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Chemical composition of *Leplaea mayombensis* (Pellegrin) Staner

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ABSTRACT

One new coumarinolignan, cleomiscosin F (**1**) along with ten known compounds namely 3,4-secotirucalla-4(28),7,24-trien-21-hydroxy-21,23-epoxy-3-oic acid (**2**), 3,4-secotirucalla-4(28),7,24-trien-3,21-dioic acid (**3**), ceramid A (**4**), ceramid B (**5**), mayombensin (**6**), aridanin (**7**), stigmasterol and β-sitosterol and their glucosides were isolated from the seeds and roots of *Leplaea mayombensis* (Meliaceae). The structures of the compounds were elucidated based on the interpretation of their spectroscopic data. Some of the isolated compounds (**3**, **4** and **5**) were tested *in vitro* against bacteria strains *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas agarici*, and *Micrococcus luteus*. Compound **3** displayed good activity against *Bacillus subtilis, Micrococcus luteus*, and *Pseudomonas agarici* with MIC values of 1.7, 2.3 and 9.8 µM, respectively; while compound **4** showed significant activity against *Micrococcus luteus* with MIC value of 11.9 µM.

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1. Introduction

Leplaea mayombensis (Pellegrin) Staner, is a plant species described for the first time by Pellegrin in the region of Mayombe Bayaka near Tchibanga in the south of Gabon (Koenen et al., 2012). It is a big tree up to 15-30 m high, belonging to the Meliaceae family and commonly distributed in tropical Africa (Shultes and Raffaud, 1990). *Leplaea* species are well known in traditional medicine in Africa for the treatment of several illnesses such as malaria, rheumatism, syphilis, leprosy and other microbial infections (Mulholland et al., 2000; Fonge et al., 2012). Their previous chemical investigations led to the report of a wide variety of secondary metabolites including sesqui-, di- and triterpenes, limonoids, steroids, flavonoids, and coumarins (Akinniyi et al., 1980; John et al., 1980; Moutoo et al., 1992; Furlan et al., 1996; Garcez et al., 1998; Pereira et al., 2012). As part of our continuous studies on this genus (Djeukeu et al., 2017), the composition of the methanol extracts of seeds and roots of *L. mayombensis* were examined. This paper describes the isolation and identification of one new compound cleomiscosin F (**1**) along with 3,4-secotirucalla-4(28),7(8),24(25)-trien-21-hydroxy-21,23-epoxy-3-oic acid (**2**) (Hernandez et al., 2018), 3,4-secotirucalla-4(28),7,24-trien-3,21-dioic acid (**3**) (Akinniyi et al., 1980), ceramid A (**4**), ceramid B (**5**), mayombensin (**6**) (Djeukeu et al., 2017), aridanin (**7**) (Feumo et al., 2016), stigmasterol, β-sitosterol and their glucosides (Chaturvedula and Prakash, 2012; Khatun et al., 2012) as well as the evaluation of antibacterial activity of compounds (**3**), (**4**) and (**5**) against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas agarici*, and *Micrococcus luteus* by microdilution method.

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2. Experimental

2.1. Apparatus

EI-MS was performed on a Finnigan MAT95 spectrometer with perfluorkerosene as reference substance. ESI-HR-MS were measured on a Bruker FTICR 4.7 T mass spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded at 500 MHz and 125 MHz, respectively, on Bruker DRX 500 NMR spectrometer with TMS as internal standard. The chemical shifts (δ) are given in ppm and coupling constant (*J*) in Hertz. Melting points were measured on a Mettler FP61 melting point apparatus. Compounds were dissolved in C_5D_5N, $CDCl_3$. Flash chromatography was performed using silica gel (Macherey Nagel & Co; Düren, Germany; 230-400 mesh). Column chromatography and vacuum liquid chromatography were carried out on silica gel (70-230 mesh, ASTM, Merck). Thin-layer chromatography was performed using Merck precoated silica gel 60 F254 and spots were visualized with UV light (254 and 365 nm) or using ceric sulfate spray reagent before heating. Gentamycin was purchased from Jinling Pharmaceutic (Group) Corp. All reagents used were of analytical grade.

