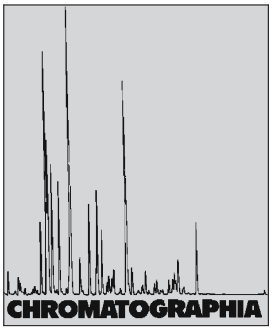
Preparative Droplet Counter-Current Chromatography for the Separation of the New *Nor-Seco*-Triterpene and Pentacyclic Triterpenoids from *Qualea Parviflora*



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Abstract

The methanolic extract of the bark of the medicinal plant *Qualea parviflora* (Vochysiaceae) contains new *nor-seco*-triterpene and pentacyclic triterpenoids. They were separated in a preparative scale using droplet counter-current chromatography. The optimum solvent used was composed of a mixture of $CHCl_3$/MeOH/H_2O (43:37:20, *v/v*) in the descending mode and led to a successful separation of the new compound 28-*nor*-17, 22-*seco*-2α, 3β, 19, 22, 23-pentahydroxy-Δ^{12}-oleanane, besides the known triterpenoids bellericagenin B, bellericaside B and arjunglucoside I . Identification was performed by ESI-MS, ^{1}H NMR and ^{13}C NMR analyses.

Keywords

Thin Layer chromatography
Droplet counter-current chromatography
Nor-seco-triterpene and pentacyclic triterpenoids
Qualea parviflora bark

Introduction

In the search for biologically active compounds from Brazilian plants, we studied the constituents of the bark of *Qualea parviflora* (Vochysiaceae). *Q. parviflora*, popular name "pau-terra-de-flor-miudinha" or "pau-terra-mirim", is a tree found in the American Continent, in the tropical zone. It is used as antiseptic, antiulcerogenic and against gastrointestinal disorders [1].

Previous studies of this species resulted in the identification of fatty acids [2] and several polysaccharides in seeds [3]. Oleanane triterpenoids attract widespread interest because they represent an important group of naturally occurring triterpenoids and many of them exhibit pharmacological properties [4]. Reviews have appeared on the antibacterial properties [5, 6], strong hepatoprotective activities [7], significant cytotoxic activity against cultured P-388 cells and significant, albeit less intense, cytotoxic with a variety of cultured human cancer cells [8] from oleanane triterpenoids.

The fractionation of polar extracts from plant origin is often a difficult task. The separation and purification of secondary metabolites using the conventional methods such as column chromatography and high-performance liquid chromatography (HPLC) require several steps resulting in low recoveries of products. Adsorption chromatographic methods almost always led to irreversible adsorption or decomposition of labile substances. To overcome these problems, we have been using droplet counter-current chromatography (DCCC) [9–11]. The separation is based on a liquid-liquid partition chromatographic method, which does not employ solid supporting matrix [12, 13]. This technique has been successfully applied to the analysis and separation of apolar as well as polar natural products [11, 14–17]. The DCCC technique in this special application was able to separate two triterpene components from two further glycosidally bound triterpene in a single chromatographic run. Applicabilty in a wide range of polarity seems to be the strength of the DCCC methodology in comparison to other CCC techniques. In this case we deal with saponin structures with possible high potential of emulsifying activity. The DCCC as not rapidly moving CCC method seems to be beneficial for this natural product class of saponins [18].

The present paper introduces a method for the separation and purification of 28-*nor*-17, 22-*seco*-2α, 3β, 19, 22,

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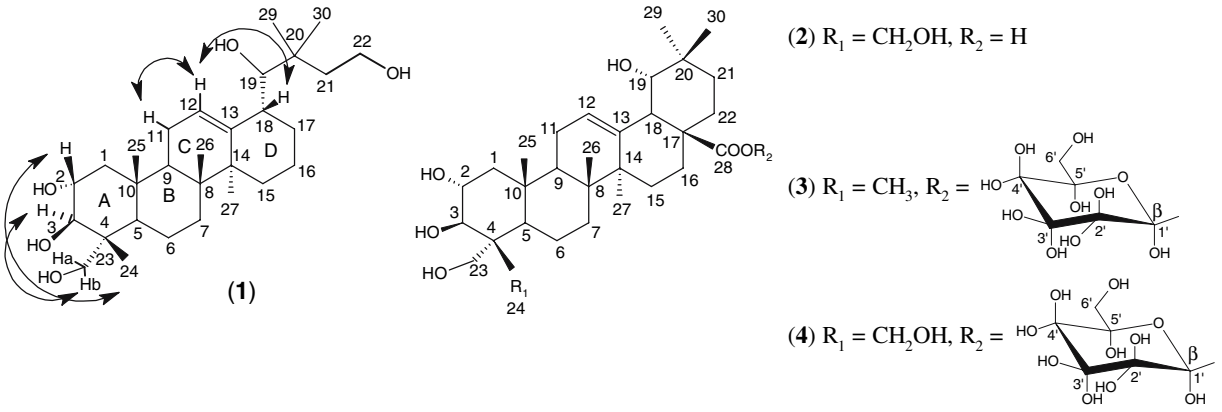

