Jean Rodolphe Chouna, Jean-de-Dieu Tamokou, Pépin Nkeng-Efouet-Alango*, Bruno Ndjakou Lenta and Norbert Sewald

Antimicrobial triterpenes from the stem bark of Crossopteryx febrifuga

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Abstract: Phytochemical investigation of the stem bark extract of Crossopteryx febrifuga resulted in the isolation of epimeric mixtures of 3β-urs-12,20(30)-dien-27,28-dioic acid and 18-epi-3β-urs-12,20(30)-dien-27,28-dioic acid (1), as well as: 3β-D-glucopyranosylurs-12,20(30)-dien-27,28-dioic acid and 18-epi-3β-D-glucopyranosylurs-12,20(30)-dien-27,28-dioic acid (2), together with some known compounds such as the monoglyceride of palmitic acid, as well as β-sitosterol and its glucoside. The structures of the isolated compounds were determined by application of spectroscopic methods. The MeOH extract and compounds 1 and 2 were examined for antimicrobial activity in in vitro assays against bacteria (Enterobacter aerogenes ATCC13048, Escherichia coli ATCC8739, Klebsiella pneumoniae ATCC11296, Staphylococcus aureus) and fungi (Candida parapsilosis, Candida albicans ATCC 9002 and Cryptococcus neoformans IP 90526). The tested samples showed selective activities. The antibacterial and antifungal activities of compound 2 (MIC=8–64 μg/mL) were in some cases equal to or even higher than those of the respective reference drugs chloramphenicol (MIC=16–64 μg/mL) and nystatin (MIC=128–256 μg/mL).

Keywords: antimicrobial; Crossopteryx febrifuga; Rubiaceae; triterpenes.

1 Introduction

Crossopteryx febrifuga (Afzel. ex G. Don) Benth. (Rubiaceae) is a deciduous tree widely used in African traditional medicine for the treatment of several diseases such as dysentery, diarrhea and fevers [1–3]. Previous work reported analgesic, anti-inflammatory, antipyretic, antimicrobial, hypoglycemic, cytotoxic, antioxidant, antiplasmodial and antitrypanosomal properties of C. febrifuga extracts [1, 3–8]. Phytochemical screening and analysis of different extracts of C. febrifuga showed the presence of tannins, alkaloids, saponins, flavonoids, steroids, carbohydrates, phenolic compounds, cardiac glycosides, and anthraquinones [6, 9–13]. However; it is not yet known which of the phytoconstituents is/are responsible for the antimicrobial effect of the bark of this plant, when it is used to cure infectious diseases. Therefore, the present study was undertaken to fill this gap and now reports the antimicrobial activity of the MeOH extract of the stem bark of C. febrifuga and the isolated triterpenes.

2 Results and discussion

2.1 Phytochemical investigation

The air-dried, powdered stem bark of C. febrifuga was extracted with MeOH. The dry crude MeOH extract obtained was re-extracted with EtOAc, and the EtOAc soluble fraction subjected to repeated column chromatography over silica gel and/or Sephadex LH20. This process yielded the known compounds monoglyceride of palmitic acid, β-sitosterol and its glucoside, and the epimeric mixtures of the new ursane-type triterpene (1) and its glucoside (2).

