First report of isolation of antibacterial ceramides from the leaves of Euclinia longiflora Salisb

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Abstract
Two phytosphingosine-type ceramides (euclinide A (1) and B (2)) were isolated alongside one fatty acid (geddic acid (3)), one fatty acid-1-glyceride ((2S)-1-O-hentriacontanoyl glycerol (4)), ten triterpenes (α-amyrin (5), α-amyrin acetate (6), ursolic acid (7), β-amyrin (8), β-amyrin acetate (9), β-amyrin palmitate (10), oleanic acid (11), maslinic acid (12), betulinic acid (13) and cylicodiscic acid (14)) and four sterols (β-amyrin (15), stigmasterol (17), and their glucosylated derivatives β-sitosterol-3-O-β-glucopyranoside (16) and stigmasterol-3-O-β-glucopyranoside (18)) from the methanol extract of the leaves of Euclinia longiflora Salisb. using routine chromatographic methods. The structures of the new ceramides and known compounds were determined by analyses of HR-FAB-MS, 1D and 2D NMR (HSQC, HMBC, and NOESY) data and confirmed, where applicable, by comparison with data reported in literature. The methanolic extract and the ceramides were evaluated for their antibacterial activities against different bacterial strains using microdilution method and the MIC values ranged from 6.25 to 50 μg/mL and were considered as moderate activity compared to ciprofloxacin. It is the first report of ceramides in Euclinia longiflora.

Keywords
Euclinia longiflora Salisb., Rubiaceae, ceramides, antibacterial activity

Introduction
Euclinia genus, belonging to the family Rubiaceae, consists of about three species, mainly distributed in tropical and subtropical regions over the world.1 Those three species of Euclinia genus were identified in Cameroon namely E. longiflora, E. squamifera, and E. marissima. The different parts of these plants are used with efficiency in traditional medicine in the form of cataplasm, infusion or decoction for the treatment of infectious diseases such as malaria, cutaneous, and subcutaneous parasitic infection.2 Euclinia longiflora Salisb. is a small tree of up to 7 m high. It is distributed in Tropical Africa, from Guinea-Bissau, Cameroon to Democratic Republic of Congo and Angola. It can be identified through its shiny green leaves and its lightly fragrant, creamy-white flowers which are trumpet-shape with five curled lobes. This species is currently treated as synonyms of Gardenia devoniania Lindl., Gardenia longiflora Salisb., Gardenia longifolia G.Don, Gardenia macrantha Shult., Randia bowiesiana A. Cunn., Ex Hook, Randia devoniania (Lindl.) Benth & Hook.f., Randia longiflora Salisb., Randia macrantha (Shult.) DC., Solena bowiesiana (A. Cunn., Ex Hook.) D. Dietr., Solena macrantha (Shult.) Dietr., Rothmannia bowiesiana (A. Cunn., Ex Hook) Benth.3 Previous phytochemical investigation of others species of same genus revealed the presence of a wide range of secondary metabolites, including alkaloids, glycosides, sapo-

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This paper investigates and reports the isolation, structural elucidation, and antibacterial evaluation of two natural ceramides products named euclinide A and B, which were isolated as chemical entities from the Eucnedia longiflora Salisb. for the first time.

Results and Discussion

The chemical investigation of the leaves extracts of Eucnedia longiflora Salisb. by usual chromatographic techniques led to the isolation of two new compounds 1 and 2 together with known compounds including one gedic acid (3),8 (26R)-1-O-hentriacontanoyl glycerol (4),9 α-amyrin (5),10 α-amyrin acetate (6), ursolic acid (7),11 β-amyrin (8), β-amyrin acetate (9), β-amyrin palmitate (10),12 oleic acid (11), maslinic acid (12),13 betulinic acid (13),14 cyclodisic acid (14),15 β-sitosterol (15) stigmasterol (17),10 and their glucosylated derivatives β-sitosterol-3-O-β-glucopyranoside (16) and stigmasteryl-3-O-β-glucopyranoside (18).17

