102.63 (d)	102.67 (d)
2'''	75.02 (d)	75.42 (d)	75.19 (d)
3'''	78.55 (d)	77.14 (d)	76.83 (d)
4'''	71.31 (d)	71.51 (d)	71.70 (d)
5'''	78.46 (d)	78.89 (d)	78.61 (d)
6'''	62.72 (t)	63.03 (t)	63.04 (t)
2''-Bz			
1		166.01 (s)	166.00 (s)
2		132.19 (s)	133.22 (s)
3		128.05 (d)	128.79 (d)
4		130.22 (d)	130.24 (d)
5		132.48 (d)	132.09 (d)
6''-Bz			
1			166.45 (s)
2			130.74 (s)
3			128.07 (d)
4			129.89 (d)
5			132.55 (d)

ppm for internal standard TMS in pyridine-d_5.

Multiplicity by DEPT experiments in parentheses, *s*, quaternary; *d*, methine; *t*, methylene and *q*, methyl carbons.

Therefore, the absolute configuration of aglycone carulumagenin was assigned to be 20(*R*), and the structure of 1 was deduced as carulumagenin 3-*O*-*β*-D-glucopyranosyl-*β*-D-digitalopyranoside-20-*O*-*β*-D-glucopyranoside.

The results described above would imply an alternative method for the determination of absolute configuration of pregnane steroids with a free hydroxyl group at the C-20 position, which are based on the fundamental principle that the free rotation of C_{17}-C_{20} bond of C_{20}-OH pregnanes was inhibited after glycosylation at C_{20}-OH. Thus, the favourable conformation could be 'frozen out' and could be suitable for spatial relationship analysis by NOED or ROESY (NOESY) experiments.

Caruntelloside IV (2) gave a positive Liebermann–Burchard test for steroids, and its FAB-MS revealed a molecular formula of $C_{48}H_{74}O_{18}$ (MW = 922), in which quasi-molecular ions were detected at *m/z* 923 $[M+H]^+$ in a positive-ion mode and *m/z* 921 $[M-H]^-$ in a negative-ion mode respectively, in combination with a ^{13}C NMR (DEPT) experiment. In the IR spectrum, bands for hydroxyl groups at 3440 cm^{-1}, a carbonyl group at 1700 cm^{-1}, and characteristic absorptions of a benzene ring at 1600 and 1450 cm^{-1} were apparent. Its mass spectrum presented a base peak fragment at *m/z* 105 (benzoyl cation) and a strong ion at *m/z* 122 (benzoic acid ion), indicative of the presence of a benzoyl group. The appearance of the ^1H and ^{13}C NMR of 2 are very similar to those of 1, and therefore, it was deduced that 2 is an analogue of 1 with an additional benzoyl group.

Following the same methodology as described for 1, the aglycone of 2 was deduced as carulumagenin and its NMR spectral data assigned. The inner sugar was shown to be *β*-D-digitalopyranose with a $^4C_1$ conformation by analysis of its ^1H coupling pattern and spatial relationship with reference to H-3, since all of the signals were well resolved.

Anomeric protons of the two glucose protons H-1'' at δ 5.14 (*d*, J = 7.6 Hz) and H-1''' at δ 5.16 (*d*, J = 7.9 Hz) could be readily differentiated by the observed ROE cross contour between H-1'' and H-4', and also the correlation of H-1'' with the ^{13}C signal of C-4' in the ROESY and HMBC spectra, respectively. In the DQF-COSY spectrum, H-1''' exhibited a cross-peak with a triplet signal at δ 5.75 (*t*, J = 7.9 Hz), assignable to a benzoylated geminal proton by its correlation with the ^{13}C signal of the carbonyl group at δ 166.11, indicating that the benzoyl group was attached to the C-2'' position of the isolated glucose. This, in turn, was attached directly to the aglycone at the C-20 hydroxyl group from the strong ROE correlations between the proton pairs of H-1'''/H_3-18, H-1'''/H_3-21 and H-1'''/H-20, and also the cross-peak between H-1''' and C-20 by the ROESY and HMBC experiments. Thus, the structure of 2 was elucidated as carulumagenin 3-*O*-*β*-D-glucopyranosyl (1→4)-*β*-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-*β*-D-glucopyranoside.

Table 2. ^{1}H NMR data of carumbelliosides III, IV and V (1–3)*

	1	2	3
1α	1.05 (*dt*, 13.5, 3.9)	0.83 (*m*)	0.85 (*m*)
1β	1.72 (*m*)	1.69 (*m*)	1.69 (*m*)
2α	2.09 (*m*)	2.10 (*m*)	2.09 (*m*)
2β	1.64 (*m*)	1.65 (*m*)	1.64 (*m*)
3α	3.86 (*m*)	3.86 (*dt*, 10.5, 7.5)	3.85 (*dt*, 11.9, 4.9)
4α	2.64 (*m*)	2.62 (*m*)	2.61 (*m*)
4β	2.33 (*br t*, 12.0)	2.30 (*m*)	2.28 (*m*)
6	5.43 (*br s*)	5.31 (*s*)	5.30 (*s*)
7α	2.02 (*m*)	2.03 (*m*)	2.03 (*m*)
7β	2.59 (*m*)	2.48 (*m*)	2.46 (*m*)
8β	1.94 (*m*)	1.87 (*m*)	1.87 (*m*)
9α	1.19 (*m*)	1.22 (*m*)	1.23 (*m*)
11α	2.12 (*m*)	2.08 (*m*)	2.07 (*m*)
11β	1.74 (*m*)	1.80 (*m*)	1.80 (*m*)
12α	1.26 (*m*)	1.26 (*m*)	1.24 (*m*)
12β	1.15 (*m*)	1.18 (*m*)	1.18 (*m*)
15-H_2	1.13 (*m*)	1.28 (*m*)	1.29 (*m*)
16α	1.69 (*m*)	1.72 (*m*)	1.72 (*m*)
16β	1.87 (*m*)	1.90 (*m*)	1.87 (*m*)
17α	1.53 (*dd*, 11.5, 6.5)	1.52 (*m*)	1.49 (*m*)
18-H_3	1.33 (*s*)	0.92 (*s*)	0.93 (*s*)
19-H_3	0.83 (*s*)	0.75 (*s*)	0.75 (*s*)
20	4.06 (*m*)	4.05 (*br q*, 6.0)	4.06 (*br q*, 6.1)
21	1.37 (*d*, 6.0)	1.43 (*d*, 6.0)	1.42 (*d* 6.1)
digitalose			
1′	4.73 (*d*, 8.0)	4.75 (*d*, 7.6)	4.75 (*d*, 8.4)
2′	4.37 (*dd*, 9.5, 8.0)	4.42 (*dd*, 9.6, 7.6)	4.41 (*dd*, 9.5, 7.8)
3′	3.52 (*dd*, 9.5, 2.5)	3.55 (*dd*, 3.2, 9.6)	3.55 (*dd*, 2.6, 9.0)
4′	4.07 (*t*, 2.5)	4.37 (*m*)	4.37 (*m*)
5′	3.69 (*q*, 6.5)	3.72 (*q*, 6.4)	3.72 (*q*, 6.3)
6′	1.52 (*d*, 6.5)	1.55 (*d*, 6.4)	1.55 (*d*, 6.3)
-OCH_3	3.63 (*s*)	3.66 (*s*)	3.66 (*s*)
glucose			
1″	5.09 (*d*, 8.0)	5.14 (*d*, 7.6)	5.14 (*d*, 7.9)
2″	3.92 (*t*, 8.0)	3.98 (*m*)	3.98 (*t*, 8.7)
3″	4.18 (*t*, 8.0)	4.21 (*m*)	4.23 (*t*, 8.7)
4″	4.12 (*m*)	4.20 (*t*, 8.0)	4.19 (*t*, 8.7)
5″	4.05 (*m*)	3.95 (*m*)	4.21 (*dd*, 8.2, 6.3)
6″	4.50 (*br d*, 10)	4.54 (*m*)	5.23 (*br d*, 11.4)
	4.29 (*br d* 10)	4.31 (*m*)	4.90 (*dd*, 11.4, 6.3)
glucose			
1‴	4.83 (*d*, 8.0)	5.16 (*d*, 7.9)	5.17 (*d*, 8.5)
2‴	3.84 (*m*)	3.75 (*t*, 7.9)	3.76 (*t*, 8.5)
3‴	3.89 (*m*)	4.35 (*m*)	4.32 (*m*)
4‴	4.05 (*m*)	4.26 (*m*)	4.19 (*t*, 8.7)
5‴	3.83 (*m*)	3.97 (*m*)	3.95 (*dd*, 8.4, 6.5)
6‴	4.45 (*d*, 11.5)	4.52 (*m*)	4.56 (*br d*, 11.2)
	4.25 (*d*, 11.5)	4.30 (*m*)	4.35 (*m*)
2‴-Bz			
3		8.20 (*d*, 7.6)	8.23 (*d*, 7.8)
4		7.33 (*t*, 7.6)	7.39 (*t*, 7.8)
5		7.44 (*d*, 7.6)	7.45 (*d*, 7.8)
6‴-Bz			
3			8.18 (*d*, 7.5)
4			7.33 (*t*, 7.5)
5			7.51 (*d*, 7.5)

* ppm from internal standard TMS in pyridine-d_5, coupling constants in Hz given in parentheses.

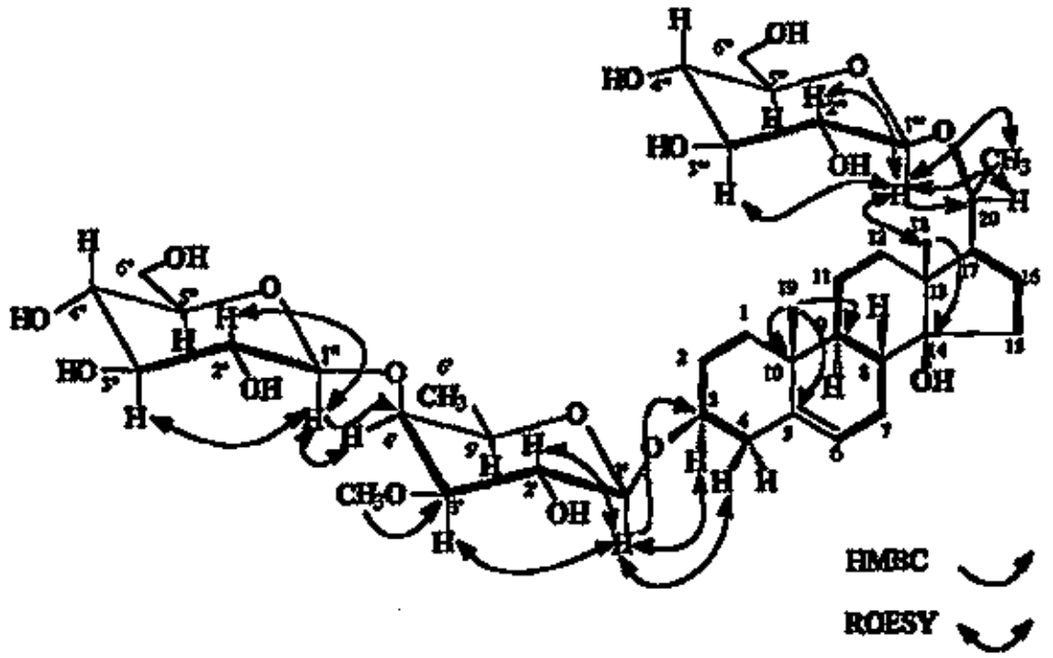

Fig. 1. The significant ROE and ^1H-^{13}C long range correlations of carumbelloside III (1) observed in the ROESY and HMBC spectra, with HOHAHA coupling fragments shown with bold C—C bonds.

Carumbelloside V (3) gave a positive Liebermann–Burchard test for steroids, and its negative-ion FAB-mass spectrum revealed a molecular formula $C_{56}H_{90}O_{19}$ (MW = 1026) based on the quasi-molecular ion $[M-H]^-$ observed at *m/z* 1025 in combination with the ^{13}C NMR (DEPT) experiment. Compound 3 exhibited two distinct sets of ^1H and ^{13}C NMR signals of a benzoyl group, besides the very close IR and UV absorption characteristics of compounds 1 and 2, implying the presence of an additional benzoyl group in 3 by comparison with 2. The aglycone of 3 was characterized as carallumagenin, the same as that in 1 and 2, with the NMR spectroscopic data completely assigned by the methods as mentioned above.

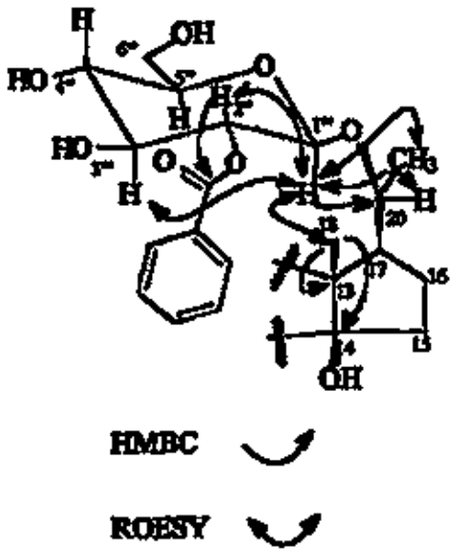

Fig. 2. Partial structure of carumbelloside IV (3) with the diagnostic ROE and ^1H-^{13}C long range correlations observed in the ROESY and HMBC spectra.

With the introduction of another benzoyl group in its molecule, compound 3 presented a more resolved ^1H NMR spectrum compared with those of 1 and 2 in the hydroxylated methine proton region, which enabled them to be assigned more readily. The ^1H NMR assignments of the inner β-D-digitalose were achieved from ROESY, DQF-COSY and HOHAHA spectral analysis using the well-defined signal of H-3 at δ 3.85 as an emanating point. Proton-3 showed a ROE cross peak with H-1′ at δ 4.75 (*d*, J = 8.4 Hz), which in turn was coupled with H-2′ and long-range coupled with H-5′ and H-4′ in the HOHAHA spectrum. Proton-5′ could only be recognized by a ROE correlation with H-1′ due to the weak coupling between H-5′ and H-4′.

The two benzoyl groups were disclosed as being associated with different spin systems from the DQF-COSY and HOHAHA spectral analysis. The doublet anomeric proton at δ 5.14 (*d*, J = 7.9 Hz), assignable to H-1″ based on the ROE cross-peak with H-4′ of the inner digitalose, showed vicinal coupling with H-2″ at δ 3.98 (*t*, J = 8.7 Hz) and relayed coupling with H-3″ at δ 4.23 (*d*, *t*, J = 8.7 Hz), which, in turn, showed vicinal coupling with H-4″, resonating coincidently with H-4‴ at δ 4.19 (*t*, J = 8.7 Hz), and was relay coupled with H-5″ at δ 4.21 (*dd*, J = 8.5 and 6.3 Hz). Thus, one benzoyl group was attached to the C-6″ hydroxyl group since H-5″ coupled with one of the H_2-6″ protons appearing as a double doublet at δ 4.90 (its partner resonating at δ 5.23 as a doublet in the

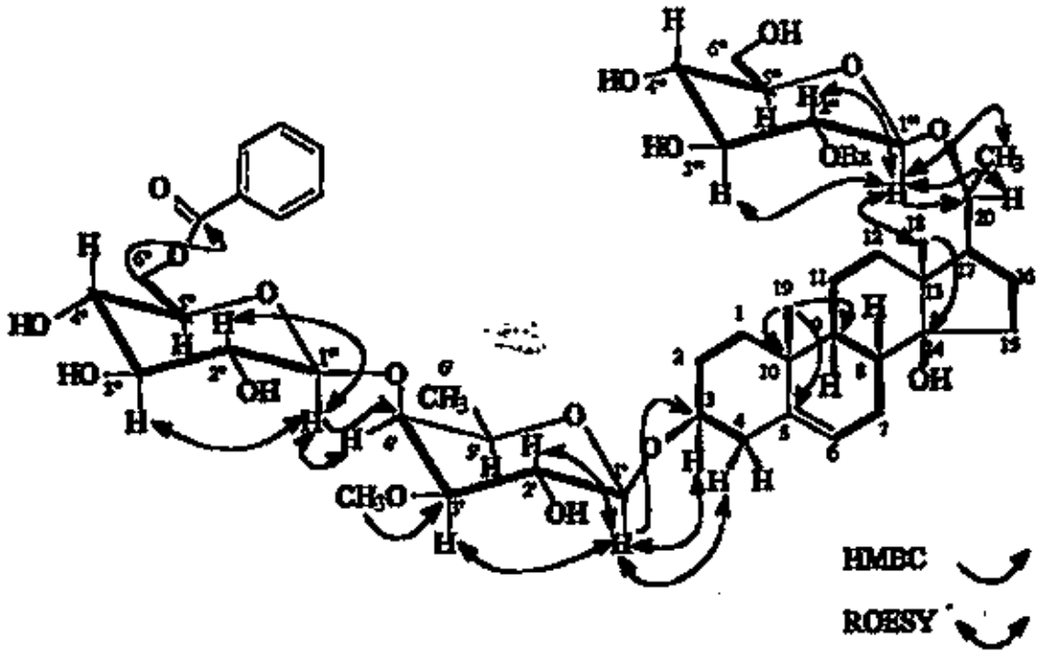

Fig. 1. The significant ROE and ^1H-^{13}C long range correlations of carumbelloside V (3) observed in the ROESY and HMBC spectra, with HOHAHA coupling fragments shown with bold C—C bonds.