Compound 1 was obtained as a brown amorphous solid. It gave a positive reaction in the Lieberman–Burchard
test, as usual for a triterpenoid. Its (-)-ESI-HRMS showed a chloride adduct ion peak at \( m/z \) 519.28973 [M+Cl]−, corresponding to the molecular formula \( C_{30}H_{48}O_{10}Cl \) (calcd. 519.28828) containing 9 degrees of unsaturation. The \( ^1H \) NMR spectrum of compound 1 exhibited characteristic signals for a pentacyclic triterpene with four methyl singlets at \( \delta \) 0.60; 0.67; 0.80; 0.88; one methyl group appearing as doublet at \( \delta \) 0.96 (J = 6.2 Hz), as well as one olefinic methine and one olefinic methylene proton at \( \delta \) 5.55 and 4.60, 4.70, respectively. The \( ^{13}C \) NMR data suggested that compound 1 consists of an ursa-12,20-diene \([\delta \ 132.2 \ (C-13); \ 128.4 \ (C-12) \ and \ \delta \ 105.8 \ (C-30); \ 152.3 \ (C-20)]\), with two carboxylic acid groups at \( \delta \ 175.9 \ (C-27) \ and \ \delta \ 177.6 \ (C-28) \), respectively [9, 14]. Additional signals of olefinic carbons at \( \delta \ 132.5 \ (C-13); \ 127.9 \ (C-12) \ and \ carboxylic \ carbons \ at \ \delta \ 176.1 \ (C-27), \ 178.2 \ (C-28) \) were also observed, which was due to the epimerization at C-18. In fact, for an oleanane-type triterpene (which may arise from some ursane-type triterpenes), the 18\( \alpha \) and 18\( \beta \) series can be recognized by inspecting the chemical shift of some characteristic carbons. The geometry of the D/E ring junction does not cause a significant alteration in the shielding of carbons in the A and B rings. The chemical shift of C-18 is sensitive to the change in the absolute configuration at C-18, and therefore influencing the chemical shift of neighbouring carbons like C-12, C-13, C-14, C-17, C-19, C-27 and C-28 [14].

The HMBC spectrum of 1 supported the proposed structure, showing correlations from H-12 to C-1, C-13, C-14, C-17, C-19 and C-28; from H-30 to C-19, C-20 and C-21; from H-26 to C-7, C-8, C-9, and C-14; and from H-23, H-24 to C-3. According to DEPT, HMQC and HMBC experiments and literature data, the structure of compound 1 was elucidated as a mixture of 18-epi-3\( \beta \)-urs-12,20(30)-diene-27,28-dioic acid and 3\( \beta \)-urs-12,20(30)-diene-27,28-dioic acid (1:3 ratio) (Figure 1A). The latter had been previously obtained after acid hydrolysis of the 28-O-\( \beta \)-D-glucopyranosyl ester of 3\( \beta \)-[\( \alpha \)-L-rhamnopyranosyl]-urs-12,20(30)-diene-27,28-dioic acid [9].

Compound 2 was isolated as a white amorphous solid. It gave a positive reaction in the Lieberman-Burchnard test, as usual for a triterpenoid. Its (+)-ESI-HRMS showed a sodium pseudomolecular ion peak at \( m/z \) 669.36128 [M+Na]+, corresponding to the molecular formula \( C_{36}H_{54}O_{10}Na \) (calcd. 669.36092), indicating 10 degrees of unsaturation. The \( ^1H \)-NMR spectrum of compound 2 shows signals for a glycosylated pentacyclic triterpene, with four methyl singlets at \( \delta \) 0.68; 0.86; 0.87; 0.88; one methyl group appearing as doublet at \( \delta \) 0.96, as well as one olefinic methine and one olefinic methylene proton at \( \delta \) 5.53 and 4.60; 4.68, respectively. Signals for the \( \beta \)-configured sugar fragment appear between \( \delta \) 4.15 and 3.01; with the anomeric proton at \( \delta \) 4.15 (1H, d, J = 7.8 Hz, H-1'). These data suggested that compound 2 contains 1 as aglycone. In fact, the \( ^{13}C \) NMR data of the aglycone moiety was almost identical to that of \( \delta \ 128.4/128.1 \ (C-12); \ 132.1/132.5 \ (C-13); \ 152.2 \ (C-20); \ 176.0/176.1 \ (C-27); \ 177.6/178.2 \ (C-28); \ 105.8 \ (C-30)]\). The structure of the sugar moiety was identified as glucose according to the \( ^{13}C \) NMR data, the 18\( \alpha \)-OH and 18\( \beta \)-OH values: 105.3 (C-1'), 76.8 (C-3'), 76.5 (C-2'), 73.9 (C-4'), 70.1 (C-5'), 61.2 (C-6') [12], and was linked at C-3 of the aglycone according to the HMBC (Figure 1B) correlation from H-3 to C-1'. From the above spectroscopic data, the structure of compound 2 was determined as a mixture of 18-epi-3\( \beta \)-D-glucopyranosyl-urs-12,20(30)-diene-27,28-dioic acid and 3\( \beta \)-D-glucopyranosyl-urs-12,20(30)-diene-27,28-dioic acid (1:3 ratio) (Figure 1A).

