A new *ent*-clerodane diterpenoid from *Crassocephalum bauchiense* Huch. (Asteraceae)

Alembert T. Tchinda[*a], Simplice R. Mouokeu[b], Rosalie A.N. Ngono[c], Madeleine R.E Ebelle[c], Aristide L.M. Kognou[a,b], Diane K. Nono[d], Joe D. Connolly[e] and Michel Frédérich[f]

[a]*Laboratory of Phytochemistry, Institute of Medical Research and Medicinal Plant Studies, (IMPM), P.O. Box 6163, Yaounde, Cameroon*

[b]*Institute of Fisheries and Aquatic Sciences, University of Douala, PO Box 2701, Douala, Cameroon*

[c]*Department of Biochemistry, Faculty of Sciences, University of Douala, PO Box 24157, Douala, Cameroon.*

[d]*Department of Organic Chemistry, Faculty of Science, The University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon*

[e]*Department of Chemistry, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, Scotland.*

[f]*Laboratoire de Pharmacognosie, Université de Liège, Centre Interdisciplinaire de Recherche*

sur le Médicament (CIRM), Département de Pharmacie, Université de Liège, B36, B-4000, Liège, Belgium

[*] Corresponding author. Email : alembertt2002@yahoo.fr

Tel: 00(237)76925929

Abstract

A phytochemical investigation of the whole plant of *Crassocephalum bauchiense* Huch. resulted in the isolation of a new clerodane diterpenoid, *ent*-2*β*,18,19-trihydroxycleroda-3,13-dien-16,15-olide (**1**) together with two known flavonoids 3',5-dihydroxy-4',5',6,7,8-pentamethoxyflavone (**2**) and 4',5-dihydroxy-3',5',6,7,8-pentamethoxyflavone (**3**). The compounds were tested against the chloroquine-sensitive 3D7 strain of *Plasmodium falciparum.* Compound **2** showed weak activity (IC_{50} = 10.1 µg/mL) whilst compounds **1** and **3** were inactive. The structures of the compounds were defined by detailed spectral analyses, especially ^{1}H- and ^{13}C- NMR, ^{1}H-^{1}H COSY, NOESY, HMBC and HR-ESIMS.

Key words: *Crassocephalum bauchiense; isolation; clerodane diterpenoid; flavonoids; antiplasmodial activity.*

1. Introduction

Crassocephalum bauchiense Hutch. is an erect bushy annual herb about 1ft. high with bright blue florets in numerous heads growing in rough open ground in Nigeria and Cameroon (Hutchison & Dalziel 1958). The plant is used in folk medicine in Cameroon to treat gastrointestinal infections, liver disorders, epilepsy, pain, arthritis, colics, behavioural disturbances in mentally-retarded children, inflammatory disorders and neuropathic pain (Arbonnier 2000; Moukeu et al. 2011). In experimental studies, the aqueous extract of the leaves showed antinociceptive activity (Taiwe et al 2012) and the fractions antibacterial and immunomodulary activities (Mouokeu et al., 2011; 2013). To the best of our knowledge, no previous phytochemical study has been done on this plant.

In our efforts to investigate Cameroonian medicinal plants for the biological activity of their constituents, we carried out a phytochemical study on the dichloromethane-methanol (1:1) extract of the titled plant and report herein the isolation and structure elucidation of a new clerodane diterpenoid *ent*-2*β*,18,19-trihydroxycleroda-3,13-dien-16,15-olide (**1**), from the whole plant of *C. bauchiense* together with two known flavonoids, 3',5-dihydroxy-4',5',6,7,8-pentamethoxyflavone (**2**) and 4',5-dihydroxy-3',5',6,7,8-pentamethoxyflavone (**3**), identified for the first time in the *Crassocephalum* genus. The isolated compounds were tested against the 3D7 strain of *Plasmodium falciparum*. Compound **2** showed weak activity.

2. Results and discussion

The crude CH_2Cl_2-MeOH (1:1) extract of the whole plant of *C. bauchiense* was subjected to a series of silica gel and sephadex LH-20 chromatographic columns to give the new diterpenoid (**1**) and the known flavonoids 3',5-dihydroxy-4',5',6,7,8-pentamethoxyflavone (or Gardenin C) (**2,** Sujata et al. 2013) and 4',5-dihydroxy-3',5',6,7,8-pentamethoxyflavone (**3,** Fushiya et al. 1999) (Fig. 1).