Fig. 1. Structure and *g*NOESY correlations for the *nor-seco*-triterpene **1** and the pentacyclic triterpenoids **2**–**4**

23-pentahydroxy-Δ^{12}-oleanane (**1**), bellericagenin B (**2**), bellericaside B (**3**) and arjunglucoside I (**4**) (Fig. 1) from methanolic extract of bark of the *Q. parviflora* using DCCC.

Experimental

Chemicals

All solvents used for DCCC and TLC were of analytical-reagent grade from Merck (Darmstadt, Germany).

Preparation of Methanolic Extract and Sample Solution

Qualea parviflora bark (500 g) were collected at Ypê Garden (Cerrado region) Porto Nacional city [Tocantins State (TO)], Brazil by Adriane R. Duarte. A voucher specimen was identified by Dr. S.F. Lolis from UNITINS, Porto Nacional city and deposited under no. 9226 at UNITINS herbarium.

The air-dried and powdered bark were exhaustively extracted (three times) with chloroform and methanol (48 h, 4 L each) successively at room temperature. Solvents were evaporated at 60 °C under reduced pressure and affording the $CHCl_3$ extract (4.1 g) and MeOH extract (10.7 g).

Preparation of Two-Phases Solvent System

The solvent system composed of $CHCl_3$/MeOH/H_2O (43:37:20, v/v) was thoroughly equilibrated overnight in a separatory funnel at room temperature and the two phases separated shortly before use.

Droplet Counter-Current Chromatography

The DCCC was a Tokyo Rikakikai Eyela model 300 DCCC chromatograph (Tokyo, Japan) with 300 tubes (400 × 2 mm i.d.) connected in series. The solvent system used was $CHCl_3$/MeOH/H_2O (43:37:20, v/v) in the descending mode. After the mobile phase front emerged and the hydrostatic equilibrium was established in the column the MeOH extract (1.00 g) was dissolved in 16 mL of a mixture consisting of 8 mL lower phase and 8 mL upper phase of the solvent system $CHCl_3$/MeOH/H_2O (43:37:20, v/v) and it was injected with a P.C. Inc. Injection Module with a 16 mL sample injection loop. The flow of the mobile phase was set to 30 mL h^{-1}. We collected 130 fractions of 6 mL each with a Pharmacia Redifrac automated fraction collector (Uppsala, Sweden). The time frame for the presented chromatographic run was one day.

Analyses and Purification of the Compounds by TLC

The collected fractions were analysed by Aldrich TLC plates (Milwaukee, USA) (silica gel on glass, 20 cm × 20 cm, 0.25 mm layer thickness, $CHCl_3$/MeOH/H_2O, 80:18:2, v/v) and the spots were visualized by spraying with anisaldehyde reagent, followed by heating at 110 °C for 5 min [19, 20]. Fractions with similar R_F were combined. Fraction 23 gave **2** (R_F 0.7, 4 mg), fraction 82 gave **3** (R_F 0.6, 8 mg) and fractions 105–110 gave **4** (R_F 0.4, 17 mg). Fractions 16–17 were submitted to Aldrich preparative TLC plates (silica gel F254 on glass 20 cm × 20 cm, 0.25 mm layer thickness, $CHCl_3$/MeOH/H_2O, 80:18:2, v/v) to give **1** (R_F 0.9, 10 mg) (Fig. 1). Beyond triterpenes and saponins isolated, we got rich fractions in ellagic acid derivatives and tannins.

Structural Identification of the Compounds

The NMR spectra in [$^2H_6$]dimethyl sulfoxide (DMSO-d_6) and pentadeuterated pyridine (Pyr-d_5) were obtained using a Varian INOVA-500 spectrometer (Palo Alto, USA), operating at 500 MHz for ^1H and 126 MHz for ^{13}C and two-dimensional NMR (*g*HMQC, *g*HMBC, *g*COSY and *g*NOESY). Chemical shifts were given in δ (ppm) using tetramethylsilane (TMS) as internal standard.