HETCOR spectrum), and which further showed a correlation contour with the carbonyl of the benzoyl group at δ 166.45 from the HMBC experiment.

The remaining benzoyl group was rationalized to be bound to the C-2‴ hydroxyl group by means of vicinal and relayed coupling pattern deciphering using DQF-COSY and HOHAHA spectral analysis, as well as the HMBC experiment. The vicinal coupling of the benzoylated methine proton revealed by the HMBC spectrum at δ 5.76 (*t*, J = 8.5 Hz) with both H-3‴ at δ 4.32 and the anomeric proton H-1‴ at δ 5.17 (*d*, J = 8.5 Hz) which showed ROE cross contours with H_3-21 and H_3-18 as anticipated, were evident in the DQF-COSY spectrum. In addition, H-5‴ at δ 3.95 (*dd*, J = 8.7 and 6.3 Hz) and the methylene proton pair H_2-6‴ could be delineated by the HOHAHA spectrum despite the overlapping signal of H-4‴. Therefore, with the accomplishment of the unambiguous assignments of the NMR signals, the structure of 3 was established as caratunagenin 3-*O*-[6-*O*-benzoyl-β-D-glucopyranosyl(1→4)]-β-D-digitalopyranoside-20-*O*-(2-*O*-benzoyl)-β-D-glucopyranoside.

It is rarely reported that C-21 steroidal glycosides contain sugar (s) at both C-3 and C-20 positions. To our knowledge, this is the first report of a C-21 steroidal bisdemosidic glycoside with acyl groups bound to the sugars rather than steroidal parent skeleta, usually to the C-11, C-12 and C-20 hydroxyl groups. This may be of some significance for the biosynthesis of pregnane steroidal glycosides and also the chemotaxonomy of the title plant.

Except for showing the potency of advanced 2D-NMR techniques in the structure elucidation of steroidal glycosides, the assignments of these compounds provide a basis for the confirmation of the structures and assignments published, and also facilitate the further structure characterization of related compounds. For example, the apparently uninterpretable ^{13}C assignments on C-3 of the pregnane aglycone and C-2′ of digitalose of caratunbensides A and B, isolated from *C. tuberculata* [8], should be revised following the current results.

Carumbellosides III (1), IV (2) and V (3) were devoid of significant cytotoxity in the UIC human cancer cell panel according to established protocols [11].

EXPERIMENTAL

General. Mps: uncorr. UV spectra were taken in MeOH soln on a Beckman DU-7 spectrometer. IR spectra were recorded in a KBr pellet on a MIDAC FT-IR interferometer. The optical rotations were measured with a Perkin–Elmer 241 polarimeter. The ^1H, ^{13}C NMR, DEPT, DQF-COSY, HOHAHA, HETCOR and ROESY spectra were recorded at 500.13 MHz for ^1H, and 125.76 MHz for ^{13}C with a GE OMEGA 500 instrument, using GE standard programs in C_5D_5N soln. HMBC experiments were carried out on a Varian Plus-400 spectrometer. FAB-MS were recorded by the direct-inlet on a VG ZAB-HS mass spectrometer using glycerol as matrix. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck) and the detection was achieved by spraying with 10% H_2SO_4 followed by heating.

Plant material. The plant material of *C. umbellata*

was collected in Tirupathi, Andhra Pradesh, India, and identified by Dr V. S. Raju, Department of Botany, Kakatiya University, Warangal, India. A voucher sample was deposited in the herbarium of the University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India.

Extraction and isolation. The fresh whole plant (5 kg) of *C. umbellata* was chopped and crushed and extracted with EtOH at room temp. for 7 days. The extract was filtered and the solvent was removed *in vacuo*. To the concentrate, 1 l of H_2O was added, and extracted successively with toluene, ether, EtOAc and *n*-BuOH. After evapn of the solvent, the EtOAc ext and the *n*-BuOH ext were subjected to flash Silica gel CC to yield compounds 1, 2 and 3, respectively.

Isolation of carumbelloside III and IV. The *n*-BuOH extract (35 g) was dissolved in MeOH and then $CHCl_3$ was added until carumbelloside I ppted out. It was removed by filtration and the filtrate was subjected to flash silica gel chromatography (10–40 μm, 500 g) using EtOAc–MeOH–H_2O (81:11:8) as the eluent and frs of 50 ml each were collected. Frs 85–120 contained carumbelloside III and were purified further by a repetition of the process. Frs 15–21 contained carumbelloside IV which was purified by re-chromatography using the same system.

Carumbelloside III (1). 1600 mg, yield 0.012%), amorphous powder, mp 183–185°; $[\alpha]_D^{25}$ −18.4° (MeOH, *c* 0.50). IR ν_{max} (cm^{-1}): 3600–3200 (OH), 980, 920, 902, 856. FAB-MS *m/z* (negative): 817 $[M-H]^-$, 655 $[M-H-162]^-$, 493 $[M-H-162-162]^-$; *m/z* (positive): 841 $[M+Na]^+$, 819 $[M+H]^+$, 801 $[M+H-H_2O]^+$, 639 $[M+H-H_2O-162]^+$, 495 $[M+H-162-162]^+$, 479 $[M+H-H_2O-162-160]^+$. ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 2.

Carumbelloside IV (2). (75 mg, yield 0.0015%), amorphous powder, mp 188–190°; $[\alpha]_D^{25}$ −36.8° (MeOH, *c* 0.50). UV λ_{max}^{EtOH} nm (log ε): 218 (4.24), 224 (4.30), 281 (4.15). IR ν_{max} (cm^{-1}): 3450, 1700, 1600, 1450, 1280. FAB-MS *m/z* (positive): 923 $[M+H]^+$, 905 $[M+H-H_2O]^+$, 743 $[M+H-H_2O-162]^+$; *m/z* (negative): 921 $[M-H]^-$, 817 $[M-H-Bz]^-$, 655 $[M-H-Bz-162]^-$. ^1H NMR and ^{13}C NMR data are shown in Tables 1 and 2.

Isolation of carumbelloside V (3). The EtOAc extract (around 10 g) was subjected to flash chromatography eluting with $CHCl_3$–MeOH (95:5). Frs were pooled and re-chromatographed using EtOAc–MeOH–H_2O (81:11:8) to yield carumbelloside V.

Carumbelloside V (3). (25 mg, yield 0.0005%), mp 160–162°; $[\alpha]_D^{15}$ −32.4° (MeOH, *c* 0.50). UV λ_{max}^{EtOH} nm (log ε): 229 (4.05), 257 (3.30), 278 (3.21). IR ν_{max} (cm^{-1}): 3420, 1700, 1600, 1450, 1280. FAB-MS *m/z* (negative): 1025 $[M-H]^-$, 921 $[M-H-Bz]^-$, 655 $[M-H-Bz-162]^-$. ^1H NMR and ^{13}C NMR spectroscopic data are shown in Tables 1 and 2.

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PREGNANE GLYCOSIDES FROM *CARALLUMA RETROSPICIENS*

AHMED F. HALIM and ASHRAF T. KHALIL*

Faculty of Pharmacy, Mansoura University, Mansoura, Egypt

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Key Word Index—*Caralluma retrospiciens*; Asclepiadaceae; pregnane glycosides; new diacylated pregnane trioside.

Abstract—Two pregnane ester glycosides were isolated and identified from the alcohol extract of the aerial parts of *Caralluma retrospiciens*. Their structures were established as 12β-benzoyloxy-20-isovaleroyloxy-8β,14β-dihydroxypregnane-3-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)-galactopyranoside] (caretroside A) and the bioside 12β-benzoyloxy-8β,14β-dihydroxypregn-20-one-3-*O*-[β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside]. They were characterized through physical and chemical methods in addition to standard spectroscopic techniques especially 2D NMR (COSY, HMQC and HMBC). This is the first report of the isolation of these compounds from a natural source.

INTRODUCTION

Caralluma retrospiciens (Ehrenb.) N.E.Br. is a rare succulent plant that grows wild on the stony grounds in the Elba region [1]. Many Asclepiadaceae plants are rich in pregnane glycosides [2], which may possess prominent antitumour activities [3, 4] and function as precursors of cardenolides [5]. Certain pregnane glycosides were recently proved to induce differentiation of the mouse myeloid leukaemia cells into phagocytic cells [6]. Several pregnane ester glycosides were isolated from a few *Caralluma* species [7–10]. In a previous w... ork by one of the authors [11], two pregnane aglycon... were isolated after acid hydrolysis of a compl... mixture of glycosides obtained from a column chro... matographic fraction of the ether extract of C. re... ...tiens. The aglycones were identified as 12β-benzoyloxy-3β... trihydroxy-pregn-20-one and ...-pregnd... isovaleroyloxy-3β,8β,14β-... ent stu... ...rned with the characteriza... ...come of the less compli... ...ane extract.

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(determined by liquid chromatography) displayed a $[M+1]^+$ ion peak at m/z 759, suggesting a molecular formula $C_{42}H_{62}O_{12}$ and a double bond equivalence = 12. The sugar moieties were represented by two anomeric carbons at δ 95.4 and 101.2, indicating the presence of two sugar residues. These sugars were identified as D-cymarose and D-oleandrose by TLC after acid hydrolysis. The aglycone was proved to be 12β-benzoyloxy-3β,8β,14β-trihydroxy-pregn-20-one based on cochromatography, UV, mass spectral, IR and ^1H NMR data [11]. The ^{13}C NMR data for the aglycone (Table 1) revealed a glycosylation site at C-3 as indicated by the downfield shift of C-3 (δ +6) and the upfield shifts of C-2 and C-4 (δ −2.3 and −4, respectively), proving that the sugar moieties are attached to C-3. In addition, the H-17 signal of the aglycone moiety appeared as a double doublet at δ 2.90 (*J* = 11 and 6 Hz) and the ^{13}C signals of both C-12 and C-18 indicated that 1 has H-17α and its C-17 side chain was in a β-configuration [12]. The ^{13}C NMR signals of the sugar moieties confirmed the identities of D-cymarose and D-oleandrose (Table 1). The D-oleandrose was identified as the terminal sugar based on the values for C-1 and C-3 (δ 101.2 and 80.3) [13]. The β-glycosidic linkage of each sugar was revealed by the high coupling constants of the anomeric protons in the ^1H NMR spectrum. Thus, the structure of 1 was established as 12β-benzoyloxy-8β,14β-dihydroxy-pregn-20-one-3-*O*-[β-D-oleandropyranosyl-(1→4)-β-D-cymaropyranoside].

Compound 2 was identified as 12β-benzoyloxy-20-isovaleroyloxy-8β,14β-dihydroxypregnane-3-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)-galactopyranoside]. The identification was based on thorough investigation of

Table 1. ^{13}C NMR data (75 MHz, $CDCl_3$) for compound 1

C atom no.	δ	C atom no.	δ
1	38.0	D-Cymarose	
2	28.8	1'	95.4
3	77.2	2'	35.6*
4	33.9	3'	77.6
5	45.3	4'	82.8
6	23.2*	5'	68.3
7	35.1†	6'	18.2
8	75.6	$-OCH_3$	57.9
9	47.4		
10	36.4	D-Oleandrose	
11	24.7*	1''	101.2
12	77.2	2''	35.9*
13	54.6	3''	80.3
14	86.2	4''	70.4
15	36.2†	5''	71.3
16	25.1*	6''	18.5
17	57.7	$-OCH_3$	56.0
18	12.6		
19	12.6		
20	217.5		
21	33.1		
Benzoyl			
1	166.3		
2	130.2		
3	129.5		
4	128.6		
5	133.2		
6	128.5		
7	129.5		

*,†Assignments are exchangeable in the same column.

chemical and spectral data including IR, FAB mass spectra, ^1H NMR, ^{13}C NMR, APT, DEPT, COSY, HMQC and HMBC.

The ^{13}C NMR data (Table 2), as well as the APT and DEPT spectra, indicated the presence of 52 carbons of which seven were methyls, 11 methylenes, 27 methines and seven quaternaries. The same spectral data revealed the absence of any steroidal olefinic bond signals as well as any signal characteristic for a carbonyl group at C-20 (δ 217.5). Instead, two carbonyl ester signals were evident at δ 167.2 and 171.4. The former was proved to be due to a benzoyl ester as indicated by an IR band at 1708 cm^{-1} and the NMR signals of the aromatic ring protons and carbons (see Experimental and Table 1). The latter was of an isovaleroyl ester as confirmed by an IR band at 1720 cm^{-1}, the COSY spectral data, which revealed two CH_2 signals at δ 0.88, each coupled to a CH multiplet at δ 1.98, which in turn was coupled to a CH_2 signal at δ 2.10 attributed to H-5, H-4, H-3 and H-2 of the isovaleroyl and in the HMQC correlated to the corresponding carbons at δ 22.1, 22.0, 25.0 and 43.1 [3, 11].

One ester moiety was attached to C-12 (δ 74.2) as evident from the downfield shift of H-12, δ 5.36 (1H, *dd*, J = 12.6 and 4.4 Hz). This large coupling constant proved the α (axial)-configuration of this proton. The other ester moiety was connected to C-20 (δ 72.2) as indicated by the downfield shift of H-20 at δ 4.90 (1H, *dq*, J = 7.7 and 6.0 Hz).

The oxygenated quaternary carbons at δ 75.9 and 82.7 were assigned to C-8 and C-14, respectively, in accordance with reported data [14]. The last oxygenated carbon in the aglycone moiety was that at C-3 to which the sugar moiety was attached.

The relative stereochemistry at the chiral centres in the aglycone moiety was deduced from comparison of the coupling constants of the protons and/or the chemical shifts of the carbons with those reported for related pregnanes [3, 9, 12, 14]. However, the geometry at C-17 and C-20 could not be assigned.

The sugar moiety was represented by three monosaccharides as demonstrated in the HMQC spectrum (^1H–^{13}C correlations) by three anomeric carbon signals at δ 100.7, 102.9 and 103.4 crossed with the anomeric protons at δ 4.14 (1H, *d*, J = 7.60 Hz), 4.35 (1H, *d*, J = 7.65 Hz) and 4.28 (1H, *d*, J = 7.74 Hz), respectively. The large coupling constants indicated β-glycosidic linkages in all cases.

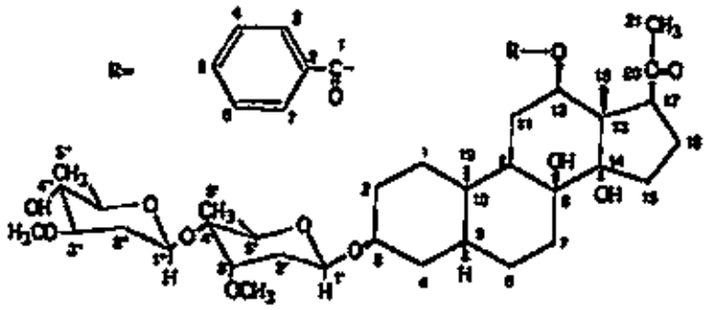
On acid hydrolysis, compound 2 yielded glucose (R_f 0.55) and another sugar (R_f 0.73) [7]. The latter was not a 2-deoxysugar, as indicated by a negative Keller–Killiani test, and it contained one methoxyl group at δ 3.35 (3H, *s*) crossed with the carbon signal at δ 57.7 and a secondary methyl group at δ 1.11 (3H, *d*, J = 6.2 Hz) crossed with δ 18.8. This monosaccharide was proved to be 6-deoxy-3-*O*-methyl-D-galactose and not 6-deoxy-3-*O*-methyl-D-allose, which is commonly present in Asclepiadaceous pregnanes. The H-3' proton absorbed as a double doublet at δ 3.03 ($J_{3',2'}$ = 9.6 and $J_{3',4'}$ = 2.80 Hz), which indicated that H-3' was axial. The distinctly smaller $J_{3',4'}$ showed that H-4' was equatorial. The latter absorbed as a broad doublet at δ 4.01; the $J_{4',5'}$ was very small and could not be calculated.