Compound **1** was isolated as an amorphous solid. Its molecular formula $C_{20}H_{30}O_5$ was deduced from the HRESIMS which showed the pseudo-molecular ion peak $[M+Na]^+$ at *m/z* 373.1970. Its IR spectrum exhibited characteristic absorptions for hydroxyl groups (3238 cm^{-1}), *α,β*–unsaturated-*γ*-lactone (1745 cm^{-1}) and double bond (1649 cm^{-1}) (Esquivel et al. 1989). The structure of the compound was established from its ^1H- and ^{13}C-NMR spectroscopic data (Table1), which suggested a clerodane diterpenoid (Esquivel et al., 1989).

The ^{13}C NMR spectrum of **1** revealed 20 C-atoms, including a lactone carbonyl and two trisubstituted double bonds, three methines (one oxygenated), eight methylenes (three oxygenated), a secondary methyl group and a tertiary methyl group and two fully substituted carbons. The presence of an α,β-unsaturated γ-lactone moiety was evident from the lowfield signal of the olefinic proton H-14 (δ_H 7.35, brs), showing HMBC correlations with the lactone carbonyl C-16 (δ_C 175.3), C-12 (δ_C 128.1), C-13 (δ_C 133.2) and C-15 (δ_C 70.6). This was further supported by the other long range correlations between the oxymethylene protons H_2-14 (δ_H 4.81, brs,) and C-13 and C-16.

The two methyls signals at δ_H 0.88 (d, J = 7.8 Hz) and δ_H 0.82 (s) in the ^1H NMR spectrum of compound **1** were assigned to Me-17 and Me-20 respectively. The characteristic Me-18 and Me-19 of clerodane diterpenoids (Merrit & Ley 1992) were oxidized to oxymethylenes which were observed in the ^{13}C NMR spectrum at δ_C 62.5 [δ_H 4.19 (H-18a) and 3.89 (H-18b), both d, J = 12.8 Hz] and δ_C 64.5 5 [(δ_H 4.02 (H-19a) and 3.71 (H-19b), both d, J = 10.8 Hz] respectively. The positioning of these methylene groups at C-4 and C-5 was based on the observation of HMBC correlations between H-18b and C-3 (δ_C 128.6) and C-4 (δ_C 147.5) and between H-19a and C-6 and C-10 (Table 1).

Compound **1** is isomeric with a previously reported clerodane, *ent*-7β,18,19-trihydroxy-cleroda-3,13-dien-16,15-olide, isolated from another source (Esquivel et al., 1989). In addition, their NMR data are very close, the difference being the position of the secondaryhydroxyl group which was fixed at C-2 (δ_C 68.1) in **1** by the HMBC correlations observed between H-2 (δ_H 4.28,) and C-1, C-3 and C-4. The HMBC correlation between H_2-1

and C-2 as well as the COSY connectivity between H_2-1 and H-2 further confirmed the attachment of the hydroxyl group at C-2.

The relative configuration of compound **1** was determined from the NOESY spectrum (Table 1) and by comparison of NMR data with those of reported analogues (Das et al. 2005; Esquivel et al. 1989; Gu et al. 2014). The important NOESY cross peaks included those between H-10 and H-2 and H-8 one one hand, Me-17 and Me-20 and H-19a on the other hand. These results revealed the trans AB-ring junction and the relative configurations at C-2, C-8 and C-9.

In vitro antiplasmodial activity of compounds **1**, **2** and **3** was evaluated against the chloroquino-sensitive 3D7 strain of *P. falciparum*. Compound **2** was moderately active (IC_{50} = 10.1 µg/mL) whilst compounds **1** and **3** were inactive (IC_{50} > 40 µg/mL). However, at higher concentrations, compound **3** inhibited 42% of the parasites. Compounds **2** and **3** have the same A and B rings. The two flavonoids differ in the positions of the hydroxy and methoxy groups in ring B. The presence of these groups at C-3' and C-4' in compound **1** may be responsible for his activity while the positions of these groups in compound **3** may account for the reduction in activity.