ESI-MS fingerprints were obtained in both the positive and negative ion modes on a Micromass Q-TOF mass spectrometer (Manchester, UK). Typical ESI-MS conditions were as follow: source temperature 100 °C, desolvation temperature 100 °C, capillary voltage 3.0 kV, and cone voltage 40 V. Sample solutions were introduced into the electrospray source using the syringe pump of the instrument at a flow rate of 8 μL min^{-1} in MeOH.

The spectral data of the four compounds are given as below:

Compound **1**: 28-*nor*-17,22-*seco*-2α, 3β,19,22,23-pentahydroxy-Δ^{12}-oleanane. $C_{29}H_{50}O_5$. ESI (+)-MS *m*/*z* (rel. int.): 479.3734 $[M + H]^+$ (100), calculated 479.3737. For ^1H and ^{13}C NMR data see Table 1.

Compound **2**: 2α, 3β, 19α, 23, 24-pentahydroxy-olean-12-en-28-oic acid.

Bellericagenin B [5]. $C_{30}H_{48}O_7$. ESI (+)-MS *m/z* (rel. int.): 519.6943 $[M - H]^-$ (100), calculated 519.6980. For ^1H and ^{13}C NMR data see Table 2.

Compound **3**: β-D-glucopyranosyl-2α, 3β,19α,23-tetrahydroxyolean-12-en-28-oate. Arjunglucoside I [21–23]. $C_{36}H_{58}O_{11}$. ESI(-)-MS *m/z* (rel. int.): 665.8298 $[M - H]^-$ (100) calculated 665.8392. For ^1H and ^{13}C NMR data see Table 2.

Compound **4**: β-D-glucopyranosyl-2α, 3β,19α,23,24-pentahydroxyolean-12-en-28-oate. Bellericaside B [5]. $C_{36}H_{58}O_{12}$. ESI(–)-MS *m/z* (rel. int.): 681.8343 $[M - H]^-$ (100) calculated 681.8391. For ^1H and ^{13}C NMR data see Table 2.

Results and discussion

A series of experiments was performed to determine a suitable two-phase solvent system for DCCC [12–13]. Small amounts of the MeOH extract (10 mg) were dissolved into 2 mL of two immiscible liquid phases consisting of organic solvent and water (e.g. $CHCl_3$/MeOH/H_2O and EtOAc/*n*-PrOH/H_2O).

From this mixture, 10 µL of the upper phase and 10 µL of lower phase were spotted onto TLC plates and eluted with several solvent systems. After elution, plates were dried and the spots were visualized by spraying with anisaldehyde reagent, followed by heating at 110 °C for 5 min to compare the intensity of the spots containing triterpenoids in each phase. TLC analyses allowed to visually estimate the distribution of the triterpenoids between the two phases. The mixture of $CHCl_3$/MeOH/H_2O (43:37:20, v/v) gave the best result, with the compounds of interest almost distributed between the two phase (partition coefficient value *K* of approximately 1). A good chromatographic resolution was obtained with the lower phase of this solvent system, with R_F values of 0.9, 0.7, 0.6 and 0.4 for compounds **1**–**4**, respectively. The relatively high proportion of $CHCl_3$ in the solvent mixture and the R_F values of the compounds indicate the presence of medium polarity triterpenoids in *Q. parviflora*. Thus, the lower phase was chosen as mobile phase and the upper phase was used as stationary phase for the DCCC separation of the MeOH extract of *Q. parviflora*.

The MeOH extract (1.00 g) was injected in DCCC, as described previously.

Table 1. ^{13}C and ^1H NMR spectroscopic data for compound **1** (δ)

Position	Multiplicity	^{13}C DMSO-d_6	^{13}C Pyr-d_5	^1H DMSO-d_6	*g*HMQC DMSO-d_6	*g*HMBC DMSO-d_6	*g*NOESY DMSO-d_6
1β	CH_2	46.6	47.8	1.70	H-1	–	H-1α, 2, 25
1α	–	–	–	0.72	–	–	–
2	CH	67.5	69.2	3.48	H-2	H-3	–
3	CH	75.7	78.5	3.17	H-3	H-23, 24	H-23a, 23b
4	C	42.5	44.0	–	–	H-23, 24	–
5	CH	46.2	48.3	–	–	H-23, 24, 25	–
6	CH_2	17.7	19.0	–	–	–	–
7	CH_2	32.2	33.3	–	–	H-26	–
8	C	39.0	40.4	–	–	H-26, 27	–
9	CH	47.4	48.3	–	–	H-26	–
10	C	37.6	38.9	–	–	H-25	–
11	CH_2	23.3	19.0	1.84	–	–	H-12, 25, 26
12	CH	121.6	–	5.18	H-12	v	H-11, 18
13	C	144.5	–	–	–	H-27	–
14	C	41.3	42.6	–	–	H-26, 27	–
15	CH_2	28.1	29.5	–	–	H-27	–
16	CH_2	27.6	28.7	–	–	–	–
17	CH_2	44.9	47.8	–	–	H-19	–
18	CH	43.5	45.1	2.98	H-18	–	H-12, 29
19	CH	80.7	81.5	3.09	H-19	H-29, 30	–
20	C	35.0	36.0	–	–	H-29, 30	–
21	CH_2	51.5	52.8	2.57	H-21	–	H-22
22	CH_2	60.3	61.6	3.43	–	H-21	–
23a	CH_2	64.0	66.7	3.03	H-23a	H-24	H-23b, 24
23b	–	–	–	3.29	H-23b	–	H-23a, 24
24	CH_3	13.6	14.6	0.53	H-24	–	H-2, 25
25	CH_3	16.7	17.6	0.90	H-25	–	H-2, 24, 26
26	CH_3	17.2	17.9	0.68	H-26	–	H- 25
27	CH_3	24.2	24.6	1.22	H-27	–	–
28	–	–	–	–	–	–	–
29	CH_3	28.2	29.2	0.82	H-29	H-30	
30	CH_3	24.8	25.1	0.87	H-30	H-29	