The sequence of attachment of the sugars was finally achieved from the HMBC spectrum of 2 (Fig. 1) where C-H long-range coupling was observed between H-1''' of the terminal D-glucose (δ 4.28) and C-6'' of the middle D-glucose (δ 68.6) and the H-1'' of the latter (δ 4.35) and C-4' of 6-deoxy-3-*O*-methyl-D-galactose (δ 73.4) [10].

Furthermore, HMBC measurements (Fig. 1) helped in the unambiguous confirmation of the sites of attachment of certain moieties to the steroidal nucleus. For example, glycosylation occurred at C-3 as observed from the coupling between H-1' of 6-deoxy-3-*O*-methyl-D-galactose (δ 4.14) and C-3 of the aglycone moiety (δ 76.1). Acylation of C-12 occurred with the benzoyl moiety as demonstrated by the coupling between H-12 (δ 5.36) and the benzoyl carbonyl (δ 167.2). This new compound was designated carotroside A.

EXPERIMENTAL

Instruments. mp: hot-stage melting point microscope (Sybron, U.S.A.). UV: Perkin-Elmer 550 S. IR: Pye



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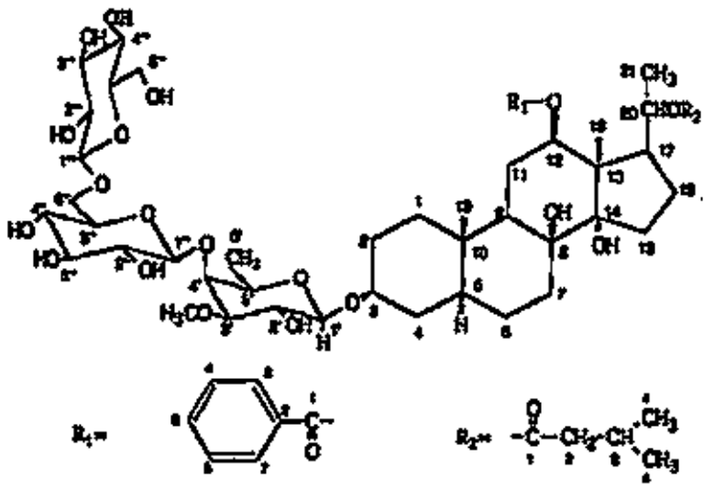

2

Unicam Sp. 1000 (Cambridge, U.K.). MS: Vestec 201 Thermospray MS system for LC-MS and JEOL JMS DX-303 for FAB-MS. Optical rotation: JASCO DIP-370 digital polarimeter at 25° in MeOH. HPLC: system equipped with M-6000A Waters Associates Chromatography pump; U6K injector; Waters 440 Absorbance Detector operating at 254 nm and HP3390 recording integrator (Howlett Packard); Bondapak (250 mm × 13 mm I.d.) C_{18} column (Waters Associates); MeCN–H_2O (1:1) as mobile phase at a flow rate of 2 ml min^{-1}. NMR: Varian VXR-300 FT Spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C including APT, DEPT, COSY and HETCOR and Bruker AM 500 for HMQC and HMBC in $CDCl_3$ or DMSO-d_6 with TMS as int. standard.

Extraction and isolation of the glycosides. The fresh plant material was collected and elaborated as described in ref. [11]. The Et_2O extract yielded a bitter residue (5.5 g) which was fractionated by silica gel CC using gradient mixts of $CHCl_3$–MeOH. The frs eluted with 4% MeOH in $CHCl_3$ afforded 1 while frs eluted with 7% MeOH in $CHCl_3$ afforded 2. Each compound was further purified by HPLC as mentioned above.

Acid hydrolysis. Each glycoside (40 mg) was treated with 0.05 N H_2SO_4 in 50% MeOH (25 ml) at 70° for 60 min. H_2O (10 ml) was then added and the mixt. was warmed at 60° for a further 30 min. After cooling, the mixt. was extracted with $CHCl_3$ (3 × 25 ml) to obtain the aglycone. The aq. phase of the hydrolysate was neutralized with sat. $Ba(OH)_2$ soln. After the inorganic ppt was filtered off, the filtrate was concd, then used for TLC examination of the sugars against authentic samples using silica gel, $CHCl_3$–MeOH–H_2O [(18:3:1) lower layer] in the case of 1 and cellulose, BuOAc–MeOH–H_2O–HOAc (13:3:3:4) in the case of 2. The plates were sprayed with aniline phthalate reagent in each case and the R_f values were as follows: 0.68 (cymarose), 0.62 (oleandrose) in the case of the first solvent system and 0.73 (6-deoxy-3-*O*-methyl-D-galactose) and 0.55 (glucose) in the case of the second.

12β-Benzoyloxy-8β,14β-dihydroxypregn-20-one-3-O-[β-D-oleandropyranosyl-(1 → 4)-β-D-cymaropyranoside] (1). Amorphous powder (80 mg); $[\alpha]_D$ −3.25 (*c* 0.65; MeOH) UV: λ_{max}^{MeOH} nm: 230, 282; LC-MS *m/z*: 759 $[M+1]^+$. $C_{42}H_{62}O_{12}$; IR ν_{max}^{KBr} cm^{-1}: 3450 (OH), 2980 (CH), 1710 (C=O of benzoyl ester), 1690 (C=O), 1600 (C=C aromatic), 1270 (ester), 715 (C-H aromatic); ^1H NMR (300 MHz, $CDCl_3$): δ 0.99 (3H, *s*, H-19), 1.22 (3H, *d*, *J* = 6.2 Hz, H-6'), 1.25 (3H, *d*, *J* = 6.2 Hz, H-6''), 1.30 (3H, *s*, H-18), 2.15

Table 2. ^{13}C NMR (75 MHz, DMSO-d_6) data for compound 2

C atom no.	δ	C atom no.	δ
1	40.8	Isovaleroyl	
2	28.5	1	171.4
3	76.1	2	43.1
4	33.9	3	25.0
5	44.3	4	22.0
6	24.4	5	22.1
7	37.4	6-Deoxy-3-methyl galactose	
8	75.9	1'	100.7
9	50.2	2'	70.3
10	36.1	3'	64.1
11	31.2	4'	73.4*
12	74.2	5'	68.9
13	46.6	6'	18.8
14	82.7	$-OCH_3$	57.7
15	33.3		
16	17.3	Glucose-1	
17	50.4	1"	102.9
18	16.9	2"	73.4*
19	12.9	3"	76.6†
20	72.2	4"	70.0‡
21	17.0	5"	75.0†
Benzoyl		6"	68.6
1	167.2		
2	128.7	Glucose-2	
3	129.6	1"'	103.4
4	128.5	2"'	73.6*
5	133.5	3"'	76.6†
6	128.5	4"'	69.2‡
7	129.6	5"'	76.2†
		6"'	61.0

*,†,‡Assignments are exchangeable in the same column.

(3H, *s*, H-21), 2.90 (1H, *dd*, *J* = 11, 6 Hz, *α*H-17), 3.38 (3H, *s*, $-OCH_3$ of cymarose), 3.44 (3H, *s*, $-O-CH_3$ of oleandrose), 3.62 (1H, *m*, H-3), 4.45 (1H, *dd*, *J* = 9.5, 2 Hz, H-1' anomeric), 4.60 (1H, *dd*, J = 9, 1.5 Hz, H-1" anomeric), 4.86 (1H, *dd*, *J* = 10.5, 4 Hz, H-12), 7.48 (2H, *t*, *J* = 7.8 Hz, H-4,6 of benzoyl), 7.61 (1H, *dd*, *J* = 7.8, 1.2 Hz, H-5 of benzoyl), 8.07 (2H, *dd*, *J* = 7.8, 1.2 Hz, H-3,7 of benzoyl). ^{13}C NMR: Table 1.

2β - Benzoyloxy - 20 - isovaleroyloxy - 5β,14β - dihydroxypregnane-3-O-[β-D-glucopyranosyl-(1→6) -β-D-glucopyranosyl-(1→4)-β-D-(3-O-methyl-6-deoxy)-galactopyranoside] (*cumaroside A*) (2). Crystals (200 mg), mp 238–242°; $[\alpha]_D$ −2.47 (*c* 0.96; MeOH); FAB-MS *m/z*: 1063 $[M + Na]^+$. $C_{55}H_{92}O_{21}$. IR ν_{max}^{KBr} cm^{-1}: 3550 (OH), 2970 (CH), 1720 (C=O of aliphatic ester), 1708 (C=O of aromatic ester), 1600 (C=C aromatic), 1270 (ester), 713 (C-H aromatic). ^1H NMR (300 MHz, DMSO-d_6): δ 0.88 (6H, *d*, *J* = 7 Hz, H-4,5 of isovaleroyl), 0.89 (3H, *s*, H-19), 1.05 (3H, *d*, *J* = 6 Hz, H-21), 1.11 (3H, *d*, *J* = 6.2 Hz, H-6'), 1.16 (3H, *s*, H-18), 1.98 (1H, *m*, H-3 of isovaleroyl), 2.10 (2H, *d*, *J* = 5.5 Hz, H-2 of isovaleroyl), 3.03 (1H, *dd*, *J* = 9.6, 2.8 Hz, H-3'), 3.35 (3H, *s*, $-OCH_3$ of 6-deoxy-3-*O*-methyl-D-galactose), 3.45 (1H, *m*, H-3), 3.55 (1H, *m*, H-6"b), 3.68 (1H, *m*, H-6"a), 4.14 (1H, *d*, *J* = 7.6 Hz, H-1' anomeric), 4.28 (1H, *d*, *J* = 7.74 Hz, H-1"' anomeric), 4.35 (1H, *d*, *J* = 7.65 Hz, H-1" anomeric), 4.90 (1H, *dq*, *J* = 7.7, 6 Hz, H-20), 5.36 (1H, *dd*, *J* = 12.6, 4.4 Hz, H-12), 7.54 (2H, *t*, *J* = 7.9 Hz, H-4,6 of benzoyl), 7.65 (1H, *dd*, *J* = 7.9, 1.2 Hz, H-5 of benzoyl), 8.12 (2H, *dd*, *J* = 7.9, 1.2 Hz, H-3,7 of benzoyl); ^{13}C NMR: Table 2; HMBC: Fig. 1.

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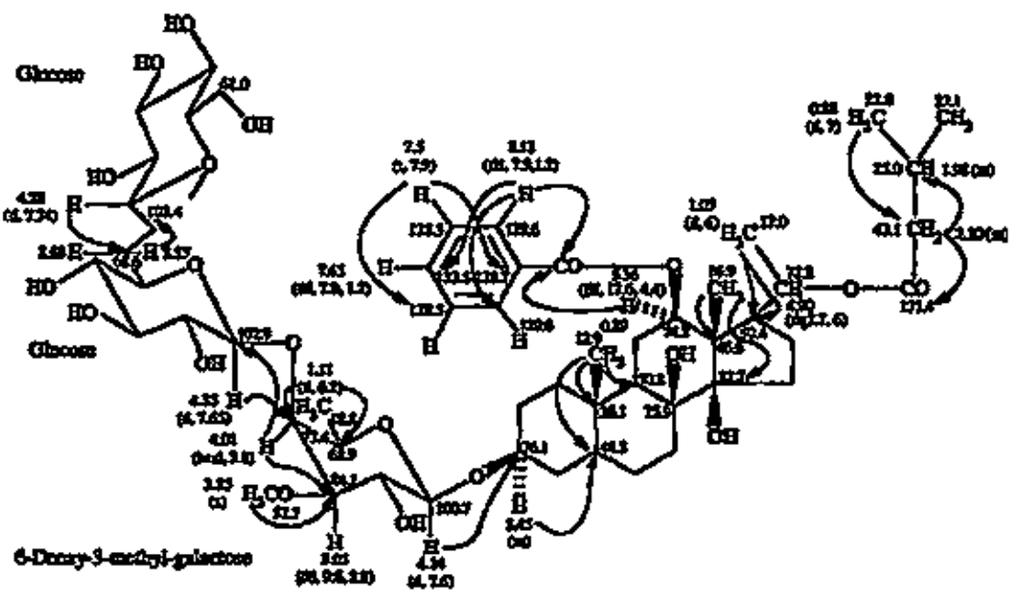

Fig. 1. Important HMBC correlations of compound 2.

sugars, and to Dr K. Takaya, Tokyo College of Pharmacy, Japan, for running the HMQC and HMBC spectra of compound 2.

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Pregnane Glycosides from *Caralluma russeliana*

Mohammed Abdul-Aziz Al-Yahya,[†] Essam Abdel-Sattar,*[,‡] and Eric Guittet[§]

Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh 11451, P.O. Box 2457, Saudi Arabia, Pharmacognosy Department, College of Pharmacy, Cairo University, Cairo 11562, Egypt, and Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, 91198 Gif-Sur-Yvette, France

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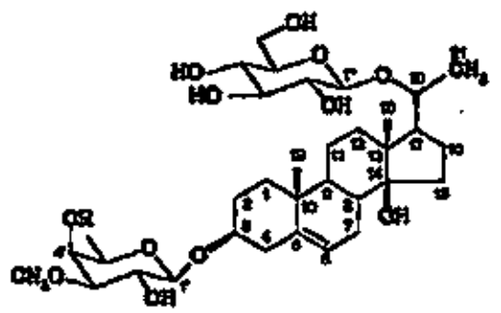
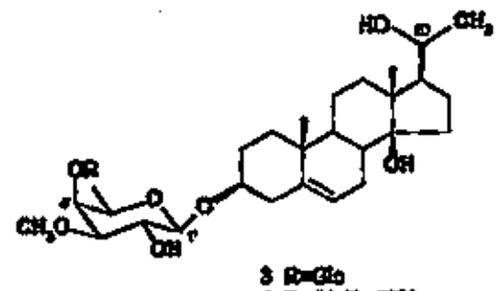
The aerial parts of *Caralluma russeliana* yielded four new pregnane glycosides, russelliosides A−D (1−4), in addition to a known flavone glycoside, luteolin 4′-*O*-β-D-neohesperidoside. The structures of compounds 1−4 were elucidated using a combination of spectroscopic methods.

The genus *Caralluma* belongs to the family Asclepiadaceae, which comprises some 200 genera and 2500 species. Plants belonging to the genus *Caralluma* are normally leafless and succulent perennial herbs that grow wild in stony habitats.[1] Several members of the genus *Caralluma* have found medicinal uses in the treatment of rheumatism, diabetes, and leprosy and as antiseptics and disinfectants.[2] Previous studies on plants in the genus *Caralluma* have reported the isolation of several pregnane glycosides or their esters,[3−7] of which some showed antitumor activity,[5,8] and others were postulated as precursors of cardenolides.[6] We report here the isolation and structure determination of four new pregnane glycosides (1−4) and a known flavone glycoside from the aerial parts of *Caralluma russeliana* (Coumb. Ex Brongn.) Gutfod.[10]



1 R=H
2 R=Glc



3 R=Glc
4 R=Glc(6′′′→1′′)Glc

The *n*-butanol fraction of the ethanol extract of the aerial parts of *C. russeliana* was chromatographed repeatedly to afford five compounds, namely, four pregnane glycosides (1−4) and a known flavone glycoside. Compounds 1−4 gave a positive test for sterols (Liebermann-Burchard reagent), and all five compounds gave a positive test for sugars and/or glycosides (Molish reagent).