3. Experimental

4.1 *General experimental procedures*

The optical activity was determined with an AA-10 Automatic (ANALIS) polarimeter The UV spectra were recorded with an U-2910 spectrometer (λ_{max} in nm). The IR spectrum was recorded on a Perkin-Elmer 1750 FTIR spectrometer. The HRESI mass spectra were recorded with a Bruker APEX-Qe 9.4T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer equipped with a hybrid quadrupole analyzer and using an electrospray source.
NMR spectra were recorded in MeOH-d_4 on a Bruker 500 MHz NMR AV II spectrometer equipped with a cryoprobe, with TMS as an internal reference. Chemical shifts were recorded in δ (ppm) and the coupling constants (J) are in hertz (Hz). Column chromatography (CC) was carried out on silica gel (70-230 mesh, Merck). TLC was performed on Merck precoated silica gel 60 F_{254} aluminum foil and compounds were detected using 10% sulfuric acid in ethanol as spray reagent.

4.2 *Plant material*

The whole plant of *C. bauchiense* was collected at Dschang, in the West Region of Cameroon. The botanical identification was done at the National Herbarium in Yaounde

(Cameroon) by referring to the sample number 7954/SRF/Cam. A voucher specimen of the plant is kept in the Herbarium of the Department of Plant Biology of the University of Dschang under the code number 0033/UDs/PB.

4.3 *Extraction and isolation of compounds*

The whole plant of *C. bauchiense* was dried at room temperature for two weeks and powdered. The powder (1.5 Kg) was macerated with a mixture of CH_2Cl_2-MeOH (4L, 1:1) for two days. After filtration, the solvent was removed under reduced pressure using a rotary evaporator (45° C) to yield of 25 g (1.6%) of the crude extract which was further fractionated by column chromatography (CC) on 200 g of silica gel 60 with hexane-EtOAc and EtOAc-MeOH mixtures of increasing polarity. Thirty-three fractions of 300 ml each were collected and pooled according to their TLC profile. Compound **2** (30 mg) crystallized from fraction 13 collected from the column with the mixture 60:40. Fractions 14-17 collected from the main column with the mixtures hexane-EtOAc 60:40 to 50:50 were further chromatographed on a silica gel CC with hex-EtOAc (90-10) to yield 29 fractions. Compound **3** (12 mg) crystallized from fraction 11. Fraction 28 obtained from the main column with the mixture hexane-EtOAc 30:70 was chromatographed on a Si gel CC eluting with $CHCl_3$-MeOH (98:2 to 90:10) to give 13 fractions. Sub-fractions 6 and 7 were regrouped and passed through several Sephadex LH-20 columns using methanol as eluent to give compound **1** (8 mg).

Compound **1**: Greyish amorphous powder; $[\alpha]^{25}_D$: +23.1 (*c* 0.03, MeOH); UV (MeOH) λ_{max} 215 nm; IR (KBr) 3238, 2952, 2912, 2869, 1745, 1720, 1649, 1448, 1386, 1350, 1209, 1083, 1039, 842 cm^{-1}; ^1H and ^{13}C NMR data see Table 1; HRESIMS: *m/z* 373.19705 ($[M+Na]^+$, calcd. for $C_{20}H_{30}NaO_5$, *m/z* 373.19855).

4.4 *In vitro antiplasmodial assay*

Continuous culture of the *P. falciparum* chloroquine sensitive 3D7 strains was assessed following the procedure already described in Frederich et al. (2002). The parasites were obtained from Prof. Grellier (Museum d'Histoire Naturelle, Paris, France). Each compound was applied in a series of eight threefold dilutions from 0.02 to 40 µg/ml for a pure substance) on two rows of a 96-well microplate and were tested in triplicate (n=3). Parasite growth was estimated by determination of lactate dehydrogenase activity as described previously in Kenmogne et al. (2006). Artemisinin (98%, Sigma–Aldrich) and chloroquine were used as positive controls (IC_{50} 4.12 ng/mL).