2.2. Plant material

Seeds and roots of *L. mayombensis* were collected in December 2014 from Mount Kala located at 3°52′ of latitude North and 11°31′ longitude East, 800 m altitude, near Yaounde, Cameroon. Their authentication was carried out by M. Victor Nana at the National Herbarium of Cameroon where a voucher specimen has been deposited (accession number: 46220HNC).

2.3. Extraction and isolation

The air-dried powders of *L. mayombensis* roots and seeds gave 4.6 and 4.3 kg, respectively. The seed powder was extracted by maceration with 10 L of methanol at room temperature for 48 h. After evaporation under reduced pressure, 142.6 g of crude extract were obtained. Crude extract was then fractionated by vacuum liquid chromatography (VLC) using the silica gel (70-230 mesh, Merck) with gradient of *n*-hexane-ethyl acetate (v:v) (9:1); (8:2); (3:2); (1:1); (2:3) and ethyl acetate. The sub-fractions were combined in two main fractions A-B, based on their TLC analysis. Fraction A (78.6 g) was subjected to column chromatography over silica gel 60 (70-230 mesh, ASTM, Merck) with a gradient of *n*-hexane-ethyl acetate; ethyl acetate-methanol. A total of 374 fractions of around 100 mL each were collected. The pure compounds were obtained by direct crystallization. Fractions 40-69 eluted with *n*-hexane-ethyl acetate (19:1) gave a mixture of stigmasterol and β-sitosterol (48 mg). The combined fractions 117-138, eluted with *n*-hexane-ethyl acetate (17:3) yielded 3,4-dimethyl-secotirucalla-4(28),7,24-trien-3,21-dioic acid (**3**) (8.6 mg). Fractions 310-335 eluted with *n*-hexane-ethyl acetate (3:2) afforded 3,4-secotirucalla-4(28),7,24-trien-21-hydroxy-21,23-epoxy-3-oic acid (**2**) (17.4 mg). Fraction B (27.0 g) was also studied using the same method, 382 fractions of 100 mL each were collected. Ceramid A (**4**) (23.2 mg) was obtained in the combined fractions 105-122, eluted with *n*-hexane-ethyl acetate (1:1), ceramid B (**5**) (15.6 mg) in the combined fractions 135-147, eluted with *n*-hexane-ethyl acetate (2:3). This same fraction B afforded glucosides of stigmasterol and β-sitosterol (14.8 mg) in the combined fractions 172-186, eluted with *n*-hexane-ethyl acetate (2:3), mayombensin (**6**) (9.6 mg) in the combined fractions 118-240, eluted with *n*-hexane-ethyl acetate (3:7), and aridanin (**7**) (27.3 mg) in the combined fractions 328-345, eluted with ethyl acetate-MeOH (39:1). Root powder of *L. mayombensis* was extracted by maceration with 12 L of MeOH at room temperature. After evaporation, 132.0 g of extract were obtained. The crude extract was subjected to column chromatography over silica gel 60 (70-230 mesh, ASTM, Merck), with a gradient system of *n*-hexane-ethyl acetate, ethyl acetate-methanol. A total of 289 fractions of *ca*. 200 ml each were collected. The pure compounds were obtained by direct crystallization. Fractions 50-67, eluted with *n*-hexane-ethyl acetate (4:1) gave 3,4-dimethyl-secotirucalla-4(28),7,24-trien-3,21-dioic acid (**3**) (13.1 mg). The combined fractions 98-106, eluted with *n*-hexane-ethyl acetate (1:1) gave cleomiscosin F (**1**) (9.6 mg). Fractions 148-161 eluted with *n*-hexane-ethyl acetate (2:3) precipitated at room temperature to give glucosides of stigmasterol and β-sitosterol (28.2 mg).

2.4. Spectroscopic data of compounds **1**-**7**

Cleomiscosin F (1) Yellow amorphous powder, R_f = 0.42 silica gel 60 F254 *n*-hexane-ethyl acetate (1:1); EI-MS *m/z* 386.1 $[M]^+$ ($C_{20}H_{18}O_8$); ^{1}H and ^{13}C NMR data (Table 1).