Compound 1 was isolated as colorless needle crystals, mp 170−172 °C, $[\alpha]^{25}_{D}$ −15°, and was assigned the molecular formula $C_{34}H_{52}O_{10}$ as shown by its ESMS data (*m/z* 619 [M + Na]$^+$) in combination with the ^{13}C NMR spectral data. Its IR spectrum showed the presence of a hydroxyl group (3450 cm^{-1}) and the absence of carbonyl groups. Compound 1 showed two anomeric protons and carbons at δ_H 4.39 and 4.32 and δ_C 104.5 and 102.9 in the ^{1}H and ^{13}C NMR spectra, respectively, suggesting it to be a diglycoside. Acidic hydrolysis of 1 yielded two sugars, of which one was identified as glucose (TLC). The structure of the second sugar was determined as 3-*O*-methyl-6-deoxygalactose by comparison with NMR data reported in the literature.[5,6,7] The signals at δ_H 1.23 (3 H, d, J = 6.2 Hz) and δ_C 17.1 and at δ_H 3.46 (3 H) and δ_C 57.3 were assigned to the secondary methyl (C-6′) and OMe groups, respectively, in 3-*O*-methyl-6-deoxygalactose. The genin was identified as calogenin from a careful analysis of its NMR spectra (COSY, HMQC, HMBC) and by comparison with literature values.[7,11−13] The double bond was positioned between C-5 and C-6 on the basis of long-range HMBC correlations between H-6 and C-4 and C-7 and between H-5 and C-4 and C-7. This genin is common to russelliosides A−D (1−4). The glycosidation site at C-3 was deduced from the ^{13}C NMR spectrum of 1 as a result of the downfield shift of C-3 and the upfield shifts of C-2 and C-4.[14] A long-range correlation between C-3 (δ_C 79.9) and H-1′ (δ_H 4.32) in the HMBC spectrum indicated the attachment of the 3-*O*-methyl-6-deoxygalactose sugar unit to C-3. The glucose unit was shown to be attached to C-20 from the downfield shift (+12 ppm) of the C-20 signal, relative to analogous data for compound 4. Further structural proof for 1 was achieved by observation of a long-range correlation between C-20 (δ_C 79.1) and H-1′′ (δ_H 4.39) in the HMBC spectrum. Compound 1 was assigned with H-17 in the α-configuration (δ_H 1.70, dd, J = 11.1, 4.7 Hz) and the side chain with an β-configuration, as deduced from the NOESY spectrum and by comparison with related compounds.[5−7,13] From the foregoing evidence, the structure of compound 1 was established as calogenin 20-*O*-β-D-glucopyranosyl-3-*O*-β-D-(3-*O*-methyl-6-deoxy)-galactoside and has been named russellioside A.

Compound 2 was isolated as colorless crystals, mp 202−204 °C, $[\alpha]^{25}_{D}$ − 15.4°, with the molecular formula $C_{28}H_{42}O_7$ determined from its ^{13}C NMR and ESMS (*m/z* 841 [M + Na]$^+$) data. Its IR spectrum was similar to that of com-

* To whom correspondence should be addressed. Tel.: (20)-2-2787284. Fax: (20)-2-3626105. E-mail: abdel-sattar@writeme.com.
† King Saud University.
‡ Cairo University.
§ Centre National de la Recherche Scientifique.

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pound 1. Acid hydrolysis of 2 showed the same aglycone and the same sugar units as those of 1 (glucose and 3-*O*-methyl-6-deoxygalactose). Compound 2 exhibited three anomeric protons and carbons at δ_H 4.35, 4.33, and 4.60 and δ_C 104.8, 104.7, and 103.4 in its ^{1}H and ^{13}C NMR spectra, respectively, suggesting it to be a triglycoside. The ^{1}H and ^{13}C NMR spectra showed the presence of an additional glucose unit attached through a 1 → 4 linkage to the 3-*O*-methyl-6-deoxygalactose unit. This was confirmed from the HMBC spectrum, which demonstrated a long-range correlation between C-1‴ (δ_C 104.67) and H-4′ (δ_H 4.16) and between C-4′ (δ_C 78.5) and H-1‴ (δ_H 4.60). Therefore, compound 2 was deduced as calogenin 20-*O*-β-D-glucopyranosyl-3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)galactoside and has been named russelloside B.

Compound 3 was isolated as colorless crystals, mp 188–190 °C, $[\alpha]^{25}_D$ −39.5°, with the molecular formula $C_{39}H_{62}O_{16}$, as deduced from its ^{13}C NMR and ESMS (*m/z* 679 [M + Na]$^+$) data. Compound 3 showed only two anomeric protons and carbons at δ_H 4.42 and 4.67 and δ_C 105.5 and 106.8 in the ^{1}H and ^{13}C NMR spectra, respectively, suggesting it to be a diglycoside. The signal at δ_C 68.8 corresponding to C-20 appeared shifted upfield (−10.8 ppm) relative to those of compounds 1 and 2, indicating the absence of glycosidation at C-20. The identity of the two sugars at C-3 and their linkages were similar to 2 and were confirmed from the ^{1}H–^{1}H COSY and HMBC spectra. Thus, compound 3 was assigned as calogenin 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)]galactoside and has been named russelloside C.

Compound 4 was obtained as amorphous powder, $[\alpha]^{25}_D$ −[illegible] and was assigned a molecular formula of $C_{39}H_{62}O_{17}$ (^{13}C NMR data and ESMS, *m/z* 841 [M + Na]$^+$). Compound 4 was shown to be a triglycoside, as it exhibited three anomeric protons and carbons at δ_H 4.59, 4.38, and 4.34 and δ_C 103.7, 105.1, and 105.9, respectively, in its ^{1}H and ^{13}C NMR spectra. In a manner similar to compound 3, no sugar unit was found to occur at C-20, as indicated by the upfield shift (−12 ppm) of C-20 (δ_C 67.1) when compared to 1. The additional sugar moiety was identified as glucose and was shown to be linked to the other glucose unit through a 1 → 6 linkage, as indicated by a downfield shift (+7.7 ppm) of C-6″ (δ_C 71.2) relative to the corresponding carbon in 2 (δ_C 63.5). Further confirmation was achieved from the HMBC spectrum, which showed correlations between C-6″ (δ_C 71.2) and H-1‴ (δ_H 4.38) and between C-1‴ (δ_C 105.9) and H-6″ (δ_H 4.15, 3.78). From the previous discussion and by comparison with data reported in the literature,[5,6] compound 4 was assigned as calogenin 3-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranosyl(1→4)-β-D-(3-*O*-methyl-6-deoxy)]galactoside and has been named russelloside D.

A prominent feature in the ^{1}H NMR spectra of 1–4 was the very small value of the vicinal H-17 to H-20 coupling constants (see Experimental Section). A detailed analysis of the conformation of the side chain was performed on russelloside A (1), and NOESY cross-peaks were observed between H-17 and H-20, Me-18 and H-20, but not between H-17 and Me-18 and Me-21. From these observations, it was concluded that the side chain is oriented with carbon C-20 having the *S* configuration.

The final isolate 5 was identified as luteolin 4′-*O*-β-D-neohesperidoside on the basis of the analysis of its spectral data and by comparison with NMR values reported in the literature.[18]

Experimental Section

General Experimental Procedures. Melting points were determined on an electrothermal melting point apparatus (Electrothermal Ltd., Southend-on-Sea, Essex, U.K.) and are uncorrected. Optical rotations were measured at ambient temperature, using a Perkin-Elmer 241 MC polarimeter. IR spectra were recorded on a Pye Unicam SP3-300 instrument. UV spectra were recorded on a Shimadzu 265 spectrophotometer. ^{1}H and ^{13}C NMR data were recorded in CD_3OD using a Bruker-400 NMR spectrometer (400.13 and 100.6 MHz, respectively) and a Varian Unity Plus 500 NMR spectrometer (500 and 125 MHz, respectively). Electrospray mass spectra (ESMS) were recorded on a quadrupolar Navigator mass spectrometer (Thermoquest).

Plant Material. The plant material was collected in March, 1995, from a rocky region south of Jeddah, in the southwest of Saudi Arabia. The plant was kindly identified by Dr. Sultan Ul-Abedin, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, and a voucher specimen deposited in the herbarium of the College of Pharmacy, King Saud University (#11677-A).

Extraction and Isolation. The dried ground aerial parts (550 g) of *C. russelliana* were percolated with ethanol (5 L) at room temperature to give a dark greenish semisolid residue (75 g) after evaporation of the solvent. The ethanolic extract (25 g) was suspended in water and defatted with petroleum ether, then shaken with EtOAc (2.3 g) and *n*-BuOH (33 g). A portion of the *n*-BuOH fraction (7 g) was chromatographed on a Si gel column (5 × 19 cm) using a mixture of $CHCl_3$–MeOH–H_2O (78:20:2) to give four main fractions (A–D). Fraction A (250 mg) was rechromatographed on a Si gel column (3.5 × 17 cm) using 20% MeOH–$CHCl_3$ as solvent system to afford fractions A-1 and A-2. Fraction A-1 was purified by medium-pressure chromatography (MPLC) over C_{18} Si gel (LiChroprep RP-18, 25–40 μm, 2 × 13 cm) using 25% MeOH–H_2O (flow rate 4 mL/min), to give 40 mg of compound 1. Compound 3 (17 mg) was isolated from fraction A-2 by chromatography over a Si gel column (3 × 18 cm) using 10% MeOH–$CHCl_3$ as solvent system. Fraction B (3.5 g) yielded pure compound 2 (2.1 g) by crystallization from MeOH/diethyl ether. Fraction C (2.3 g) was treated with MeOH/diethyl ether to give an additional amount of compound 2 (700 mg). The mother liquor left from fraction C was evaporated, and the residue left was rechromatographed on a Si gel column (2.5 × 15 cm) using EtOAc–MeOH–H_2O (100:20:12) as solvent system to remove a contaminating flavonoid. Fraction C-1 (810 mg) was further purified on another Si gel column (3 × 50 cm) using $CHCl_3$–MeOH–H_2O (80:10:1), to afford compound 4 (105 mg). Luteolin 4′-*O*-β-D-neohesperidoside (70 mg) was precipitated as a yellowish amorphous powder upon concentration of fraction D.

Acid Hydrolysis of Compounds 1–4. Compounds 1-4 were hydrolyzed according to the procedure reported by Ahmed et al.[3]

Russelloside A (1): colorless needles, mp 170–172 °C (MeOH/diethyl); $[\alpha]^{25}_D$ −13° (*c* 0.08, MeOH); IR (KBr) ν_{max} 3450 (OH), 2900, 1100 cm^{-1}; ^{1}H NMR (400.13 MHz, CD_3OD) δ 5.41 (1 H, br m, H-6), 4.39 (1 H, d, *J* = 7.9 Hz, H-1″), 4.33 (1 H, d, *J* = 7.9 Hz, H-1′), 4.01 (1 H, br q, *J* = 6.4 Hz, H-20), 3.84 (1 H, dd, *J* = 2, 12 Hz, H-6″A), 3.83 (1H, br d, *J* = 3.2 Hz, H-4′), 3.83 (1 H, dd, *J* = 12 Hz, 5.4, H-6″B), 3.55 (1 H, m, H-3), 3.46 (3 H, s, MeO), 3.17 (1 H, dd, *J* = 7.9 Hz, H-3″), 3.13 (1 H, dd, *J* = 9.6 Hz, 4, H-2″), 2.43 (1 H, dd, *J* = 13 Hz, 4.8, H-4α), 2.03 (1 H, bt, *J* = 12 Hz, H-4β), 2.34 (1 H, m, H-7α), 1.30 (3 H, d, *J* = 6.4 Hz, H-21), 1.29 (3 H, d, *J* = 6.2 Hz, H-6′), 1.11 (3 H, s, H-18), 1.02 (3 H, s, H-19); ^{13}C NMR, see Table 1; ESMS *m/z* 679 [M + Na]$^+$.

Russelloside B (2): colorless crystals, mp 202–204 °C (MeOH/diethyl); $[\alpha]^{25}_D$ −15.4° (*c* 0.1, MeOH); IR (KBr) ν_{max} 3420 (OH), 2930, 1100 cm^{-1}; ^{1}H NMR (400.13 MHz, CD_3OD) δ 5.42 (1 H, br m, H-6), 4.60 (1 H, d, *J* = 7.7 Hz, H-1‴), 4.33 (1 H, d, *J* = 7.9 Hz, H-1″), 4.35 (1 H, d, *J* = 7.7 Hz, H-1′), 4.16 (1 H, br d, *J* = 3.1 Hz, H-4′), 4.01 (1 H, br q, *J* = 6.4 Hz, H-20), 3.85 (2 H, dd, *J* = 12, 3.5 Hz, H-6″, H -6‴A), 3.52 (1 H, m, H-3),

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mdelawcol@cable.net.co
mdeabogados@cable.net.co

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Atentamente,

JAIME EDUARDO DELGADO VILLEGAS
C.C. No. 19'270.955 de Bogotá
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Short communication

Antinociceptive and anti-inflammatory activity of carumbelloside-I isolated from *Caralluma umbellata*

Mullangi Ramesh [a,*], Yepuri Nageshwar Rao [a,1], Meda Rama Kumar [a], Achanta Venkata Narasimha Appa Rao [a], M.C. Prabhakar [a], B. Madhava Reddy [b]

[a] *University College of Pharmaceutical Sciences, Kakatiya University, Warangal 506 009, India*
[b] *G. Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad 500 028, India*

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Abstract

The phytochemical study using *Caralluma umbellata* (Asclepiadaceae) whole plant allowed the isolation of a novel pregnane glycoside named carumbelloside-I (3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-3β,14β-dihydroxypregn-5-en-20-one). Carumbelloside-I was evaluated for both antinociceptive activity and anti-inflammatory activity. The antinociceptive activity was evaluated in mice using the writhing test method, while the anti-inflammatory activity was evaluated in rats using the paw edema test with carrageenin. Carumbelloside-I has significant antinociceptive action. It has no anti-inflammatory activity. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Caralluma umbellata*; Asclepiadaceae; Pregnane glycoside; Antinociceptive activity; Anti-inflammatory activity; Carumbelloside-I; 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-3β,14β-dihydroxypregn-5-en-20-one

1. Introduction

Caralluma umbellata Haw. [Syn.: *Boucerosia umbellata* W&A, *Stapelia umbellata* Roxb., *Caralluma campanulata* N.E.Br.] (Asclepiadaceae), is a thick, erect, leafless, branching, succulent perennial herb. It grows wild in dry and arid regions of Chittoor district and several districts of Andhra Pradesh, India. Stem juice warmed and mixed with turmeric powder is given in stomach disorders and abdominal pains (Vedavathy et al., 1997). From this plant we have isolated five novel pregnane glycosides viz., carumbellosides-I and -II (Lin et al., 1994); carumbellosides-III, -IV and -V (Qin et al., 1997) and a known flavone glycoside, i.e. luteolin-4′-*O*-neohesperidoside

* Corresponding author. Present address: Dr. Reddy's Research Foundation, Miyapur, Hyderabad 500 050, India. Tel.: +91-40-304-5439; fax: +91-40-304-5438.
E-mail address: mullangir@hotmail.com (M. Ramesh)
[1] Present address: Dr. Reddy's Research Foundation, Miyapur, Hyderabad 500 050, India.

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(Ramesh et al., 1999). This flavone glycoside possess potent anti-inflammatory activity (Ramesh et al., 1998). To the best of our knowledge no pharmacological work was carried out on *C. umbellata*. Carumbelloside-I, the major phytochemical constituent of *C. umbellata* was subjected to preliminary pharmacological screening to clarify the utility of this plant in treating abdominal pains and *stomach disorders*.

2. Materials and methods

2.1. Plant material

Fresh whole plants of *Caralluma umbellata* were collected in Tirupathi, Andhra Pradesh, India by Dr D. Rangachary, Department of Botany, S.V. University, Tirupathi and identified by him. A voucher specimen (AVN-CU-94) is deposited in the herbarium of University College of Pharmaceutical Sciences, Kakatiya University, Warangal, India.

2.2. Extraction and isolation

The fresh whole plants (5 kg) of *C. umbellata* were chopped and crushed in a mixer and extracted with ethyl alcohol at room temperature for 7 days. The extract was filtered and the solvent was removed under vacuum. To the concentrate, water (1 l) was added, extracted successively with toluene, ether, ethyl acetate and *n*-butyl alcohol. The yield of butanolic extract was 35 g. The butanolic extract was dissolved in methanol and chloroform was added to precipitate out 9.4 g of carumbelloside-I (Qin et al., 1997). Carumbelloside-I (CU-I, 1) (Fig. 1) was identified by extensive 2D-NMR methods and molecular modeling (Lin et al., 1994).

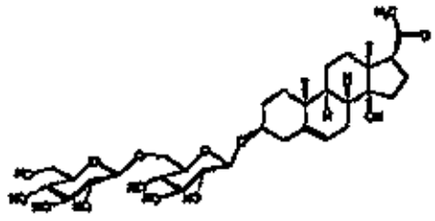

Fig. 1. Carumbelloside-I: 3-*O*-(β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl)-3β,14β-dihydroxypregn-5-en-20-one.