### 2.1.1 Antimicrobial assays

The MeOH extract and compounds 1 and 2 were examined in in vitro assays against bacterial and fungal species and the results are compiled in Table 1. Generally, the MeOH extract and the tested compounds 1 and 2 exhibited...
selective activities; their inhibitory effects being noted on 7 of 7, 5 of 7, and 7 of 7, respectively, of the studied microorganisms. *Klebsiella pneumoniae* ATCC11296 and *Cryptococcus neoformans* IP 90526 were the most sensitive bacteria and yeasts, respectively, while compound 2 was the most active sample tested. The lowest MIC value (8 μg/mL) was obtained with compound 2 on *K. pneumoniae* ATCC11296. This compound (MIC = 8–256 μg/mL) and the MeOH extract (MIC=256–1024 μg/mL) displayed antimicrobial properties against all the tested microorganisms. The antibacterial and antifungal activities of compound 2 (MIC=8–64 μg/mL) were in some cases equal or even higher than those of the two reference drugs, i.e. chloramphenicol (MIC=16–64 μg/mL) and nystatin (MIC=128–256 μg/mL), thereby highlighting its potent antimicrobial activity. Phytochemical screening by Halilu and coworkers [6] had previously revealed the presence of saponins and other metabolites; they also demonstrated the antimicrobial activities of the root bark extract of *C. febrifuga*. The present results not only confirm the claimed antimicrobial activity underlying the bark’s traditional uses, but also corroborate the results of Halilu and coworkers [6]. In addition, this work proves that the isolated ursane-type triterpenes and their β-glucosides are at least partially responsible for the antimicrobial activity of the extract on bacterial and fungal species.

### Table 1: Minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC) (μg/mL) of the crude extract and compounds 1 and 2 against fungal and bacterial species.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Inhibition parameters</th>
<th>MeOH extract</th>
<th>Compound 1</th>
<th>Compound 2</th>
<th>Reference drugs*</th>
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<tbody>
<tr>
<td><em>Enterobacter aerogenes</em> ATCC13048</td>
<td>MIC</td>
<td>1024</td>
<td>128</td>
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<td>64</td>
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<tr>
<td></td>
<td>MBC</td>
<td>1024</td>
<td>128</td>
<td>64</td>
<td>64</td>
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<tr>
<td></td>
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<td>1</td>
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<td>1024</td>
<td>128</td>
<td>32</td>
<td>64</td>
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<tr>
<td></td>
<td>MBC</td>
<td>2048</td>
<td>128</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC11296</td>
<td>MIC</td>
<td>512</td>
<td>64</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td>512</td>
<td>64</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>MBC/MIC</td>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em>**</td>
<td>MIC</td>
<td>1024</td>
<td>64</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td>1024</td>
<td>128</td>
<td>64</td>
<td>64</td>
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<tr>
<td></td>
<td>MBC/MIC</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>Candida parapsilosis</em>**</td>
<td>MIC</td>
<td>256</td>
<td>&gt;256</td>
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<td>256</td>
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<td></td>
<td>MFC</td>
<td>256</td>
<td>Nd</td>
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<tr>
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<td>MFC/MIC</td>
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<td>Nd</td>
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<td>1</td>
</tr>
<tr>
<td><em>Candida albicans</em> ATCC 9002</td>
<td>MIC</td>
<td>512</td>
<td>&gt;256</td>
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<td>MFC</td>
<td>512</td>
<td>Nd</td>
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<tr>
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<td>256</td>
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<td>128</td>
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<tr>
<td></td>
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</tr>
</tbody>
</table>

*nystatin for fungi and chloramphenicol for bacteria; nd, not determined.