The positions of these four hydroxyl groups were further confirmed from the HMBC correlations [Figure 2]. The chain length of the fatty acid was determined by the characteristic fragmentation ions observed in the EI mass spectra at m/z 367 [CH3(CH2)11(CHOH)(CO)]+, and m/z 384 [CH3(CH2)16(CHOH) NH2CO + H]+ [Figure 3]. In addition, NMR and MS data of compound 1 corresponded very well to that of the synthetic ceramide [2S,2′R,3,4,R]-2-[2-hydroxytetradecanoylaminoo] octadecane-13,4-triol, with respect to the signals due to H-1a, H-1b, H-2, H-3, H-4, and [H-2′] [Table 1].22 The fact that the rotational optical rotation of this compound [2S]D20 to 34 [c = 0.0001, MeOH] was different with that reported in the literature suggested it was new. Thus, the structure of compound 1 is suggested to be [2S,2′R,3,4,R]-2-[2-hydroxytetradecanoylaminoo] octadecane-13,4-triol to which the trivial name euclinide A was assigned.

Compound 2 was isolated from the leaves of Eucnedia longiflora as a white solid. Optically active compound, with [2S]D20 + 40 (c = 0.0001, MeOH), which reacted positively to the Dragendorff test, indicating the presence of a nitrogen atom. Its molecular formula, C40H82NO5, implying one degree of unsaturation, was determined from its high-resolution FAB mass spectrum which showed in positive mode, the pseudo-molecular ion peak [M + H]+ at m/z 656.6193 (Calcd C40H82NO5 656.5727). The set of NMR data of compound 2 are quite identical with those of compound 1. However, compared to compound 1, and according to the MS data, compound 2 has 2 carbons and 4 hydrogen atoms less than compound 1.

The main difference between the two compounds is the length of the long-chain amino base, which in compound 2 has twenty-two carbons instead of twenty-four as in compound 1. This was confirmed by the characteristic fragment ions observed in the EI mass spectrum of compound 2 at m/z 399 [CH3(CH2)18(CHOH)CO]+, characteristic of the long-chain acid base. The ion fragment at m/z 283[CH3(CH2)14(CHOH)CO]+ (Figure 4), suggests that the length of the fatty acid moiety bearing 18 carbons did not change. Analysis of the HSQC and HMBC spectra led to the assignment of proton and carbon signals for compound 2. The amino base and fatty acid of compound 2 were assigned as 2-amino-docosane-13,4-triol and 2-hydroxyoctadecanoic acid, respectively. In the case of compound 1, the stereochernistry at C-2, C-3, C-4, and C-2′ were determined and suggested to be 2R, 3S, 4R for these carbons, respectively. The NMR data and comparison of the optical rotation of compound 2 (+ 40) and the synthetic ceramide (+9.1)23 as well as with related naturally occurring ceramides suggested that compound 2 has the same absolute configuration for the core structure in the 2,3,4,2′ part. On the basis of this evidence, the structure of compound 2 is suggested to be (2S,2′R,3,4,R)-2-[2-hydroxytetradecanoylamino] octadecane-13,4-triol to which the trivial name euclinide B was assigned (Table 1).

The two ceramides were screened for their antimicrobial activity against a wide range of microorganisms including Streptococcus pneumoniae ATCC49619, Staphylococcus aureus ATCC43300, Klebsiella pneumoniae ATCC700603, Haemophilus influenzae ATCC49247, Escherichia coli ATCC25922, Pseudomonas
aeruginosa HM601, *Staphylococcus aureus* BAA 977, *Streptococcus pneumoniae* ATCC49619 and showed moderate activity with MIC (minimal inhibitory concentration) range from 6.25 to 50 μg/mL (Table 2). The most active metabolite is the euclinide A (1) with MIC values of 6.25 μg/mL and 17.5 μg/mL against *Klepsiella pneumoniae* and *Haemophilus influenzae*, respectively. The second ceramide (Euclinide B [2]) was less active compared to the previous one with MIC of 12.5 μg/mL and 50 μg/mL. It is worth mentioning that these compounds were also active against *Escherichia coli* and *Staphylococcus aureus*. For euclinide A, the MIC values against these strains were 5 μg/mL and 6 μg/mL, respectively. Concerning euclinide B, the MIC values were both 7 μg/mL. In view of these results, it is possible that the amide and hydroxyl groups present in the structures of these ceramides are responsible for the activities observed. The large family of ceramides which includes the isolated compounds, namely euclinide A and euclinide B, are well known for their interesting biological activities especially antimicrobial activities.\textsuperscript{24,25} Regarding the extracts, a significant activity is observed with regards to the strains *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* with MICs varying between 100 and 200 μg/mL.