2.3. Test animals

Male Wistar rats (150–200 g) and Swiss Albino mice of either sex (22–25 g) were used in experiments. The animals were maintained under standard environmental conditions and had free access to feed (Lipton India Ltd., Bangalore) and tap water *ad libitum* during the *quarantine period*. Groups of six test animals were used.

2.4. Writhing test

The Siegmund et al. (1957) technique modified by Koster et al. (1959) was adopted to assess the *antinociceptive activity* in *pre-screened* mice. After 16 h fast mice were divided into five groups of six each. Group I served as a control group received carboxy methyl cellulose 1 ml/100 g of 0.5% w/v, orally. Groups II–IV received CU-I at a dose of 1, 3 and 10 mg/kg, respectively, as a fine suspension in 0.5% w/v carboxy methyl cellulose, orally. Group V was orally administered analgin or metamizol (monohydrate sodium [*N*-(2,3-dihydro-1,5-*dimethyl*-3-*oxo*-2-*phenyl*-1H-*pyrazol*-4-yl)-*N*-methylamino]methanesulphonate) at a dose of 50 mg/kg as a standard drug. An hour after the administration, the animals were given an intraperitoneal injection of 0.6% v/v acetic acid solution (volume of injection 0.1 ml/10 g). The number of writhes produced in these animals were counted for 30 min.

2.5. Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the carrageenin-induced edema in the rat paw according to the technique of Winter et al. (1962). After a 16 h fast, rats were divided into five groups of six each. Group I served as a control group and received carboxy methyl cellulose at 1 ml/100 g of 0.5% w/v, orally. Groups II–IV re-

Table 1
Effect of CU-1 on acetic acid-induced writhings in mice[a]

S. No.	Treatment	Dose (mg/kg, p.o.)	Writhings (mean ± S.E.M.)	% Inhibition
1	Control		17.6 ± 1.24	
2	CU-1	1	11.8 ± 0.58	32.95[b]
3	CU-1	3	9.8 ± 0.37	44.32[**]
4	CU-1	10	7.8 ± 0.58	55.68[**]
5	Amigin	50	7.16 ± 2.21	59.32[**]

[a] n = 6;
[b] $P < 0.02$;
[**] $P < 0.01$, compared to control.

Table 2
Effect of CU-1 on carrageenin-induced edema in rats[a]

S. No	Treatment	Dose (mg/kg, p.o.)	Mean edema (ml) ± S.E.M.	% Inhibition
1	Control		17.93 ± 0.738	
2	CU-1	1	15.35 ± 0.63[**]	15.35
3	CU-1	3	14.65 ± 0.56[**]	18.36
4	CU-1	10	13.97 ± 0.50[**]	22.20
5	Hydrocortisone	6	10.28 ± 0.65[b]	42.76

[a] n = 6;
[b] $P < 0.01$;
[**] $P > 0.05$, compared to control.

ceived CU-1 at a dose of 1, 3 and 10 mg/kg, respectively, as a fine suspension in 0.5% w/v carboxy methyl cellulose, orally. Group V was orally administered hydrocortisone at a dose of 6 mg/kg as a standard drug. An hour after the administration edema was induced by injecting 0.1 ml of 1% carrageenin in normal saline into the plantar aponeurosis of the right hind paw. The measurement of the paw volume was carried out by means of a Ugo Basile Plethysmograph model 7150, immediately and 3 h after carrageenin injection.

2.6. Statistical analysis

All results were expressed as means ± S.E.M. The differences between experimental groups were compared by one-way ANOVA (control vs. treatment, Bonferriouni's method) (using Jandal Scientific, Sigmastat statistical software, version 1.0) and were considered statistically significant when $P < 0.05$.

3. Results

3.1. Antinociceptive activity

Table 1 shows the results of antinociceptive activity. CU-1 exhibited significant dose dependent antinociceptive activity at the doses tested. The antinociceptive activity of CU-1 at 10 mg/kg is comparable with the reference antinociceptive agent used in this study.

3.2. Anti-inflammatory activity

Table 2 shows the results of anti-inflammatory activity. These results indicate that CU-1 has no

significant anti-inflammatory activity at the doses tested.

4. Discussion

The juice of *C. umbellata* warmed and mixed with turmeric powder is given in stomach disorders and abdominal pains (Vedavathy et al., 1997). Phytochemical investigations of *C. umbellata* have resulted in isolation of CU-I, as the major chemical constituent. Pharmacological screening results of CU-I presented here may help to establish the scientific basis for the utility of *C. umbellata* for stomach disorders and abdominal pains. The findings of this study have demonstrated that CU-I has significant antinociceptive activity. Our present efforts are directed to test the CU-I antinociceptive activity in other models of analgesia and also elucidate the mechanism of action.

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CHARACTERIZATION OF CHEMICAL CONSTITUENTS AND TOXICITY EVALUATION OF SOME POISONOUS PLANTS

K. Usmanghani

A summary of the research work carried out by our group on the isolation and structure elucidation of chemical constituents of three suspected poisonous plants (*Caralluma tuberculata*, Asclepiadaceae; *Primula denticulata*, Primulaceae; and *Castanospermum australe*, Papilionaceae) is reported. *Primula denticulata* is a very common and abundant weed found in the northern foothill zone and throughout the Himalayas from 1300-4300 m. *P. denticulata* appears in open spaces (such as forest clearings) by melting snow and in damp meadows; the mauve to pinkish-blue flowers always attract livestock and other animals (1). *Caralluma tuberculata*, a succulent perennial herb that occurs wild and cultivated, is found throughout the plains. It is also very common and abundantly available; because of its succulent stem, stock will graze it (2). *Castanospermum australe*, a large tree known as black bean or Moreton Bay chestnut that occurs in Queensland and New South Wales, is cultivated in the gardens of Pakistan; plants are found growing in the Karachi area as well (3).

Since these three species belong to the families Primulaceae, Asclepiadaceae, and Papilionaceae, as well as by judging their chemical history to elaborate toxicants, *P. denticulata* and *Castanospermum australe* were studied for sapogenin constituents; *Caralluma tuberculata* was selected for the study of pregnane glycosides. Saponins and their genins haemolyze red blood cells and paralyze the gills of fish. These are bitter compounds that affect palatability and feed intake, have growth-depressing properties in poultry and swine, and have been implicated in bloat in ruminants (4,5). Saponin glycosides (triterpenes) of *Holothuria mexicana* and *Cucumaria frandatrix* showed higher toxicity than the glycosides of plant origin; this was attributed to the presence of four units of monosaccharide attached to the aglycone. It is also reported that monodesmosidic saponins are more haemolytic than bidesmosidic saponins (6).

PRIMULA DENTICULATA

The literature search on *Primula* showed that the glandular trichomes on calyx and flowering stalks of *P. obconica* contain the skin irritant benzoquinone derivative, primin (2-methoxy, 6-n-pentyl-p-benzoquinone). *Cyclamen purpurascens* contains the triterpenoid saponin cyclamin, which exhibits local irritant action and acute toxicity in the form of gastrointestinal upset, leading to convulsion and death (7). *P. denticulata* was selected for the study of sapogenins and saponins. The saponins isolated from the alcoholic extract of the plant were hydrolysed; the resulting sapogenin mixture contained several triterpenoids (Figure 1). Five sapogenins were isolated (8) through column chromatography and were designated as pridentigenin A-E in increasing order of polarity on the thin-layer chromatographic plate.

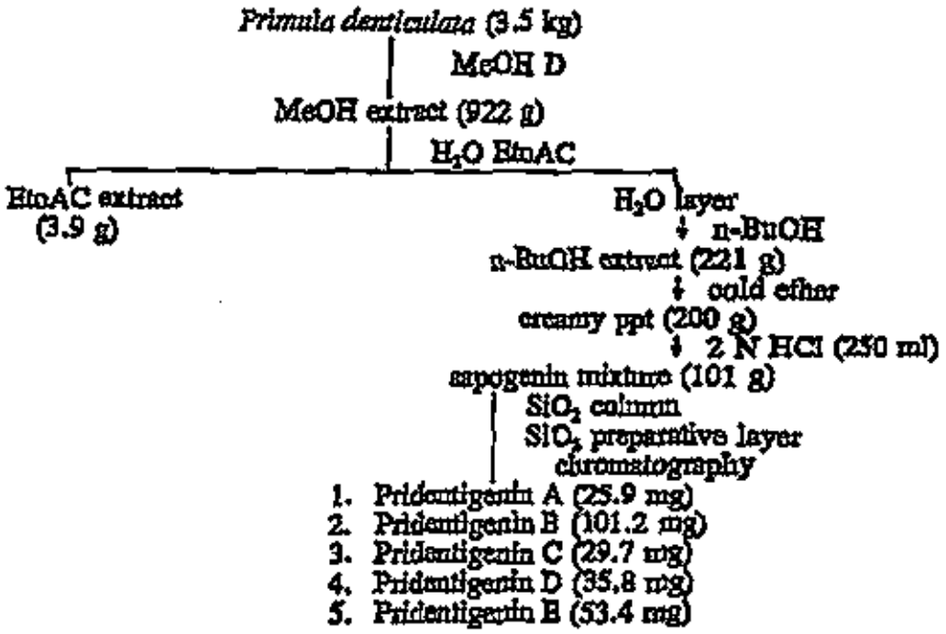


FIGURE 1
Extraction of *Primula denticulata*.

Pridentagenin A

The least polar sapogenin, it was crystallised from ether in the from of colourless needles. Melting point was 308-310 C. NMR spectrum ($CDCl_3$) of pridentigenin A showed the presence of six tertiary methyl groups through singles at δ0.70 (3H, 1xCH_3), δ0.84 (6H, 2xCH_3), δ0.97 (3H, 1xCH_3), δ1.03 (3H, 1xCH_3), and δ1.24 (3H, 1xCH_3). There was a singlet equivalent to six protons at δ3.51 indicating the presence of two equivalent OCH_3 groups in the compound. Another singlet at δ4.30 was ascribed to H-30. The mass spectrum showed molecular ion peak at m/z 516 corresponding to the molecular formula $C_{32}H_{52}O_5$. Other important peaks were at m/z 485 (base peak, M^+-OCH_3), 484 (M^+-CH_3OH), 466 (M^+-2CH_3OH), 454 (M^+-OCH_3+CH_2OH), 452 (M^+-CH_3OH), 308 (retro-

Diels-Alder fragment a) (9), 277 (a-OCH_3), 276 (a-CH_3OH), 207 (retro-Diels-Alder fragment b), and 189 (b-H_2O). Pridentigenin A (I) closely resembled cyclamigenin D reported earlier by Dorchai *et al.* (10). No authentic sample was available for comparison; however, it is very likely that the two compounds are identical.

Pridentigenin B

Pridentigenin B (II) was analysed for $C_{31}H_{50}O_4$. MS of this compound displayed a very low intensity molecular ion peak at m/z 486 which became somewhat stronger when the field desorption technique was used. Its UV spectrum had a λ_{max} at 212-213 nm in methanol, which showed the presence of conjugated double bonds. In the IR spectrum (KBr), a strong peak at 3367 cm^{-1} (OH) and a peak of medium intensity at 1630 cm^{-1} (C=C stretching) were visible and no signal due to carbonyl was present. It formed a diacetate m.p. 220 C. On the basis of the spectral data and different reactions, we proposed that pridentigenin B had the structure as given (II). It was, therefore, a homologue of cyclamigenin A (or C) isolated by Dorchai *et al.* (10). The stereochemistry at C-30 could not be determined with certainty, but it was most likely the thermodynamically more stable equatorial 30 epimer. The structure proposed for pridentigenin B found a measure of support from ^{13}C-NMR spectra of $CDCl_3$ + CD_3OH, analogous with the spectra of olean-12-enes (11), with peaks due to C-12 and C-13 at 121.5 and 142.1 ppm present. The C-3 and C-16, bearing the hydroxyl groups, showed peaks at 78.93 and 77.43 ppm, respectively, whereas the peaks of C-28 (attached to an ether function) occurred at 70.1 ppm. The peak due to C-30, which was attached to two oxygen atoms, was shifted to 109.7 ppm. The assignment of ^{13}C-NMR peaks were reported earlier (12).

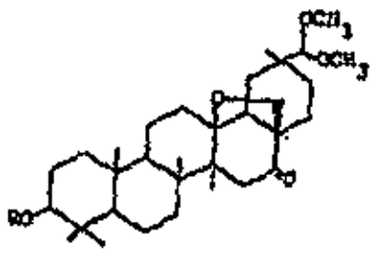
Pridentigenin C

Pridentigenin C (II) was crystallized from methanol as colourless crystal, m.p. 152-154 C. MS of pridentigenin C ($CDCl_3$) showed the presence of six methyl groups at δ0.69 (3H,1xCH_3), δ0.81 (6H,2xCH_3), δ0.87 (6H,1xCH_3), δ1.2 (3H, 1xCH_3). A methoxy peak appeared at δ3.51 (3H,OCH_3), a proton in the ring at 4.13 (1H) was attached at C-30, and another broad signal at δ5.35 was due to a proton at C-12. The NMR spectrum and MS fragmentation pattern of pridentigenin C was very similar to that of Pridentigenin B, so it appeared that pridentigenin C was a stereoisomer of pridentigenin B at C-30.

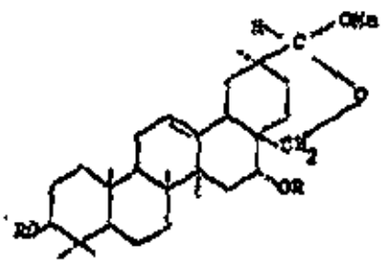
Pridentigenin D

Pridentigenin D (III) was found to be a new sapogenin and was crystallized from ether into small colourless crystals, m.p. 170 C. The IR spectrum displayed a broad band at 3360 cm^{-1} due to the OH group and a sharp peak at 1700 cm^{-1} due to a carbonyl function. MS showed a medium intensity molecular ion peak at m/z 472.35097, corresponding to molecular formula $C_{30}H_{48}O_4$ (calculated to be 472.35520). The NMR

spectrum (400 MHz) of pridentigenin D ($CDCl_3$) showed five singlets at δ0.78 (3H,s,24-Me), δ0.88 (3H,s,26-Me), δ0.94 (3H,s,25-Me), δ0.97 (3H,s,29-Me), and δ1.05 (3H,s,23-Me), and δ1.23 (3H,s,27-Me), representing six methyl groups. The double doublet at δ3.19 (J=11.5 Hz, 5.5 Hz) was due to H-3 and therefore the compound had a 3-β-OH. There were two doublets at δ2.14 and δ2.71 (J=16 Hz) assigned to the two H-15 protons in the vicinity of carbonyl group at C-16. The higher field doublet showed at further long range coupling (J=2 Hz) with a methyl group, and each wing was split into a quartet. A double doublet at δ3.47 and δ3.87 (J=8 Hz each) was assigned to the two H-30 protons, which were magnetically nonequivalent, indicating a hindered rotation of the C(20)-C(30) bond. Dreiding model showed that the hinderance in rotation was possible only if the OH group would be attached to C-30 and not to C-29. There was no signal due to olefinic protons. Pridentigenin D was found to form diacetate with a molecular peak at m/z 556. On the basis of the spectral data, structure III was proposed for pridentigenin D.

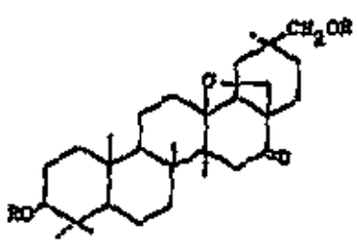


(I) Pridentigenin A
a,R=H
b,R=Ac

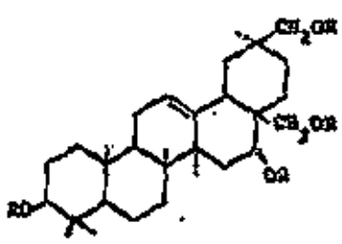


(II) Pridentigenin B
a,R=H
b,R=Ac

Pridentigenin C (epimer)



(III) Pridentigenin D
a,R=H
b,R=Ac



(IV) Pridentigenin E
a,R=H
b,R=Ac

Pridentigenin B

Pridentigenin B (IV), crystallized from methanol, had m.p. of 268-270 C and was analyzed for $C_{30}H_{50}O_4$. *MS* showed a molecular ion peak at m/z 476. The NMR spectrum in C_5H_5N showed four signal in the upper field which corresponded to six methyl groups at δ1.08 (6H,2xCH_3), δ1.2 (3H,1xCH_3), δ1.31 (3H,1xCH_3), and δ1.84 (6H,2xCH_3). Two signals at δ3.71 and δ3.97 corresponded to four protons due to CH_2OH; the latter signal was assigned to the functional group at C-28. A broad signal at δ4.64 was due to a vinyl proton at C-12. From NMR and other spectral data, it was concluded that pridentigenin B was a pentacyclic triterpenoid. The NMR spectrum showed six methyl groups without coupling. MS fragmentation pattern, in which the fragment ion at m/z 203 was stronger than the peak at m/z 191, showed that pridentigenin B was a β-amyrin type of triterpene (9). It was suggested that pridentigenin B had the structure shown (IV), which is identical to dihydrocyclamiratin D, prepared by Barton *et al.* (13) and Tschesche (14). The compound had also been isolated by Ito *et al.* (15) from *Cyclamen europea*. Recently, some of the saponins from *Primula denticulata* have also been elucidated.