**Pasteur Centre collection.

### 3 Materials and methods

#### 3.1 General experimental procedures

The EIMS spectra were recorded on a double focusing mass spectrometer (Varian MAT 311A). ESI-HRMS spectra were recorded on an Apex III (Bruker Daltonik) 7 Tesla (ESI-FT-ICR-MS). Optical rotations were recorded in MeOH solution on a Jasco DIP-360 digital polarimeter. The 1H NMR spectra and 13C NMR spectra were recorded on a Bruker DRX spectrometer operating at 500 MHz and 125 MHz, respectively, with TMS as an internal standard. Silica gel Merck 60 (0.063–0.200 mm) was used for column chromatography. Percolated aluminum backed silica gel 60 F254 sheets were used for TLC. Spots were visualized under UV light (254 nm) and (365 nm) or using a solution of molybdate/Ce4+ reagent followed by heating.

#### 3.2 Plant collection and identification

The stem bark of *C. febrifuga* was collected on February 2011 in Foumban (Western Region, Cameroon), and a specimen was identified and deposited in the Cameroon National Herbarium in Yaoundé (ref. Number 41791/HNC).
3.3 Extraction and isolation of compounds

The air dried stem bark of *C. febrifuga* (3 kg) was ground and exhaustively extracted by maceration in 11 L MeOH at room temperature for 72 h. The filtrate was evaporated to dryness to afford 223 g of extract. The MeOH extract was re-extracted by EtOAc to give a dry residue (50 g) after evaporation. The EtOAc extract (30 g) was subjected to silica gel column chromatography (0.063–0.200 mm, Merck, 206) eluted with a mixture of n-hexane-EtOAc (from 1 to 100 %, v/v) and EtOAc-MeOH (from 1 to 10 %) in increasing polarity, to yield 50 fractions of 150 mL each. Fractions 1 to 10 yielded the monoglyceride of palmitic acid (90 mg). Fractions 15–17 yielded β-sitosterol. Fractions 45–49, after column chromatography on silica gel (EtOAc-MeOH, from 1 to 10 %) followed by column chromatography on Sephadex LH20 yielded compound 1 (78 mg) in the fraction eluting with 4 % MeOH.

**Compound 1**: \( [\alpha]_D^0 = +106 \) (c 0.65 mg/mL, MeOH) \(^{13}C\)-NMR (DMSO ; 600 MHz): 175.9, 176.1 (C-27); 152.2 (C-20); 132.1, 132.5 (C-13); 128.4, 127.9 (C-12); 105.8 (C-30); 76.9 (C-5); 55.5, 55.5 (C-18); 54.9 (C-5); 55.2, 55.2 (C-14); 47.3, 47.2 (C-17); 66.0 (C-9); 39.0 (C-1); 38.4 (C-8); 38.3 (C-4); 38.0 (C-22); 36.2 (C-7); 36.2 (C-10); 34.8 (C-19); 31.3 (C-21); 28.2 (Me-23); 26.9 (C-25); 25.0 (C-16); 23.9 (C-15); 22.3 (C-11); 18.1 (Me-25); 17.9 (C-6); 16.0 (Me-26); 15.9 (Me-24); 15.8 (Me-29); 171.3 (IM). The EtOAc extract (30 g) was subjected to silica gel chromatography (0.063–0.200 mm, Merck, 206) eluted (50 g) after evaporation. The EtOAc extract (30 g) was subjected to silica gel chromatography. Fractions 39–42 yielded the glucoside of β-sitosterol. Fractions 45–49, after column chromatography on silica gel (EtOAc-MeOH, from 1 to 10 %) followed by column chromatography on Sephadex LH20 yielded compound 2 (78 mg) in the fraction eluting with 4 % MeOH.