3,4-secotirucalla-4(28),7,24-trien-21-hydroxy-21,23-epoxy-3-oic acid (**2**) White amorphous powder, R_f = 0.53 silica gel 60 F254 *n*-hexane- ethyl acetate (3:2); HR-ESI-MS *m/z* $[M+Na]^+$ 493,3309 ($C_{30}H_{46}O_4$). ^{1}H NMR (500 MHz, C_5D_5N) 5.71 (d, 2.35, H-21); 5.50 (d, 8.15, H-24); 5.26 (d, H-7); 5.09 (m, H-23); 4.92, 4.90 (brs, H-28); 2.45 (m, H-20); 2.25 (m, H-22); 1.78 (s, H-29); 1.69 (s, H-26); 1.59 (s, H-27); 1.00 (s, H-30); 0.91 (s, H-18); 0.86 (s, H-19); 13C NMR (125 MHz, C5D5N) 176.0 (C-2); 149.4 (C-4); 145.9 (C-8); 131.9 (C-25); 126.4 (C-24); 118.0 (C-7); 113.8 (C-28); 102.0 (C-21); 74.3 (C-23); 49.3 (C-5); 51.3 (C-14); 51.2 (C-17); 49.7 (C-20); 43.7 (C-13); 40.8 (C-9); 39.4 (C-22); 36.9 (C-10); 34.0 (C-15); 32.6 (C-1); 32.0 (C-6); 28.8 (C-16); 27.6 (C-2); 27.2 (C-30); 25.5 (C-26); 22.8 (C-18); 22.5 (C-29); 18.2 (C-27); 18.0 (C-11); 15.8 (C-19).

3,4-secotirucalla-4(28),7,24-trien-3,21-dioic acid (3) White crystals; (-)-ESI-MS 469.4 $[M-H]^-$ ($C_{30}H_{46}O_4$) mp 231-233 °C; ^{1}H NMR (500 MHz, $CDCl_3$) 5.23 (brs, H-7); 5.08 (d, 8.15, H-24); 4.81, 4.80 (brs, H-28); 2.23 (m, H-20); 1.77 (s, H-29); 1.68 (s, H-26); 1.58 (s, H-27); 0.98 (s, H-30); 0.90 (s, H-18); 0.86 (s, H-19); ^{13}C NMR (125 MHz, $CDCl_3$) 183.4 (C-21); 181.2 (C-2); 145.8 (C-8); 145.5 (C-4); 144.1 (C-28); 132.5 (C-25); 123.5 (C-24);

Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound 1 in $CDCl_3$.

Position	^{1}H(m, *J* in Hz)	^{13}C
2	-	161.2
3	6.32 (d, 9.8)	113.6
4	7.66 (d, 9.8)	144.3
5	6.54 (s)	100.1
6	-	146.0
7	-	137.2
8	-	132.1
9	-	138.6
10	-	111.5
1′	-	126.8
2′	6.93 (s)	110.1
3′	-	146.7
4′	6.93 (s)	114.9
5′	-	147.1
6′	6.93 (s)	120.1
7′	4.99 (d, 7.9)	77.1
8′	4.08-4,11(m)	78.8
9′	3.54 (dd, 12.5 ; 7.0)	60.8
	3.82 (dd, 12.5 ; 3.5)	
OMe-6	3.90 (s)	56.3
OMe-5'	3.87 (s)	56.0