CARALLUMA TUBERCULATA

The pregnane derivatives occur in the members of the Asclepiadaceae family. Some of the plants belonging to this family are reputed for their poisonous property; in Pakistan, these include *Calotropis procera, Sarcostemma stocksii*, and *Leptadenia pyrotechnica*. There is no doubt that many plants of Asclepiadaceae found in Pakistan possess acrid and poisonous properties, but no definite information is available about their toxicity. *Caralluma edulis* and *C. tuberculata* are also mentioned.

Very little information is available on the toxicity of pregnane derivatives of plant origin. The derivatives of the pregnane nucleus having an amino substituent at either C-3 or C-20 or both (*i.e.*, funtumine, irehine, and kurchessine) have received considerable attention. Funtumine has a molecular formula of $C_{21}H_{35}NO$ and weight of 317.50; funtumidine has a molecular formula of $C_{21}H_{37}N_3$ (16). 3-amino-5α-pregnane-20α-ol has an LD_{50} (iv) in mice of 21 mg/kg.

We investigated *Caralluma tuberculata*. In 1967, Nikaido *et al.* (17) reported the isolation and structure elucidation of two genins, boucerin (X) and dihydroboucerin (XI), from *Boucerosia aucherlana*, a synonym of *C. tuberculata*. The alcohol extract (Figure 2) of the whole plant of *C. tuberculata* yielded, after evaporation, fractionation, and repeated chromatographic separations on silica gel columns, a mixture of caratubersides A-D. This was separated by preparative HPLC employing a Bond-Pak C_{18} reversed phase column, with MeOH-H_2O (80/20) as the mobile phase.

Caratuberside A

Caratuberside A (V) was obtained as a white, crystalline substance, m.p. 170-171 C, $(\alpha)^{20}D$+60 o (c=0.66 in MeOH). On acidic hydrolysis

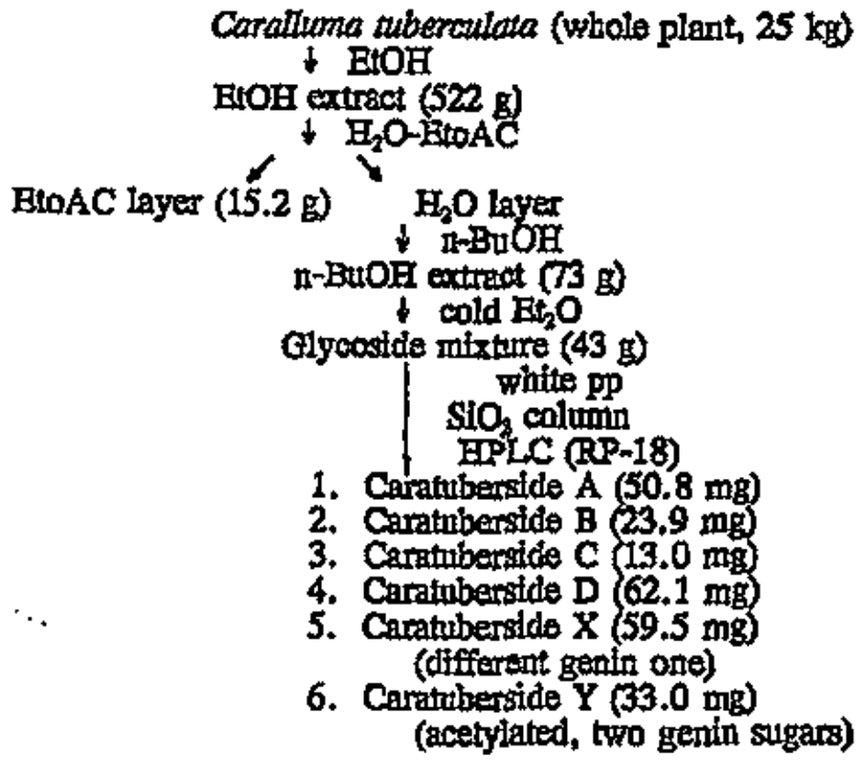


FIGURE 2
Extraction of *Caralluma tuberculata*.

with 1% HCl, caratuberside A yielded a crystalline genin as well as glucose and another sugar. Glucose was identified by TLC and HPLC. The second sugar was determined to be 6-deoxy-3-O-methyl-β-D-galactose on the basis of extensive NMR studies. Caratuberside A displayed no absorption in the UV region, indicating the absence of any conjugated double bonds in the molecule. The IR spectrum (KBr) revealed peaks at λ_{max} 3300 (OH), 2900 (CH_2), 1690 (C=O), and 1100 (C-O) cm^{-1}. In the negative ion FABMS, the compound showed an (M-H) peak at m/z 655. In the EIMS spectrum, the highest peak at m/z 477 was due to the removal of the terminal glucose unit, indicating the 6-deoxy-3-O-methyl-D-galactose is directly attached to the aglycone. There was also a strong peak at m/z 317 due to the aglycone without oxygen at C-3 and a base peak at m/z 299 $(317-H_2O)^+$.

The aglycone was obtained as a crystalline compound by acidic hydrolysis, and it structure was confirmed by spectroscopy. Because *Caralluma tuberculata* belongs to the Asclepiadaceae, which are know to contain pregnane glycosides, and because the pregnane genins boucerin and dihydroboucerin have been isolated from the acid hydrolysate of the glycosides of this plant, it was expected that the aglycone could possibly be a pregnane-type steroid. The ^1H- and ^{13}C-NMR spectrum of caratuberside A confirmed this assumption. However, the ^{13}C-NMR spectrum showed that an oxygen function at C-12, commonly found in

Asclepiadaceae, is missing (C-12, 39.44 ppm). The chemical shift of C-13 at 49.49 ppm with only one hydroxyl-bearing vicinal carbon atom (C-14, 84.76 ppm) and the chemical shift of C-11 at 21.63 ppm also support the absence of oxygen function in the neighboring C-12. This is further confirmed in the mass spectral data discussed above.

The ^{1}H-NMR of caratuberside A in CD_3OD reveals methyl singlets at δ0.83 (3H,H-10), δ1.08 (3H,H-18), and δ2.33 (3H,H-21). The last peak indicates the presence of a $COCH_3$ group in the compound. A doublet at δ1.28 (3H,J=6Hz) is assigned to the secondary methyl group in the 6-deoxy sugar, and a singlet at δ3.49 is assigned to the OMe group of the methylated sugar. A triplet at δ2.90 (J=5.6 Hz) can be assigned to H-17 vicinal to the ketonic group. This chemical shift and coupling are very close to those reported for H-17 in 13β,14-dihydroxy-14β-pregn-5-en-20-one (18). Therefore, we concluded that the configuration of caratuberside A at C-17 is the same as in this latter compound, *i.e.*, the side chain at C-17 is β-oriented. The spectrum further shows two anomeric proton signals at 4.35 (d,J=7.7 Hz) and 4.60 (J=7.6 Hz). The latter is assigned to the anomeric proton of glucose, whereas the former is assigned to the H-1 signal of the methylated 6-deoxy sugar. In both cases, the coupling constants indicate diaxial coupling, hence β-glycosidic linkages. It also proves that the hydroxylic groups at the neighboring carbon atoms in the two sugars (C-2' and C-2") have the same equatorial configuration. It may be noted here that the Keller-Killiani test for 2-desoxy sugars gave negative results with caratuberside A. This means that the 6-desoxy sugar possessed an OH group at C-2'. Decoupling experiments, as well as COSY-45 experiments, show that the H-1' doublet at δ4.35 is coupled with a double doublet at δ3.59 (J=7.7,10 Hz) due to H-2'. This means that J-2',3' is equal to 10 Hz, and therefore, H-3' must also be axially oriented. The H-3' signal becomes clear only in the ^{1}H-NMR spectrum of caratuberside A pentaacetate where this signal is not shifted downfield because it bears a methoxy rather then a hydroxy group. It absorbs as a double doublet at δ3.23 (J=10,2.5 Hz), which indicates that the methoxy group at C-3' is equatorial and H-3' is axial. The smaller J-3',4' (2.5 Hz) also shows that H-4' is equatorial. H-4' absorbs as a broad doublet at δ4.17; the J-4',5' is very small and could not be calculated. H-5' absorbs as a broad quartet at δ3.65 (J=6.3 Hz) which is coupled to the doublet at δ1.28 as shown by the decoupling experiment or irradiation of the methyl doublet at δ1.28 when this quartet is converted into a broad singlet.

The configuration of the methyl group (H-6') was determined by nOe difference measurements of caratuberside A pentaacetate. Irradiation of the H-5' quartet at δ3.49 resulted in 13.6% increase of the signal at δ3.2 (H-3') and 10.2 % increase in the intensity of the doublet at δ4.37 (H-1'). The intensity of the methyl doublet at δ1.2 also increased by 19.3%. On the other hand, irradiation of the H-3' double doublet at δ3.2 led to 5.6% nOe of H-1' at 4.37 but no increase in the intensity of methyl doublet at δ1.2. This indicates H-1', H-3', and H-5' are axial and 6-Me

is equatorial. Thus, the methylated sugar was identified as 6-deoxy-3-O-methyl-D-galactose. The ^{13}C-NMR spectrum of caratuberside A supports its proposed structure (V). It clearly shows the absence of olefinic carbon atoms, indicating that there is no carbon double bond in the compound. The assignments were aided by DEPT experiments as well as correlation with ^{13}C-NMR spectra of similar types of compounds (18).

Caratuberside B

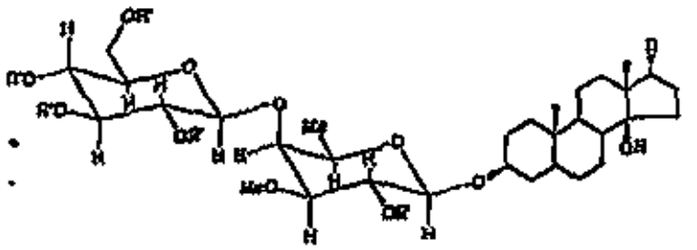
Caratuberside B (VI) was also obtained as a white crystalline compound, m.p.182 C (dec.), which on acidic hydrolysis gave a pure genin as well as glucose and 6-deoxy-3-O-methyl-β-D-galactose. Caratuberside B does not show a carbonyl absorption in the IR spectrum. In the ^{13}C-NMR spectrum, the peak assigned to the carbonyl carbon (C-20) of caratuberside A is missing. Instead, a peak at 65.30 ppm is present, indicating an additional hydroxyl-bearing carbon which, according to the DEPT spectrum, is secondary. These observations, together with the $(M-H)^+$ peak at m/z 657 in the negative ion FAB spectrum, suggested that the carbonyl groups in caratuberside A has been reduced to a secondary alcohol in caratuberside B. This is supported by the observation that the peak at 63.01 ppm (C-17) is shifted upfield to 57.08 ppm and the C-21 methyl peak from 32.37 ppm to 21.53 ppm in caratuberside B. The correlation between caratubersides A and B was confirmed by reduction of V to VI with $NaBH_4$. Thus, the structure of caratubersIde B is proved to be 3-O-(B-D-gluco-pyranosyl)-(1→4)-β-D-(3-O-methyl-6-desoxygalactopyranosyl)-14,20-dihydroxy-14β-pregnane(19).

Caratuberside C

Caratuberside C (IX) was isolated and the structure of the aglycone was found to be similar to that of caratuberside A except that in the case of caratuberside C, it had three units of sugar, two of which were observed to be glucose and the third as 6-dexyl-3-O-methylgalactose. In addition, caratuberside C was reduced with $NaBH_4$ and this compound was found to be similar to that of caratuberside D. On this basis, and on spectral data, the structure was suggested to be 3-O-(β-D-glucopyranosyl-(1→4)-3-O-(β-D-glucopyranosyl-(1→4)-β-D-(3-O-methyl-6-deoxy)-galactopyranosyl)-14-hydroxy-14β-pregnane-2-one.

Caratuberside D

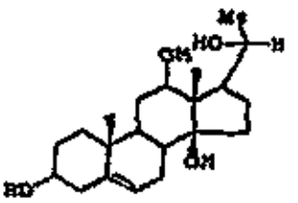
Caratuberside D (VIII) was obtained as white crystalline needles, m.p. 266.8 C(dec.), which on acid hydrolysis yielded an aglycone moiety along with two unites of glucose and one unit of 6-deoxy-3-O-methyl-D-galactose. Caratuberside D does not exhibit any carbonyl absorption in the IR scanning. The peak due to the carbonyl carbon at C-20 is missing in the ^{13}C-NMR spectrum; a peak at 65.31 ppm is present, owing to the presence of OH group. The spectral characterization was concluded by suggesting that caratuberside D can be best represented as 3-O-(β-D-glucopyranosyl)-(1→4)-3-O-(β-D-gluco-pyranosyl)-(1→4)-β-D-(3-O-methyl-6-deoxygalactopyranosyl)-14,21-dihydroxy-14β-pregnane.



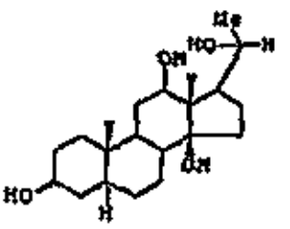
(V) Castanoside A, R=CCH_3, R'=H

(VI) Castanoside B, R=$CH(OH)CH_3$, R'=H

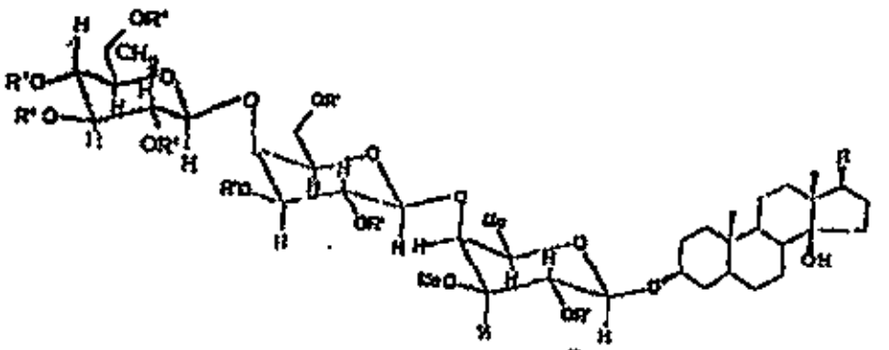
(VII) R=CCH_3, R'=Ac



(VIII) Soyasapogenin



(IX) Dihydrobarringtogenol



(X) Castanoside D, R=CCH_3, R'=H

(XI) Castanoside C, R=$CH(OH)CH_3$, R'=H

CASTANOSPERMUM AUSTRALE

Castanospermum australe has been classified as poisonous in many literature citations (20,21). Pods of *C. australe* are extremely poisonous and the entire plant demonstrated toxic effects when fed to cattle and horses. The chemical literature survey revealed the isolation of castanogenin (22), bayogenin (23,24), and bayin (8C-β-D-glucopyranosyl-7-4'-dihydroxy flavone) (25) from wood; castanogenol (triterpene, sapogenin) (26) from bark; and castanospermine (1,6,7,8-tetrahydroxy-octa-hydroindolizine alkaloid) (27) from seeds. The alkaloid constitutes approximately 0.1% of the dry seed weight and 0.05% of the dry leaf weight. In the literature, it was suspected that poisoning may be due to

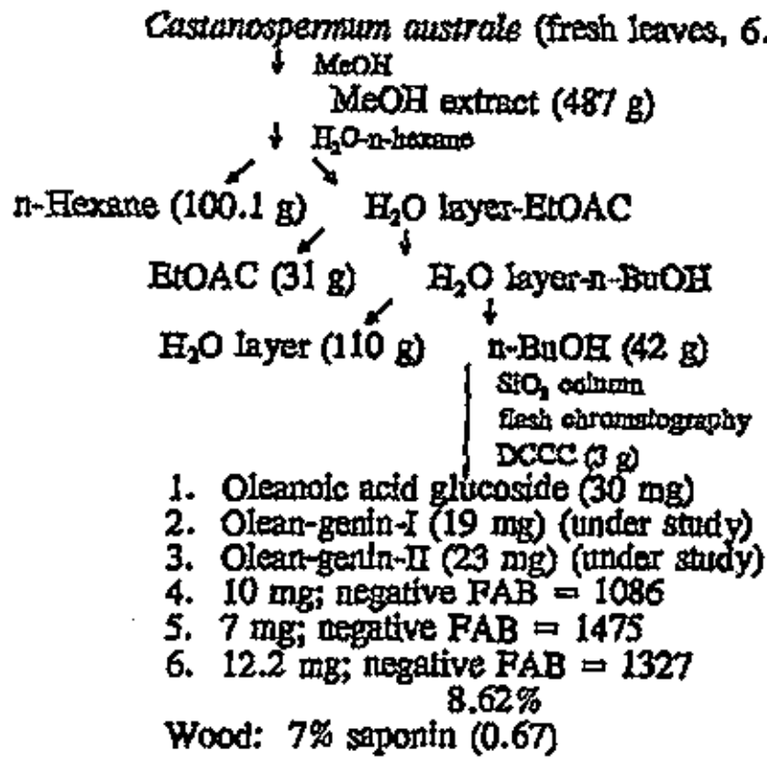


FIGURE 3
Methanolic extraction of *Castanospermum australe* leaf.

the saponin and indolizidine contents. A detailed work on castanospermine has appeared in the proceedings of the 1984 Australia-U.S.A. Poisonous Plant Symposium (28). Therefore, an attempt has been made to isolate and characterize saponins from leaves.