After the DCCC separation, the collected fractions were monitored by TLC and combined. Two triterpenoid aglycones and two glucosides were separated. Compound **1** (R_F 0.9) was the first substance to be eluted due to its lower polarity when compared to compound **2** (R_F 0.7), that has a COOH group at C-28. Despite their similar polarity, triterpenoid glycosides (**3**) and (**4**) were well separated. Compared to **3** (R_F 0.6), triterpenoid glycosides **4** (R_F 0.4) is more polar, since it bears an extra hydroxyl group in C-24, and thus was the last compound to be eluted.

Therefore, our results showed that the used conditions provided a very efficient method for the separation of the triterpenoids from *Q. parviflora*.

Structural Elucidation of Compound 1

Spectroscopic and spectrometric analyses allowed to identify four main compounds (Fig. 1). Compound **1** was obtained as a white solid and showed a bright purple spot on TLC observed with anisaldehyde reagent.

The ESI-MS exhibited only the deprotonated molecule of *m/z* 479.3734 $[M + H]^+$ (100), compatible with the molecular formula $C_{29}H_{50}O_5$.

The ^{13}C NMR spectrum showed a total of 29 signals for **1**, and a DEPT 135 experiment allowed to identify the signals as belonging to six methyl, ten methylene, seven methine and six quaternary carbons (Table 1). Two of these signals are typical of sp^2 carbons δ 121.6 (C-12) and 144.5 (C-13), suggesting the presence of a Δ^{12}-oleanane triterpene [4]. Carbon signals with chemical shifts at δ 60.3, 64.0 67.5, 75.7 and 80.7 suggested the presence of five carbinolic groups with their multiplicity defined through DEPT 135 experiments as being to two methylenic carbinolic groups (δ 60.3 and 64.0) and three methynic carbinolic groups (δ 67.5, 75.7 and 80.7). The *g*HMQC experiment allowed to establish the direct ^1H-^{13}C correlations, while the connecting position of the methyl groups was established by using *g*HMBC and NOESY experiments (Table 1, Fig. 1). Correlations in the *g*HMBC spectrum were observed between the methyl group

Table 2. ^{13}C NMR chemical shifts (δ) of pentacyclic triterpenoids **2–4** in DMSO-d_6

Position	(2)	(3)	(4)
1	46.5	46.6	46.9
2	67.3	67.4	67.6
3	77.2	77.7	77.8
4	46.4	42.5	45.4
5	46.7	46.2	46.6
6	18.5	17.5	18.5
7	32.4	28.3	32.5
8	40.4	40.4	40.0
9	47.5	47.3	47.7
10	37.2	37.4	37.4
11	23.4	23.3	23.6
12	122.1	122.3	122.4
13	143.5	143.2	143.3
14	41.1	41.3	41.2
15	27.2	31.8	28.0
16	28.4	27.8	28.5
17	44.7	45.2	48.5
18	43.2	43.1	43.3
19	80.1	80.0	80.3
20	34.8	34.7	34.9
21	27.9	27.0	27.8
22	32.2	31.8	32.0
23	62.5	63.9	62.7
24	60.6	13.6	60.8
25	16.6	16.6	16.7
26	16.4	16.7	16.6
27	24.1	24.2	24.3
28	179.1	175.8	176.1
29	28.0	28.0	28.2
30	24.5	24.5	24.7
1’	–	94.1	94.3
2’	–	72.4	72.5
3’	–	76.7	77.4
4’	–	69.5	69.7
5’	–	76.7	76.8
6’	–	60.7	60.4