In conclusion, from the methanol extract of the leaves of *Euclinia longiflora*, two phytosphingosine-type ceramides and four steroids were isolated and identified. Existing chemical studies of the species of *Euclinia* genus are scarce and poorly reported. All of the compounds reported in this work were isolated for the first time from this species. The bioactivity study of
the isolated ceramides indicated that these compounds displayed adequate and appreciable activity against *Klebsiella pneumoniae* and *Haemophilus influenzae*. The large family of ceramides which includes the isolated compounds, namely euclinide A and euclinide B, are well known for their interesting biological activities especially antimicrobial activities. It may therefore be responsible in part or in whole for these activities, hence validating the uses of this plant in traditional medicine.

**Experimental**

*General Experimental Procedures*

IR spectra were recorded on a Shimadzu 8900 FT-IR spectrophotometer in KBr disks. The NMR spectra in DMSO-$d_6$ and pyridine-$d_5$ were obtained using Bruker Av-400 and Avance-500 Cryo-Probe instruments, operating at 400 and 500 MHz for $^1$H-NMR and 100 and 125 MHz for $^{13}$C-NMR. Chemical shifts are given in $\delta$ (ppm) using tetramethysilane (TMS) as internal standard. EI-MS, HR-EI-MS, and Fast atom bombardment mass spectra (FAB-MS) were obtained with a JEOL JMS-600H mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on precoated silica gel plates (Merck 60 F254 20×20, 0,25 mm). Column chromatography was carried out using silica gel (70-230 mesh; Merck). Chromatograms were visualized by spraying with a solution of H$_2$SO$_4$ 10% or under ultraviolet lamp (254 and 365 nm).

*Plant Material*

The leaves of *Euclinia longiflora* were collected in April 2017 at mount Kala, in a locality of the Center Region of Cameroon (geographical coordinates: 3°51 North, 11°22 East). Plant
material was identified by Mr. Ngansop Eric, plant taxonomist at the National Herbarium of Cameroon (NHC), where a voucher specimen was preserved under the reference number 67215/NHC.

**Extraction and Isolation**

The leaves of *Euclia longiflora* (0.6 Kg) were dried in the shade at room temperature, away from sunlight. After this period, the dried leaves were crushed and ground to a homogeneous fine powder. The powder was fractionated over silica gel using *n*-hexane/EtOAc gradient to afford three main fractions labeled S1 (5 g; *n*-hexane/EtOAc 1:1), S2 (7.4 g; *n*-hexane/EtOAc 1:1), S3 (8.1 g; pure EtOAc).

Fraction S1 (5 g) was further chromatographed on silica gel, and also eluted with *n*-hexane/EtOAc gradient to afford three main fractions labeled S1 (5 g; *n*-hexane/EtOAc 4:1), S2 (7.4 g; *n*-hexane/EtOAc 1:1), S3 (8.1 g; pure EtOAc) and eluted with *n*-hexane/EtOAc gradient to afford three main fractions labeled S1 (5 g; *n*-hexane/EtOAc 4:1), S2 (7.4 g; *n*-hexane/EtOAc 1:1), S3 (8.1 g; pure EtOAc).

Fraction S1 (5 g) was further chromatographed on a silica gel column eluted with a mixture of *n*-hexane/EtOAc of increasing polarity. A total of 142 fractions, each of 175 mL, were collected and combined based on their TLC profiles to afford seven main subfractions (A1–A6). Subfractions A1 and A2 were combined based on their TLC profiles and chromatographed over silica gel column, and also eluted with *n*-hexane: EtOAc (1% to 100%) to afford eight compounds: α-amyrin acetate (6) (2.4 mg), ursonic acid (7) (3.6 mg), β-amyrin acetate (9) (2.7 mg). Subfraction T4 (10 g) was also purified to give euclinide A (1) (2 mg) and euclinide B (2) (5 mg). The treatment of subfraction T4 led to the obtention of stigmasterol (16) (3.8 mg) and its glucosylated derivative (18) (2.9 mg).