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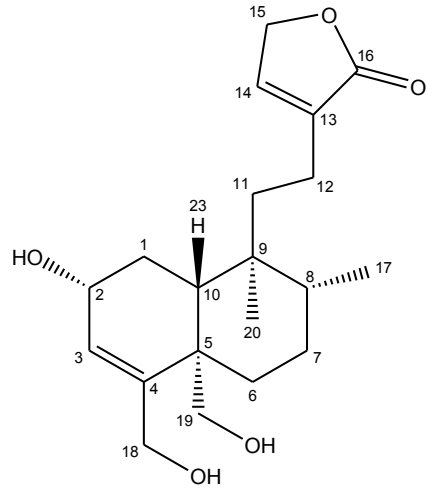
Table 1: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **1** (recorded in MeOH-d_4)

Pos.	δ_H	Mult., *J* (Hz)	δ_C	HMBC (^{1}H-^{13}C)	^{1}H-^{1}H COSY	NOESY
1	1.95	ov	27.1	C-2, C-3, C-10	H-2, H-10	H-2
2	4.28	brt (7.5)	68.1	C-1, C-3, C-4	H-1, H-3	H-1, H-3, H-10
3	5.70	brs	128.6	C-1, C-5, C-18	H-2	H-2, H-18b
4	-	-	147.5	-	-	-
5	-	-	42.9	-	-	-
6a	2.20	ov	30.6	C-8	H-12b, H-6b	H-6b
b	1.17	dt	-	C-4, C-5, C-7, C-19	H-7, H-6a	H-6a, H-10
7	1.45	m	26.4	-	H-6b	Me-17
8	1.59	ov	35.8	-	H-17	Me-17
9	-	-	38.1	-	-	-
10	1.58	ov	45.0	C-8, C-12	H-9, H-11	H-2, H-6b, H-8,
11a	1.66	ov	35.8	C-9, C-13, C-12	H-10, H-11	-
b	1.54	ov				Me-17
12a	2.17	ov	18.14	C-8, C-10, C-13	H-12b	-
b	2.02	ov			H-12a	
13	-	-	133.2	-	-	-
14	7.35	brs	145.9	C-12, C-13, C-15, C-16	H-15	H_2-15
15	4.81	brs	70.6	C-13, C-16	H-14	H-14
16	-	-	175.3	-	-	-
17	0.88	d(6.6)	14.7	C-7, C-8, C-9	H-8, H-11	H-7, H-8, , H-11b, H_3-20
18a	4.19	d(12.8)	62.2	C-3, C-4, C-5	H-18b	H-18b
18b	3.89	d(12.8)		C-4, C-5	H-18a	H-18a, H-3
19a	4.02	d(10.8)	64.5	C-4, C-5, C-6, C-10	H-19b	H-19b, H_3-20
19b	3.71	d(10.8)		C-4, C-6, C-10	H-19a	H-19a
20	0.82	s	17.8	C-8, C-9, C-10	H-17	H-19a, H_3-17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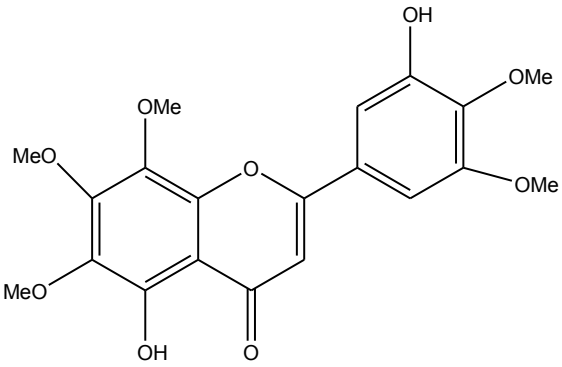
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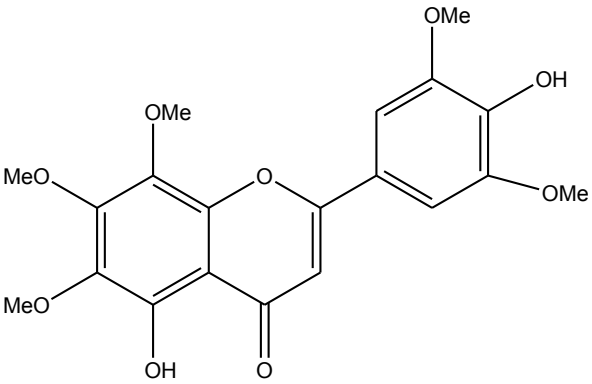
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Fig. 1 : Structures of the isolated compounds