118.0 (C-7); 51.6 (C-14); 50.4 (C-5); 50.1 (C-17); 49.0 (C-20); 43.5 (C-13); 40.9 (C-9); 36.9 (C-10); 33.7 (C-15); 32.5 (C-22); 30.7 (C-1); 30.4 (C-6); 30.3 (C-12); 27.9 (C-2); 27.9 (C-16); 27.2 (C-30); 26.2 (C-23); 25.9 (C-26); 22.0 (C-29); 21.6 (C-18); 18.3 (C-11); 17.9 (C-27); 16.4 (C-19). **Ceramid A (4)** White amorphous powder; (+)-HR-ESI-MS *m/z* 704.6138 $[M+Na]^+$ ($C_{42}H_{83}NO_5$); ^{1}H NMR (500 MHz, C_5D_5N) NH 8.58 (d, 9); 5.10 (m, H-2′); 4.45 (dd, 3.9, 9, H-3′); 4.32 (d, 4.5, H-2); 4.24 (m, H-4′); $(CH_2)_n$ 1.21-2.27 ; CH_3 0.89 (6H). **Ceramid B (5)** White amorphous powder; (-)-HR-ESI-MS *m/z* 696.6103 $[M-H]^-$ ($C_{42}H_{82}NO_6$); ^{1}H NMR (500 MHz, C_5D_5N) NH 8.58 (d, 9); 5.10 (m, H-2′); 4.45 (dd, 3.9, 9, H-3′); 4.34 (m, H-3); 4.32 (d, 4.5, H-2); 4.24 (m, H-4′); $(CH_2)_n$ 1.21-2.27 ; CH_3 0.89 (6H). **Mayombensin (6)** White crystals; (-)-HR-ESI-MS 593.2583 $[M-H]^-$ ($C_{30}H_{42}O_{12}$); mp 209-210 °C. ^{1}H NMR (500 MHz, C_5D_5N) 7.33 (dq, 7.1, 1.4, H-3′); 6.54 (s, H-21); 6.11 (brs, H-11); 6.01 (t, H-1); 4.96 (brs, H-7); 4.96 (brs, H-15); 4.67-3.75 (d, 7.2, H-28); 4.56-3.57 (dd, 11.3, 2.7, H-23); 4.34-3.48 (m, H-22); 4.22 (d, 2.9, H-19); 4.16 (d, 12.6, H-5); 4.06 (brs, H-3); 3.97 (m, H-6); 3.48 (dd, 11.4, 2.0, H-22); 3.47 (brs, H-9); 2.66 (d, 5.23, H-17); 2.38 (s, H-18); 2.22 (brs, H-16); 1.81 (ddd, 11.6, 5.5, 2.9, H-16); 1.55 (s, H-30); 1.02 (s, H-29) ^{13}C NMR (125 MHz, C_5D_5N) 167.6 (C-1′); 137.8 (C-3′); 130.1 (C-2′); 101.9 (C-11); 95.0 (C-21); 93.1 (C-20); 79.6 (C-15); 77.9 (C-28); 75.6 (C-6); 75.4 (C-1); 74.5 (C-7); 72.3 (C-19); 70.8 (C-3); 70.6 (C-14); 60.0 (C-23); 66.8 (C-13); 59.5 (C-22); 53.1 (C-17); 50.6 (C-9); 49.9 (C-10); 46.0 (C-8); 44.8 (C-4); 35.2 (C-5); 28.1 (C-16); 32.6 (C-2); 22.7 (C-30); 20.5 (C-29); 18.7 (C-18); 14.7 (C-4′). **Aridanin (7)** White crystals; mp 278-280ºC ^{13}C NMR (125 MHz, C_5D_5N) 180.1 (C-28); 144.8 (C-13); 122.6 (C-12); 104.8 (C-1′); 89.2 (C-3); 78.2 (C-5′); 76.1 (C-3′); 72.8 (C-4′); 63.1 (C-6′); 58.2 (C-2′); 55.8 (C-5); 48.0 (C-9); 46.7 (C-17); 46.5 (C-19); 42.2 (C-14); 42.0 (C-18); 39.8 (C-8); 39.3 (C-4); 38.6 (C-1); 37.0 (C-10); 34.3 (C-21); 33.3 (C-22); 33.3 (C-29); 33.2 (C-7); 31.0 (C-20); 28.3 (C-15); 28.2 (C-23); 26.4 (C-2); 26.2 (C-27); 23.8 (C-16); 23.7 (C-11); 23.7 (C-30); 18.6 (C-6); 17.4 (C-26); 17.0 (C-24); 15.4 (C-25).