The fraction S2 was also separated, chromatographed on silica gel, and eluted with *n*-hexane/EtOAc (1% to 100%) to afford 8 subfractions (A1–A8). Subfractions A1 and A2 were combined based on their TLC profiles and chromatographed over silica gel column, and also eluted with *n*-hexane: EtOAc (1% to 75%) to give eight compounds: β-amyrin palmitate (10) (3.4 mg), oleic acid (11) (5.2 mg), maslinic acid (12) (3.3 mg), betulinic acid (13) (7 mg), clycicosid acid (14) (6 mg), β-sitosterol (15) (4.5 mg) and β-sitosterol-3-O-β-glucopyranoside (16) (10 mg).

**Euclinide A** (2S,3S,4R,2′R-2′-hydroxyxetacosanoyl octadecane-13,4-triol) (1). White amorphous powder. [α]D384 4 0.0001, MeOH). IR (KBr): 3339, 3219 (OH), 2919, 2850, 1620 (N=C=O), 1543 (NH), 1466, 1382, 1277, 1107, 1070, 1024, and 723. 1H and 13C NMR (500 and 100 MHz, pyridine-d5) see Table 1 (δ = 0.0 ppm). HMBC of compound 1 and 2.

**Table 1.** Spectra Data 1H-NMR (400 MHz) Pyridine-d5, 13C NMR (100 MHz) Pyridine-d5, and HMBC of Compound 1 and 2.

| Position | δH (multi., J in Hz) | δC | δH (multi., J in Hz) | δC |
|----------|----------------------|----|----------------------|----|
| NH       | 8.53 (d, 8.8)        | −  | 8.53 (d, J = 9)      | −  |
| 1a       | 4.51 (dd, 4.0, 10.8) | 62 | 4.52 (dd, J = 4.5, 11) | 61.9 |
| 1b       | 4.42 (dd, 4.0, 10.8) | 62 | 4.43 (dd, J = 4.5,11) | 61.9 |
| 2        | 5.10 (m)             | 53 | 5.13 (m)             | 52.9 |
| 3        | 4.36 (bs)            | 76.8 | 4.37 (bs)            | 76.6 |
| 4        | 4.29 (m)             | 73 | 4.30 (m)             | 72.9 |
| 5        | 2.25, 2.01 (m)       | 34.2 | 2.25, 1.93 (m)       | 34 |
| 6        | −                    | 26.8 | −                    | 26.7 |
| Jul-15   | 1.25 (bs)            | 30.1 | 1.29 − 1.24 (bs)    | 29.6 − 30.3 |
| 16       | 1.25 (m)             | 32.2 | 32.1                 | 22.9 |
| 17       | 1.23 (m)             | 23 | 23                   | 14.3 |
| 18       | 0.85 (m)             | 14.3 | 0.85 (m)             | 175.3 |
| 1′       | 175.3                | −  | 175.2                | −  |
| 2′       | 4.62 (bs)            | 72.5 | 4.62 (bs)            | 72.4 |
| 3′       | 2.25, 1.97 (m)       | 35.8 | 2.25, 1.93 (m)       | 35.7 |
| 4′=20′   | 1.24 (s)             | 30.1 | 1.29 − 1.24 (bs)    | 29.6 − 30.3 |
| 21′      | 1.24 (s)             | 30.1 | 1.73 (m)             | 25.8 |
| 22′      | 1.24 (s)             | 30.1 | 0.85 (m)             | 14.3 |
| 23′      | 1.73 (m)             | 25.9 | −                    | −  |
| 24′      | 0.85 (m)             | 14.3 | −                    | −  |
| OH–1     | 6.19 (bs)            | −  | 6.25 (bs)            | −  |
| OH–2′    | 7.59 (bs)            | −  | 7.65 (bs)            | −  |
| OH–3     | 6.67 (bs)            | −  | 6.73 (bs)            | −  |
| OH–4     | 6.67 (bs)            | −  | 6.73 (bs)            | −  |
Euclinide B \((\{2S,3S,4R,2'-R\}-2-[2'-\text{hydroxydocosanoylamino}]\text{octadecane-13,4-triol})\) (2). White amorphous powder. \([\alpha]_D^{20} + 40^\circ\) (c 0.0001, MeOH). IR (KBr) 3338, 3216 (OH), 2919, 2850, 1620 (N–C=O), 1544 (NH), 1487, 1356, 1277, 1070, 1023, 873, 723. 1H and 13C NMR (500 and 100 MHz, pyridine-d5) see Table 1; (+)–HR–FAB–MS \(m/z\) 656.6193 ([M+H]+, C40H82NO5; calcd. 656.5727). EI-MS: 681 (absent, \(M^+\)), 619 (12), 394 (25), 384 (21), 351 (27), 339 (100), 227 (9).