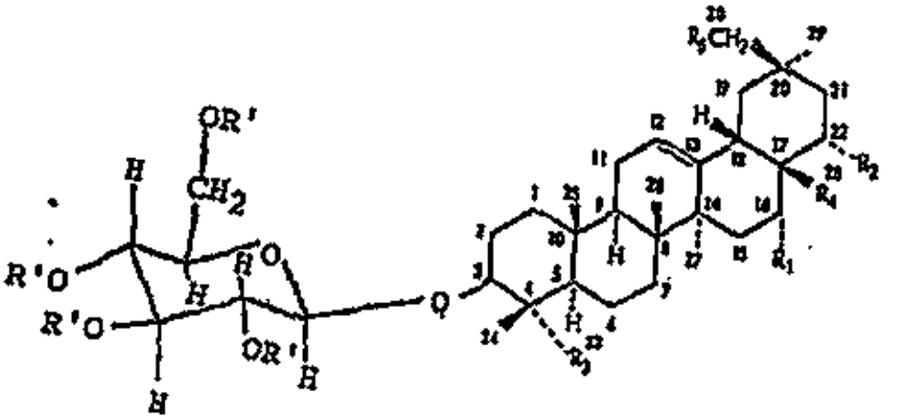
Oleanolic Acid Glucoside

The methanolic extract of the fresh leaves (Figure 3), after removal of neutral substances through ethyl acetate, was partitioned with n-butanol. After column and flash column chromatography, it afforded the major constituent (XII). On the basis of chemical evidence and spectral data (negative ion FABMS, CMR, and PMR), it was concluded that the compound was 3-O-(β-D-glucopyranosyl)-olean-12-en-28-oic acid.

TOXICITY TESTING

The toxicity of *Primula denticulata*, *Castanospermum australe*, and *Caralluma tuberculata* was determined by LD_{50} studies in mice. Ethanolic or methanolic extracts and total glycosides were given to groups of five mice each in a geometrical progression: 200, 300, 400, 500, 600, and 700 mg per kg by oral administration and 20, 25, 30, 35, 50, 75, and 100 mg per kg ip. The results are in Table 1.

In the case of *Caralluma tuberculata*, our results agree with the results of the toxicity evaluation on *Caralluma penicillata* (29). The



(XII) Oleanolic acid glucoside

results on the triterpenoid saponin mixture obtained from *P. denticulata* and *Castanospermum australe* differ somewhat from the LD_{50} values afforded by saponins of *Aralia manschurica* (150 mg/kg) (30); *Momordica chinensis* (37 mg/kg); *Anagallis arvensis* (675 mg/kg os and 30.4 mg/kg sc); and *Sapindus mukorossi* (1675 mg/kg os, 650 mg/kg iv, and 276 mg/kg ip) (31).

TABLE 1
LD_{50}s of *Primula denticulata*, *Castanospermum australe*, and *Caralluma tuberculata* in mice

Plant	Extract EtOH/MeOH Oral (mg/kg)	Glycosides Saponins/Pregnanes ip (mg/kg)
Primula denticulata	400	35
Castanospermum australe	400	30
Caralluma tuberculata	600	75

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L. F. JAMES
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Poisonous Plant Research Laboratory
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United States Department of Agriculture
Logan, Utah

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BIOLOGICAL EFFICACY OF THE EXTRACTS AND CONSTITUENTS OF Caralluma tuberculata AND C. edulis

Ghazala H. RIZWANI*°, K. USMANGHANI*, Mansoor AHMAD*, Shahid RASHID°°, Viqar Uddin AHMAD*°°

Key Word Index :

Caralluma tuberculata, Caralluma edulis, Asclepiadaceae, toxicity, hypoglycaemic, cardiovascular, antibacterial, platelet activities.

ABSTRACT

Biological activity of the ethanolic and aqueous extracts of both plants as well as caratuberosides were evaluated. Brine shrimp (LC_{50}) bioassay of ethanolic and ethyl acetate extracts of both plants showed toxicity whereas aqueous extract was found to be non-toxic. The aqueous extract of Caralluma tuberculata was found to possess hypoglycaemic activity in alloxan treated rats. However, ^{3}H-ouabain binding assay with caratuberosides A-C, and an inhibitory effect of ethanolic extract of C. edulis on adrenaline induced platelet aggregation essentially exhibited no response.

INTRODUCTION

Caralluma tuberculata N.E. Brown, and C. edulis Edgew (Asclepiadaceae) succulent perennial herbs are widely used as

(*) Department of Pharmacognosy, Faculty of Pharmacy University of Karachi, Karachi 75270 PAKİSTAN.

(**) Dept. of Pharmacology.

(***) H.E.J. Research Institute of Chemistry, University of Karachi.

antidiabetic. In the previous communications[1-4], we have already reported the active constituents including the caratubersides from this plant.

RESULTS AND DISCUSSION

The pharmacological activities of the extract and its chemical components were tested. The ethanolic extracts of C. tuberculata, containing pregnane glycosides, were subjected to different biological evaluation tests including the brine shrimp bioassay, hypoglycaemic activity, and cardiovascular profile. Moreover, pregnane-^{3}H-ouabain binding assay of the isolated pregnane glycosides from C. tuberculata, inhibition of, epinephrine-induced platelet aggregation on the ethanolic extract of C. edulis, were also investigated and reported in this communication.

Brine shrimp (LC_{50}) bioassay method was used to testify the efficacy of the extracts of C. tuberculata. Three fractions (i.e., ethanolic, ethyl acetate and aqueous) were subjected to brine shrimp bioassay as shown in Table-1. Ethanolic and ethyl acetate fractions displayed 35.36 LC_{50} at a concentration of 10, 100 and 1000µg/ml, while non-toxic effect (>1000 µg/ml) was observed in aqueous fraction. In other words we can say that it is a neutral fraction of C. tuberculata, the brine shrimp (LC_{50}) bioassay of ethanolic and ethyl acetate extract showed toxicity whereas aqueous extract was found to be non-toxic.

Hypoglycaemic activity of C. tuberculata ethanolic and aqueous extract was tested by determining the blood sugar lowering effect. The ethanolic and aqueous extracts of C. tuberculata showed hypoglycaemic activity at a dose of 71.42 mg/kg in alloxan fed diabetic male albino rats. The blood sugar level was reduced from 278.61 mg/100 ml to 248.37 mg/100 ml in ethanolic extract treated diabetic rats, while in aqueous extract treated rats blood sugar level was lowered from 317.63 mg/100 ml to 295.64 mg/100 ml after 30 to 60 minutes of extract administration, and these extracts raised the blood sugar after 3 hours. In aqueous extract treated rats the blood sugar level was significantly reduced from 314.63 mg/100 ml to 233.62 mg/100 ml after 24 hours. The fasting blood sugar in normal ethanolic extract treated rats was raised from

Table 1. Brine Shrimp Bioassay of Ethanolic, Ethyl Acetate And Aqueous Extracts of Caralluma tuberculata.

Types of extract	10 µg/ml		100 µg/ml		1000 µg/ml		Concentration LC_{50} µg/ml
	alive	death	alive	death	alive	death	
1 Ethanolic	16	14	5	25	—	30	35.3693
2 Ethyl acetate	26	4	5	25	—	30	8.9389
3 Aqueous	32	—	30	—	26	4	>1000

85.85 mg/100 ml to 138.46 mg/100 ml at 3-3.5 hours and 177.63 mg 100 ml after 24 hours.

The aqueous extract treated normal rats also showed similar effects at the same time intervals. Results of the mean blood glucose level in normal and alloxan fed rats are shown in Table-2. Among the two models used, the plant extracts showed a significant (<0.005) blood sugar lowering potency in fasted and alloxan fed models.

Akhtar[5] worked on the antidiabetic activity of C. edulis, and found that it does not lower the blood sugar level in rabbits. Whereas Ahmad et al[6]. reported that C. tuberculata aqueous extract significantly reduced the blood glucose level. However, the results of our experiments proved that it possess an antidiabetic activity in albino rats.

The results of the effects of ethanolic extract of C. edulis (i) on force of contraction on left ventricle, (ii) heart rate, (iii) coronary flow on isolated perfused rabbit heart were (in doses between 0.12 - 1000 µg) found to possess no effect on the cardiovascular system in terms of change of the contractile force, cardiac frequency and coronary out flow during one minute after administration (Table 3). It seems that the over all effect of the crude extract of C. edulis on these parameters is rather insignificant.

Table 2. Mean Blood Glucose Levels mg/100 ml ± S.E.M. of Normal And Alloxan-Diabetic Rats At Vartous Time Intervals After Oral Administration of Caralluma tuberculata N.E. Brown.

uralluma berculata xtract o. of periments = 4	Normal albino rats				No. of experiments n = 4	Alloxan-Diabetic albino rats			
	B.Adm.	0 h	3 h	24 h		B.Adm.	0 h	3 h	24 h
ntrol	92.12 (±3.13)	100.75 (±1.71)	80.75 (±1.56)	115.16 (±1.87)		608.65 (±7.62)	488.25 (±10.19)	544.48 (±3.47)	742.86 (±6.34)
hanolic tract	85.85 (±1.99)	138.46** (±3.18)	142.63*** (±1.83)	177.63* (±1.90)		278.61*** (±4.34)	248.37*** (±3.27)	270.51*** (±2.60)	285.72*** (±2.83)
ueous tract	111.92* (±2.61)	142.54** (±2.19)	138.29*** (±2.14)	146.60 (±2.63)		317.63*** (±3.57)	295.64*** (±5.48)	314.22*** (±3.00)	233.62*** (±4.10)

Adm. = Before drug administration

, 3 h, 24 h = After 0, 3, 24 hours drug administration

l values represent glucose level in mg/100 ml as mean ± S.E.M.

gnificant relative to control *P<0.1, **P<0.01, ***P<0.005, n/ group = 4.

Table 3. Effect of Aqueous Extract of Caralluma Edulis On Force of Cardiac Contraction, Cardiac Frequency And Coronary Out Flow of Isolated Perfused Rabbit Herat.

	Percent change from the control		
Dose (μg)	Inotropic effect	Chronotropic effect	Effect on coronary out flow
0.1-1000	8.5 ± 1.54	18.2 ± 1.17	19.25 ± 2.46

n = 4

The results were expressed as mean ± standard error (S.E.M.).

Table 4. ^{3}H-Qubain-Binding Assay With Caratuberosides.

Compound	IC_{50} (μM)
Caratuberoside A	260
Caratuberoside B	270
Caratuberoside C	580
5A	7600
Caratuberoside A + B	91
Progestrone	56
Quabain	0.012

The strength to this contention has been kindly provided by Professor LaBella, who very kindly tested the pregnane glycosides isolated from C. tuberculata and C. edulis as shown in Table 4. The isolated pregnanes caratuberoside A, caratuberoside B, caratu-

Table 5. Effects On Aqueous Extract of Caralluma Edulis On Adrenaline Induced Aggregation of Human Platelets.

	% inhibition of adrenaline induced platelet aggregation			IC_{50} mg/ml
	Dose A (0.625 mg/ml)	Dose B (1.25 mg/ml)	Dose C (2.50 mg/ml)	
[aqueous (n = 4)]	-8.22 ± 5.96*	-0.82 ± 7.33*	7.81 ± 8.88*	

The results were expressed as mean ± standard error (S.E.M.).

*Not significantly different from control (P>0.05).

beroside C, and a mixture of caratuberoside A and B in ^{3}H-Ouabain-binding assay were found to be essentially inactive.

The aqueous extract of C. edulis was tested on adrenaline induced aggregation of human platelets. In n = 4 experiments, the dose A (0.625 mg/ml) showed -8.22 ± 5.96 % inhibition of adrenaline induced platelets aggregation, while dose B (1.25 mg/ml) -0.82 ± 7.33, and dose C (2.50 mg/ml) 7.81 ± 8.88. All these results were not significantly different from control (P>0.05). The extract alone, however, have no direct aggregatory effect (Table 5).

On these biological evaluation findings it is therefore concluded that caratuberoside molecules are basically inactive. Nonetheless the aqueous extract of C. tuberculata showed hypoglycaemic activity, and that is why this herb drug is used for lowering blood sugar level in folkloric medicine.

EXPERIMENTAL

The different extracts and caratuberosides were obtained according to the methods as given in the literature[1-4].

i) Brine Shrimp LC_{50} Bioassay Method :

Three different extracts of C. tuberculata, i.e. ethanolic, ethyl acetate and aqueous extracts were prepared for the estimation of LC_{50} activity in brine shrimp. The procedure described by Meyer et al[7]. and followed by McLaughlin et al[8]. and Alkofahi et al[9]. was adopted for this work.

Samples and discs of three different concentrations, 10, 100, 1000 µg/ml were prepared according to the direction as given in the literature[7-9]. Brine shrimp (Artemia salina) nauplii were hatched in a specific tank. Ten shrimps were transferred to each sample vial, and then sea water was added to make the volume 5 ml. Later on dry yeast suspension was added as food to each vial including control. The vials were kept for 24 hours, thereafter the active nauplii were counted, and death percentage was calculated at each dose.

ii) Hypoglycaemic Effects :

The ethanolic (EE) and aqueous (AE) extracts of C. tuberculata were diluted in distilled water, respectively, and then were evaluated for the hypoglycaemic activity in both normal and alloxan treated male albino rats of 200-250 g weight. The hyperglycaemia was induced by administering alloxan monohydrate (Ogawa Seiki Co. Ltd., Tokyo, Japan) (150 mg/kg, i.p. body weight). Rats were maintained on diet soaked with hydrogenated oil.

The ethanolic (EE) and aqueous (AE) extracts were administered at a dose of 100 mg/kg dissolved in water. The blood glucose levels were estimated by the method of Nelson and Somogyi as given by Varley[10] on 0.1 ml of blood drawn from animals at 0,3 and 24 hours' interval. The results were recorded as shown in the Table 2.

iii) Cardiovascular Profile :

The whole plant material of C. edulis was collected from Karachi in September 1988. Fresh plant material after chopping was soaked in EtOH for about four weeks. The alcoholic extract was evaporated under reduced pressure and the syrupy residue so obtained

was dissolved in small quantity of H_2O and subjected to freeze drying. 25 g powder of the crude extract so obtained was used for cardiovascular screening.