**Compound 2**: \( [\alpha]_D^0 = +62 \) (c 0.6 mg/mL, MeOH) \(^{13}C\)-NMR (DMSO ; 600 MHz): 175.9, 176.1 (C-27); 152.2 (C-20); 132.1, 132.5 (C-13); 128.4, 127.9 (C-12); 105.3 (C-30); 87.9 (C-5); 55.5, 55.5 (C-18); 55.2 (C-5); 55.2, 55.2 (C-14); 47.3, 47.2 (C-17); 46.0 (C-9); 38.5 (C-1); 38.5 (C-8); 38.4 (C-4); 38.0 (C-22); 36.1 (C-7); 36.1 (C-10); 34.9 (C-19); 31.3 (C-21); 27.6 (Me-23); 25.5 (C-16); 25.0 (C-2); 23.9 (C-15); 22.3 (C-11); 18.0 (Me-25); 17.7 (C-6); 16.4 (Me-24); 16.0 (Me-26); 15.8 (Me-29); 105.2 (C-1′); 76.8 (C-3′); 76.5 (C-5′); 73.9 (C-4′); 70.1 (C-2′); 61.2 (C-6′). The EtOAc extract (30 g) was subjected to silica gel chromatography. Fractions 39–42 yielded the monoglyceride of palmitic acid (90 mg). Fractions 15–17 yielded β-sitosterol. Fractions 45–49, after column chromatography on silica gel (EtOAc-MeOH, from 1 to 10 %) followed by column chromatography on Sephadex LH20 yielded compound 2 (78 mg) in the fraction eluting with 4 % MeOH.

3.4 In vitro antimicrobial assay

3.4.1 Bacterial and fungal strains: The studied microorganisms were both reference (from the American Type Culture Collection) and clinical (from the Pasteur Institute Paris, France) strains of *E. aerogenes*, *E. coli*, *K. pneumoniae*, *Candida albicans*, and *C. neoformans*.

Also included were two clinical isolates of *C. parapsilosis* and *Staphylococcus aureus* collected from the Pasteur Centre (Yaounde, Cameroon). The bacterial and fungal species were grown at 37 °C and 28 °C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants, respectively.

3.4.2 Preparation of microbial inoculum: The inocula of fungi and bacteria were prepared from overnight cultures by picking numerous colonies and suspending them in sterile saline (NaCl) solution (0.9 %). Absorbance was measured at 530 nm for fungi or at 600 nm for bacteria and the densities adjusted with saline solution to match that of a 0.50 McFarland standard solution. From the prepared microbial solutions, further dilutions were prepared with saline solution to give a final concentration of 10⁶ cells/mL for fungi and 10⁶ CFU/mL for bacteria [15, 16].

3.4.3 Antimicrobial assay: The antimicrobial activity was investigated by determining the minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFCs).

MICs were determined by broth micro dilution [17, 18]. Stock solutions of the tested samples were prepared in 10 % v/v aqueous dimethylsulfoxide (DMSO) solution (Fisher Chemicals, Strasbourg, France) at a concentration of 4096 µg/mL. These were twofold serially diluted in Mueller-Hinton Broth (MHB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi to obtain a concentration range of 2048–0.25 µg/mL. For every experiment, a sterility check (10 % aqueous DMSO and medium), negative control (10 % aqueous DMSO, medium and inoculum) and positive control (10 % aqueous DMSO, medium, inoculum and water-soluble antibiotics) were included. One hundred microliters of each concentration was introduced into a microtiter plate well containing 90 µL of SDB or MHB, and 10 µL of inoculum was added to obtain a final concentration range of 4096–0.125 µg/mL. The plates were covered with a sterile lid, and incubated on a shaker at 37 °C for 24 h (bacteria) or 48 h (yeasts). MICs were assessed visually after the corresponding incubation period and were taken as the lowest sample concentration at which there was no growth or virtually no growth. Assays were performed in triplicate.

For determination of the minimum microbicidal concentration (MMC, MBC/MFC), 10 µL aliquots from each well in which no growth of microorganisms was observed, were plated on Mueller-Hinton Agar or Sabouraud Dextrose Agar and incubated at 37 °C for 24 h (bacteria) or 48 h (yeasts). The lowest concentration permitting no growth after sub-culture was taken as the respective MBC or MFC. Chloramphenicol (Sigma-Aldrich, Steinheim, Germany) for bacteria, and mycstatin (Sigma-Aldrich) for yeasts were used as positive controls.

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