**Antibacterial Assay**

The minimum inhibitory concentrations (MICs) of extracts and compounds were determined according to the Clinical Laboratory Standards Institute M07-A9 microdilution method using 96-wells microtitre plates with slight modification.\(^{26,27}\)

Six human pathogenic bacteria (\textit{Streptococcus pneumoniae} ATCC49619, \textit{Staphylococcus aureus} ATCC 43300, \textit{Klebsiella pneumoniae} ATCC 700603, \textit{Haemophilus influenza} ATCC49247, \textit{Escherichia coli} ATCC 25922, \textit{Staphylococcus aureus} BAA 977) obtained from BEI resources and the American Type Culture Collection were used for the test. 100 mL of two-fold diluted extracts/compounds and reference drugs in Muller Hinton Broth (Sigma Aldrich) were added to the wells, followed by addition of 100 mL of bacteria inoculum standardized at 1.5 \(\times 10^6\) cells/mL. A blank column was included for sterility control. The concentration range was 12.5 mg/mL to 500 mg/mL for crude extracts and 0.125 to 50 mg/mL for compounds. In each microtiter plate, a column with broad-spectrum antibiotic (Ciprofloxacin) with the concentration range from 0.5 mg/mL to 64 mg/mL was used as positive control. After 24 h of incubation at 37 °C, the turbidity was observed as an indication of growth. MIC was defined as the lowest concentration inhibiting the visible growth of bacteria. All tests were performed in triplicate.

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**Table 2. Antibacterial Activity (MIC, \(\mu\)g/mL) of Isolated Ceramides (Compounds 1 and 2) and the Extract of Leaves of \textit{E. longiflora}**

|                     | \textit{Streptococcus pneumoniae} ATCC49619 | \textit{Staphylococcus aureus} ATCC 43300 | \textit{Klebsiella pneumoniae} ATCC 700603 | \textit{Haemophilus influenza} ATCC49247 | \textit{Escherichia coli} ATCC 25922 | \textit{Staphylococcus aureus} BAA 977 |
|---------------------|---------------------------------------------|-------------------------------------------|------------------------------------------|----------------------------------------|--------------------------------------|--------------------------------------|
| Compound 1          | >50                                         | 25 ± 0.00                                 | 6.25 ± 0.00                              | 17.5 ± 0.00                            | 37.5 ± 0.00                          | 25 ± 0.00                            |
| Compound 2          | >50                                         | 37.5 ± 0.00                               | 12.5 ± 0.00                              | 50 ± 0.00                              | 37.5 ± 0.00                          | 50 ± 0.00                            |
| Extract of leaves   | 100 ± 0.00                                  | >500                                      | >500                                     | 100 ± 0.00                             | >500                                 | 250 ± 0.00                           |
| Ciprofloxacin       | 8 ± 0.00                                    | 8 ± 0.00                                  | 0.5 ± 0.00                               | 1 ± 0.00                               | 4 ± 0.00                             | 16 ± 0.00                            |

Antibacterial activity (MIC, \(\mu\)g/mL) of isolated ceramides (compounds 1 and 2) and the extract of leaves of \textit{E. longiflora}.
Supplemental Material

Please see supplementary document for the HRFABMS, 1D and 2D NMR spectra of compound 1 and compound 2.

Ethical Approval

Ethical Approval is not applicable for this article.

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Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Author Contributions

AM Munvera performed the extractive and separative experiments, resolved the structures of the compounds, analysed the antibacterial assay data and wrote the manuscript. JN Nyemb, AT Ngenge and S Nuhzat assisted in the structural analyses of the isolated compounds and manuscript, and MAF Mafo helped with the antibacterial assay. AE Nkengfack supervised this research.

Declaration of Conflicting Interests

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Trial Registration

Not applicable, because this article does not contain any clinical trials.

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