In this study, isolated perfused rabbit's heart method as described by Langendroff[11] with modification was used[12]. Briefly healthy rabbits (1-2 kg) of either sex were used. A blow on the neck of the rabbit made them unconcious. The chest was open and the heart was carefully removed in a petridish filled with McEwen's solution (NaCl : 7.60 g/l, KCl : 0.42 g/l, $CaCl_2$: 0.24 g/l, $NaHCO_3$: 2.10 g/l, glucose : 2.00 g/l, sucrose 4.50 g/l), and bubbled with oxygen. Isolated heart was mounted on the apparatus with the help of glass cannula and perfused with McEven's solution at 37°C. Recordings were made by the help of isotonic transducer (Harvard) on chart recorder (Eyla). Parameter measured were : (1) force of contraction (height in mm), (2) heart rate (beats/min), (3) coronary flow (ml/min). Atmospheric pressure on the heart was kept at 45 mm Hg, approximately. No changes weer introduced during these experiments (Fig. 1, Table 3).

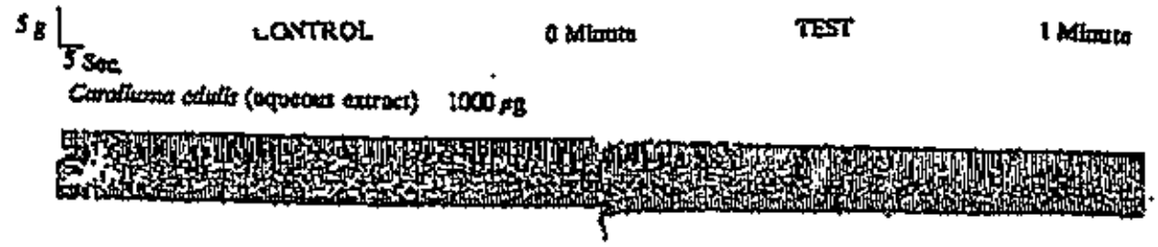

Fig. 1 : Effects of aqueous plant extract (Caralluma edulis) on spontaneous contractions of isolated perfused rabbit heart. A constant dose of 1000 µg was used for all experiments. Dose administration occurred at 0 minute.

iv) Pregnane ^{3}H-Ouabain Binding Assay :

Prof. Dr. LaBella[13] was kind enough to carry out the isolated pregnanes caratuberoside A caratuberoside B caratuberoside C and a mixture of caratuberoside A and B in ^{3}H-Ouabain binding assay (Table 4).

v) Inhibition of Epinephrine Induced Platelet Aggregation :

The experiments on the ethanolic extract of C. edulis for its inhibition of platelet aggregation property were performed by the method of adrenaline (epinephrine) induced platelet aggregation[14, 15].

Preparation of the test solution :

Plant extract was dissolved in 0.2 % w/v sodium carbonate (E. Merck, Germany) to provide a stock solution of 60 mg/ml (c). Subsequent dilutions of 30 mg/ml^{-1} (b) and 15 mg/ml (a) were prepared. About 10 µl of each solution was added to 225 µl of platelet rich plasma, sot hat the final concentrations were 2.5 mg/ml (Conc. C) 1.25 mg/ml (Conc. B) and 0.625 mg/ml (Conc. A), respectively. The pH of the solution C was noted using a pH paper (Machery-Nagel Co., Germany). The test solutions A, B and C were freshly prepared for all experiments.

Preparation of aggregating agent :

A 4 mM solution of adrenaline bitartarate (MW = 333.33, Sigma, St. Louis, USA) was prepared in 0.9 % w/v sodium chloride in distiled water. After use, it was stored at -20°C.

Platelet rich plasma (PRP) and Platelet poor plasma (PPP) :

Platelet rich plasma (PRP) was prepared from whole blood taken from a healthy person, who has denied receiving any medicine within 14 days, by centrifugation for 10 minutes at 1200 rpm at room temperature (20-25°C) in HIMAC centrifuge (Hitachi, Japan) HIMAC and was used within 3 hours after blood collection. The settled portion of blood, from which PRP had been removed, was then centrifuged at 3200 rpm for 15 minutes and the supernatant PPP was obtained.

Platelet aggregation :

Platelet aggregation was perfomed in NKK Hema Tracer (Model PAT 4M, Niko Bioscience Inc., Japan) equipped with a four channel chart recorder. The instrument was first calibrated with PRP at zero % and PPP at 100 % light transmission.

Incubation with test substances and aggregations were carried out at 37°C with continuous stirring at 1000 rpm. About 10 µl of test solution 'A' (15 mg/ml), 'B' (30 mg/ml) and 'C' (60 mg/ml) were added to curvettes containing 225 µl PRP and allowed to incubata for about 2 minutes before challenging with 5 µl of 4 mM adrenaline solution. The control response was also measured along with the response of conc. A (0.625 mg/ml), conc. B (1.25 mg/ml) and conc. C (2.5 mg/ml) for a period of 5 minutes (Fig. 2, Table 5).

For each experiment the height in chart units was measured at the end of the fifth minute and determined and the results were expressed as percentage inhibition from the control, according to the following formula :

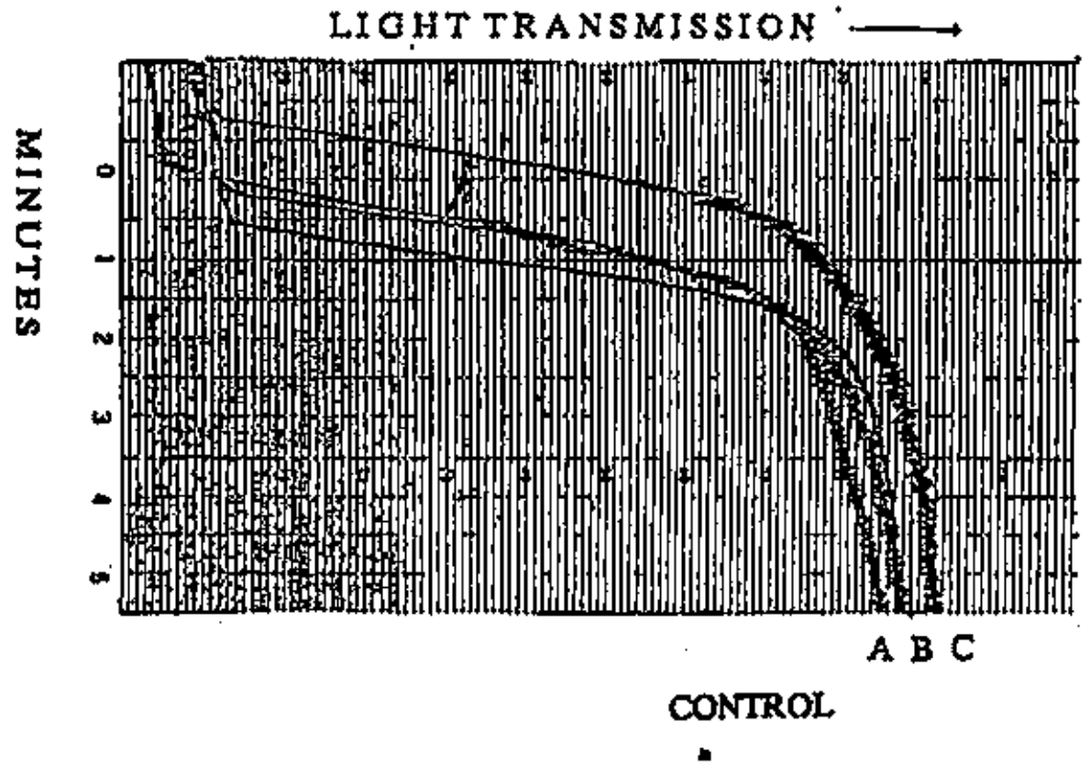

Fig. 2 : Representative tracing showing the effect of concentration A (0.625 mg/ml), B (1.25 mg/ml) and C (2.5 mg/ml) of aqueous extract of Caralluma edulis (upper panel) on adrenaline-induced aggregation of human platelets. RRP was incubated for 2 minutes with the plant extract before adding adrenaline (4 mM).

$$\% \text{ inhibition} = \frac{C - T}{3} \times 100\ \%$$

where C = % aggregation of the control.

T = % Aggregation after 2 minutes incubation with test solution.

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NEW PREGNANE GLYCOSIDES FROM *CARALLUMA TUBERCULATA*

VIQAR UDDIN AHMAD,*

H.E.J. Research Institute of Chemistry

KHAN USMANGHANI, and GHAZALA H. RIZWANI

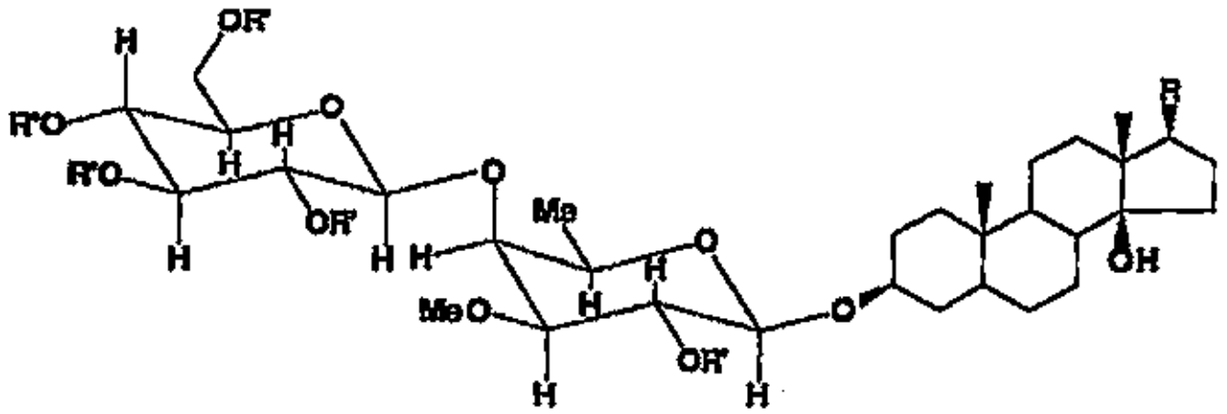
Department of Pharmacognosy, Faculty of Pharmacy, University of Karachi, Karachi-32, Pakistan

ABSTRACT.—Two new pregnane glycosides, named caratubersides A and B, were isolated from the whole plant of *Caralluma tuberculata*. Their structures were determined as 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxy)-galactopyranosyl]-14-hydroxy-14β-pregnane-20-one [1] and 3-*O*-[β-D-glucopyranosyl-(1→4)-β-D-(3-*O*-methyl-6-deoxygalactopyranosyl)]-14,20-dihydroxy-14β-pregnane [2] mainly on the basis of spectroscopic studies. Their stereochemistry has been determined by nOe difference, COSY 45, and 2D *J*-resolved NOESY ^{1}H-nmr and DEPT ^{13}C-nmr experiments.

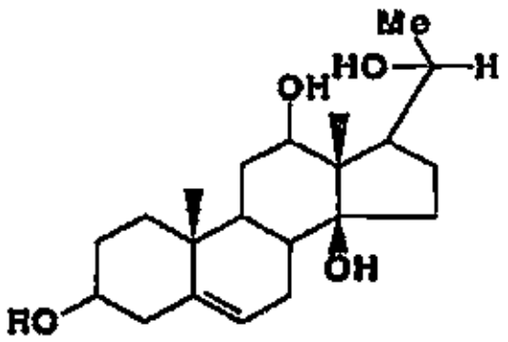
Caralluma tuberculata N.E. Brown (Asclepiadaceae) (syn. *Boucerosia aucheriana* Decn.) is a succulent perennial herb occurring wild and also cultivated throughout Pakistan (1–3). This plant is eaten raw or cooked as a vegetable and is also reputed to be a cure for diabetes and rheumatism (4). In 1967, Nikaido *et al.* (5) reported the isolation and structure elucidation of two genins, boucerin [4] and dihydroboucerin [5], from *B. aucheriana* Decn., a synonym of *C. tuberculata*. After hydrolysis of the glycosides, these authors also identified cymarose, sarmentose, oleandrose, and digitoxose in the hydrolysate. Apart from this work no other chemical investigation on this species is reported in the literature. This paper presents the isolation and characterization of two new pregnane glycosides.

RESULTS AND DISCUSSION

The EtOH extract of the whole plant of *C. tuberculata* yielded, after evaporation, fractionation, and repeated chromatographic separation on Si gel columns, a mixture of **1** and **2**. The mixture gave positive tests with Liebermann-Burchard reagent (for sterols), vanillin sulfate (for genins), and Molisch reagent (for sugars). Glycosides **1** and **2** were separated by preparative hplc employing a Bond-Pak C-18 reversed-phase column, with MeOH-H_2O (80:20) as the mobile phase.



1 R=-CCH_3, R'=H
2 R=-$CH(OH)CH_3$, R'=H



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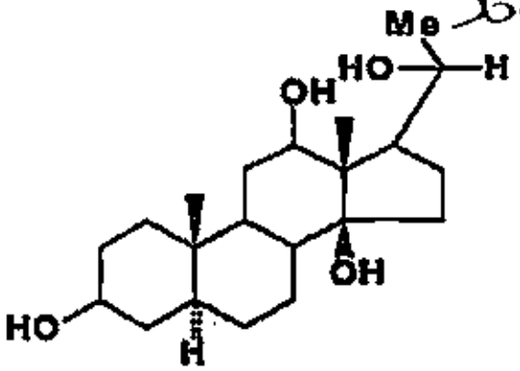

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Caratubenside A [1] was obtained as a white, crystalline substance, mp 170–171°, $[\alpha]^{20}D$ +60° (c = 0.66 in MeOH). On acidic hydrolysis with 1% HCl, 1 yielded a crystalline genin as well as glucose and another sugar. Glucose was identified by tlc as well as hplc. The structure of the second sugar was determined as 6-deoxy-3-*O*-methyl-β-D-galactose on the basis of extensive nmr studies including ^{1}H nmr (COSY-45, *J*-resolved, NOESY, nOe difference) and ^{13}C nmr (broad band and DEPT experiments) on 1 as described below.

Caratubenside A [1] displayed no absorption in the uv region indicating the absence of any conjugated double bonds in the molecule. The ir spectrum (KBr) revealed peaks at max 3300 (OH), 2900 (CH_2), 1690 (C=O), 1100 (C-O) cm^{-1}. In the negative ion fabms the compound showed an $[M-H]^-$ peak at *m/z* 655. In the eims spectrum the highest peak at *m/z* 477 was due to the removal of the terminal glucose unit, indicating the 6-deoxy-3-*O*-methyl-D-galactose is directly attached to the aglycone. There was also a strong peak at *m/z* 317 due to the aglycone without oxygen at C-3 and a base peak at *m/z* 299 $[317-H_2O]^+$.

The aglycone was obtained as a crystalline compound by acidic hydrolysis, and its structure was confirmed by spectroscopy. Because *C. tuberculata* belongs to the Asclepiadaceae, which are known to contain pregnane glycosides, and because the pregnane genins, boucerin and dihydroboucerin, have actually been isolated from the acid hydrolysate of the glycosides of this plant, it was expected that the aglycone could possibly be a pregnane type steroid. The ^{1}H- and ^{13}C-nmr spectra of 1 confirmed this assumption. However, the ^{13}C-nmr spectrum showed that an oxygen function at C-12 commonly found in pregnane aglycones of the Asclepiadaceae is missing (C-12, 39.44 ppm). The chemical shift of C-13 at 49.49 ppm with only one hydroxyl-bearing vicinal carbon atom (C-14, 84.76 ppm) and the chemical shift of C-11 at 21.63 ppm also support the absence of oxygen function in the neighboring C-12. This fact is further confirmed in the mass spectral data discussed above.

The ^{1}H-nmr of 1 in CD_3OD reveals methyl singlets at δ 0.83 (3H, H-19), 1.08 (3H, H-18), and 2.33 (3H, H-21). The last peak indicates the presence of a $COCH_3$ group in the compound. A doublet at δ 1.28 (3H, J = 6 Hz) is assigned to the secondary methyl group in the 6-deoxy sugar, and a singlet at δ 3.49 is assigned to the OMe group of the methylated sugar. A triplet at δ 2.90 (J = 5.6 Hz) can be assigned to H-17 vicinal to the ketonic group. This chemical shift and coupling are very close to those reported for H-17 in 13β,14-dihydroxy-14β-pregn-5-en-20-one (6). It is, therefore, concluded that the configuration of 1 at C-17 is the same as in this latter compound; i.e., the side chain at C-17 is β-oriented. The spectrum further shows two anomeric proton signals at δ 4.35 (d, J = 7.7 Hz) and 4.60 (J = 7.6 Hz). The latter is assigned to the anomeric proton of glucose, whereas the former is assigned to the H-1 signal of the