2.5. Evaluation of the antibacterial activities

Microbial suspensions of *Escherichia coli* (DSMZ 1058), *Bacillus subtilis* (DSMZ 704), *Pseudomonas agarici* (DSMZ 11810) and *Micrococcus luteus* (DSMZ 1605) obtained from the Deutche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany were subcultured in tubes containing nutrient broth. After 24 h, a quantity was introduced in 6 mL of distilled water, homogenized, turbidity adjusted to 0.5 McFarland standard, to obtain young colonies. The disc diffusion method was used to determine the susceptibility of microbial strains to compounds **3**, **4** and **5** dissolved in DMSO. The plates were pouring with 15 mL of nutrient broth, 1 mL of inoculum was then added uniformly, and discs previously impregnate with 25 µL of samples were placed on the medium. The plates were preincubated for 60 min in fridge to facilitate diffusion of compounds and then incubated at 37 °C for 24 h. The inhibition diameter was measured in mm, with gentamycin as positive control. Minimum inhibition concentration (MIC) of compounds and the positive control drug gentamycin were measured by the microdilution broth susceptibility assay (CLSI, 2017) against the same bacteria strains. The inocula were prepared from 12 h broth cultures, and suspensions were adjusted to 0.5 McFarland standard turbidity. The samples were dissolved in DMSO (10%) and diluted two-fold in sterile 96-well microtiter plates in duplicate, using brain heart infusion (BHI) broth. Standardized inocula of test strains were added, and after incubation at 37 °C for 24 h on a rotary shaker at 200 rpm, MICs were read as the lowest concentration with inhibition of the growth of the test organisms, compared to the positive control gentamycin and BHI broth containing DMSO (10%) as negative control.

3. Results and Discussion

The methanol extracts of seed and root of *L. mayombensis* were separated by column chromatography on silica gel to afford one new coumarinolignan cleomiscosin F (**1**), and ten known compounds which were identified by comparison with the reported data as 3,4-secotirucalla-4(28),7(8),24(25)-trien-21-hydroxy-21,23-epoxy-3-oic

acid (**2**) (Hernandez et al., 2018), 3,4-secotirucalla-4(28),7,24-trien-3,21-dioic acid (**3**) (Akinniyi et al., 1980), ceramid A (**4**), ceramid B (**5**), mayombensin (**6**) (Djeukeu et al., 2017), aridanin (**7**) (Feumo et al., 2016), stigmasterol, β-sitosterol, stigmasterol-3-*O*-β-D-glucopyranoside and β-sitosterol-3-*O*-β-D-glucopyranoside (Chaturvedula and Prakash, 2012; Khatun et al., 2012). To the best of our knowledge, this is the first report of a coumarinolignan in *Leplaea mayombensis* and in the genus. These results confirmed the phytochemical diversity of the plant on one hand with the presence of coumarinolignan and ceramid and in the order hand the occurence of bioactive terpenoids precisely secotirucallane and limonoids in the genus and in the Meliaceae family.