CH_3-24 (δ 0.53) and the carbons signals at δ 75.7 (C-3), δ 64.0 (C-23), 46.2 (C-5) and δ 42.5 (C-4); the methyl group CH_3-26 (δ 0.68) and the carbons signals at δ 39.0 (C-8), δ 47.4 (C-9) and δ 41.3 (C-14); the methyl group CH_3-29 (δ 0.82, s) and the carbons signals at δ 80.7 (C-19), δ 35.0 (C-20) and δ 24.8 (C-30); the methyl group CH_3-30 (δ 0.87) and the carbons signals at δ 80.7 (C-19), δ 35.0 (C-20) and δ 28.2 (C-29); the methyl group CH_3-25 (δ 0.90) and the carbons signals at δ 46.2 (C-5) and δ 37.6 (C-10); the methyl group CH_3-27 (δ 1.22) and the carbons signals at δ 39.0 (C-8), δ 144.5 (C-13), δ 41.3 (C-14) and δ 28.1 (C-15), the H-3 (δ 3.17) and the carbons signals at δ 67.5 (C-2), the H-19 (δ 3.09) and the carbons signals at δ 44.9 (C-17), and the H-21 (δ 2.57) and the carbons signals at δ 60.3 (C-22).

The ^1H-^1H *g*COSY experiments showed interactions between the signals at δ 0.72 (Hα-1) with δ 3.48 (H-2); δ 1.70 (H-1β) with δ 3.48 (H-2); δ 3.48 (H-2) with δ 3.17 (H-3); δ 1.84 (H-11) with δ 5.18 (H-12); δ 2.98 (H-18) with δ 3.09 (H-19); and δ 2.57 (H-21) with δ 3.43 (H-22). These data, in connection with the *g*HMQC experiment, helped to establish the ^{13}C NMR chemical shifts for C-11 and C-22 and confirmed the chemical shifts for C-1, C-2, C-3, C-12, C-18, C-19 and C-21.

A series of NMR experiments registered in pentadeuterated pyridine indicated the presence of the terminal –CH_2-CH_2-OH unit at C-21/C-22 position. The ^1H NMR spectrum allowed to identify two triplets (J = 5.5 Hz) at δ 3.02 and δ 3.99, which were confirmed by *g*HMBC and *g*COSY experiments. The NMR ^1H spectrum also allowed to recognize signals at δ 4.19 (*d*, J = 10 Hz) and at δ 3.73 (*d*, J = 10 Hz), which were assigned to H-3 and H-2, respectively; the coupling constant value agrees with a *trans* diaxial orientation, thus indicating the α-equatorial configuration of the hydroxyl group at C-2 and the β-equatorial configuration of the hydroxyl group at C-3. The absence of the CH_3-28 and the presence of the –CH_2-CH_2OH portion is characteristic of a *nor*-28-s*eco*-17, 22-triterpene.

The *g*NOESY experiments helped to clarify the relative stereochemistry of compound **1**. Irradiation at δ 5.18 (H-12) showed interactions with the signals at δ 1.84 (H-11) and at δ 2.98 (H-18), thus indicating a β-configuration to H-18 and, hence the C-19 in C-18 was placed in α. Irradiation at δ 0.53 (CH_3-24) showed interations with δ 3.48 (H-2) and δ 0.90 (CH_3-25), suggesting the α-configuration for the OH in C-2. Irradiation at δ 3.17 (C-3) showed interations with δ 3.03 (H-23a), δ 3.29 (H-23b) thus indicating the β-configuration for the OH in C-3.

Therefore, compound **1** is determined as being 28-*nor*-17, 22-*seco*-2α, 3β, 19, 22, 23-pentahydroxy-Δ^{12}-oleanane.

Compounds **2–4** were identified as bellericagenin B **(2)**, arjunglucoside I **(3)** and bellericaside B **(4)** by comparison of their physical data with literature values [5, 21–23] (Table 2).

However, these triterpenoids are being described for the first time in *Q. parviflora*.

Conclusions

The results of our studies clearly demonstrate the potential of DCCC for the preparative isolation of 28-*nor*-17, 22-*seco*-2α, 3β, 19, 22, 23-pentahydroxy-Δ^{12}-oleanane (**1**), bellericagenin B (**2**), bellericaside B (**3**) and arjunglucoside I (**4**) from *Q. parviflora* bark in only one step without need of time-consuming clean-up and minimizing the loss of material due to decomposition. In particular, preparative DCCC is a very efficient method for the separation and purification of natural products.

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