3.1. Structure elucidation of compound **1**

Compound **(1)** was obtained as yellow amorphous powder. The molecular formula was assigned as $C_{20}H_{18}O_8$ containing twelve degrees of unsaturation, by analysis of the EI-MS spectrum ($[M]^+$ *m/z* 386.1), ^1H and ^{13}C NMR spectrum. **1** gave positive respond to phenol test. It ^1H NMR spectrum showed the presence of AB system of two olefinic proton at δ_H 6.32 and 7.66 (^1H each, d, *J* = 9.8 Hz), one aromatic proton at δ_H 6.54 (s) which suggested the presence of 6,7,8 trioxygenated coumarin moiety (Rakesh and Mahendra, 2009). The ^1H NMR spectrum of **1** also displayed a singlet of three aromatic protons at δ_H 6.93 due to a 1,3,5 trisubstituted aromatic ring and upfield signals at δ_H 3.54 (dd, *J* = 12.5; 7.0 Hz); 3.82 (dd, *J* = 12.5; 3.5 Hz); 4.08-4.11 (m); 4.99 (d, *J* = 7.9 Hz) suggested the presence of a phenylpropanoid part (Rakesh and Mahendra, 2009). On this ^1H NMR spectrum, two singlets of three protons each at δ_H 3.87 and 3.90 corresponding to two methoxy groups are also presented. All these spectral data suggested that 1 possess a coumarinolignan skeleton (Ray et al., 1980; Kumar et al., 1988). This was further confirmed by the ^{13}C NMR spectrum which displayed 20 carbon signals, assignment by the aid of DEPT spectrum presented signals of two methoxy groups at δ_C 56.0 and 56.3, seven quaternary carbons amongst which one ester carbonyl at δ_C 161.2, eight methines with four aromatic carbons at δ_C 100.1, 110.1, 114.9, 120.1, two olefinic carbons of the AB system at δ_C 113.6 and 144.3, two sp3 oxymethine at δ_C 77.1, 78.8, and one hydroxymethylene at δ_C 60.8. Presence of two oxymethines confirmed the oxide linkage between coumarin moiety and the phenylpropanoid part, emphasized by cross peak on HMBC spectrum between proton at δ_H 4.99 (H-7′) with carbon at δ_C 120.1 (C-6′) and 110.1 (C-2′). The value of the coupling constant between protons H-7′ and H-8′ (*J* = 7.9 Hz) confirmed the trans position of these protons. The location of the two methoxy groups were determined by means of HMBC spectrum where cross peaks are observed between protons at δ_H 6.49 (H-5), 3.90 (OMe) and carbon at δ_C 146.0 (C-6) as well as between protons at δ_H 6.93 (H-6′), 3.87 (OMe) and carbon at δ_c 147.1 (C-5′), confirming positions C-6 and C-5′ for the two methoxy groups respectively. From all above, structure **1** was elucidated and given the trivial name cleomiscosin F.

3.2. *In vitro* antimicrobial activity

The isolated compounds (**3**), (**4**) and (**5**) were evaluated for their antibacterial potency against Gram positive and Gram-negative bacteria. The disk diffusion method was used first to select the more sensitive strains towards compounds. The results showed that none of the compounds was active against *Escherishia coli* while *Bacillus subtilis*, *Pseudomonas agarici*, and *Micrococcus luteus* were sensitive towards compounds (**3**) and (**4**). Compound (**5**) was inactive on all bacteria strains. The MIC values were then recorded for compounds (**3**) and (**4**), results are consigned in Table 2.

Table 2
Minimum inhibition concentrations (in μM) of compounds **3** and **4**.

Sample	Microorganims		
	B. subtilis	*P. agarici*	*M. luteus*
3	1.7	2.3	9.8
4	>250	11.9	>250
Gentamycin	2.5	8.2	17.1

Considering that the antimicrobial activity is significant if MIC < 25 μM (Cos et al., 2006), compound (**3**) has an activity comparable to gentamycin on *Bacillus subtilis* (MIC 1.7 μM), and is most active than gentamycin on *Pseudomonas agarici*, and *Micrococcus luteus* with MIC of 2.3, 9.8 μM respectively. These results are in accordance with previously reported works on secotirucallane compounds (Mambou et al., 2018). The side lipophilic chain of terpenoids is known to induce antibacterial activities (Sidjui et al., 2015). Compound (**4**) showed a significant activity on *Pseudomonas agarici* with MIC of 11.9 μM, no activities were observed on *Bacillus subtilis* and *Micrococcus luteus*. Ceramides are not well known for their antibacterial activities (Wouamba et al., 2017). The observed effect can be due to amide and hydroxyl groups for compound (**3**) while in compound (**4**) there are steric hindrance.

4. Concluding remarks

The chemical investigation of the methanol crude extract of seeds and roots of *Leplaea mayombensis* led to the isolation of eleven compounds including one new coumarinolignan. Two of the tested compounds were active against *Bacillus subtilis*, *Pseudomonas agarici*, and *Micrococcus luteus* with compound (**3**) displaying good activities comparable to positive control gentamycin.

Conflict of interest

The authors declare that there is no conflict of interest.

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