In vitro antimalarial, antitrypanosomal and HIV-1 integrase inhibitory activities of two Cameroonian medicinal plants: *Antrocaryon klaineanum* (Anacardiaceae) and *Diospyros conocarpa* (Ebenaceae)

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Abstract

Antiplasmodial, antitrypanosomal and anti-HIV-1 activities of crude extracts, fractions and some isolated compounds from two Cameroonian medicinal plants: *Antrocaryon klaineanum* Pierre (Anacardiaceae) and *Diospyros conocarpa* Gürke ex K. Schum. (Ebenaceae) were assessed. The phytochemical studies led to the isolation of eight compounds (**1**-**8**) from *Diospyros conocarpa* and six compounds (**6, 9**-**13**) from *Antrocaryon klaineanum*. These compounds were identified as mangiferolic acid (**1**), 3β, 22(S)-dihydroxycycloart-24E-en-26-oic acid (**2**), lupeol (**3**), aridanin (**4**), betulin (**5**), betulinic acid (**6**), bergenin (**7**), D-quercitol (**8**), entilin C (**9**), entilin A (**10**), antrocarine A (**11**), 7R,20(S)-dihydroxy-4,24(28)-ergostadien-3-one (**12**) and stigmasterol glucoside (**13**). The criteria for activity were set as follows: an IC_{50} value < 10 µg/mL for crude extracts and < 1 µg/mL for pure compounds. The hexane/ethyl acetate (1:1) fraction of *A. klaineanum* root bark (AKERF1) and the hexane/ethyl acetate (1:1) fraction of *A. klaineanum* trunk bark (AKETF1) presented the strongest antiplasmodial activities with IC_{50} values of 0.4 and 4.4 µg/mL, respectively. Aridanin (**4**) and antrocarine A (**11**), as well as the crude extract of *D. conocarpa* roots (EDCR), AKERF1 and AKETF1 showed moderate trypanocidal effects. The crude extract of *A. klaineanum* root bark (AKER) and AKETF1 exhibited attractive activities on HIV-1 integrase with IC_{50} values of 1.96 and 24.04 µg/mL, respectively. The results provide baseline information on the use of *A. klaineanum* and *D. conocarpa* extracts, as well as certain components, as sources of new antiplasmodial, antitrypanosomal and anti-HIV drugs.

Keywords: antimalarial; antitrypanosomal; HIV-1; *Antrocaryon klaineanum*; *Diospyros conocarpa*

1. Introduction

Nowadays, parasitic diseases continue to be a threat to public health. In particular, two of these diseases cause serious mortality and morbidity in humans, the African trypanosomiasis or sleeping sickness, caused by *Trypanosoma brucei*, and the malaria, transmitted by *Plasmodium* species, of which the deadliest is *Plasmodium falciparum*. With the increase in the resistance of *P. falciparum* to several chemotherapeutic agents (Wellems and Plowe, 2001), malaria remains an important health concern in the countries infected by this scourge, particularly in Africa.

On the other hand, acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus type-1 (HIV-1), is still a serious threat to public health, with more than 35 million people infected worldwide (WHO, 2017). The African continent, and more particularly sub-Saharan Africa, is the most affected region, accounting for almost two thirds of the global total of new HIV-1 infections. In the fight against AIDS, inhibition of the HIV-1 integrase (IN) activity effectively suppresses virus replication, resulting in sustained clinical efficacy. However, there are only few HIV-1 IN inhibitors that are currently approved for clinical use (Pandey and Grandgenett, 2008). Searching for other IN inhibitors from natural sources could be a useful approach (Suedee et al., 2014).

Indeed, plant biodiversity and knowledge of traditional healing can potentially open up new ways in the field of drug discovery, as exemplified by the successful case of artemisinin. In this work, we investigated the activity of two Cameroonian plants selected based on ethno-botanical uses and bibliographical studies, namely *Antrocaryon klaineanum* Pierre (Anacardiaceae) and *Diospyros conocarpa* Gürke ex K. Schum. (Ebenaceae).

Antrocaryon klaineanum is recommended in traditional medicine to treat chlamydiae infections, wounds, back pain, liver diseases and female sterility (Betti, 2002; Matig et al., 2006). In Gabon, the powdered bark is used to cure liver complaints and to facilitate the production of

breast milk, while the roots are used to treat abdominal and liver complaints (Kémeuzé and Nkongmeneck, 2011).

A chemical investigation of the stem bark of *A. klaineanum* was undertaken and resulted in the characterization of six new sterols (Douanla et al., 2015) and the isolation of two known compounds, 7,20S-dihydroxyergosta-4,24(28)-dien-3-one (Tchouankeu et al., 1996) and 20S-hydroxyergosta-4,6,24(28)-trien-3-one, described previously in the literature as a semi-synthetic product (Roy et al., 1982). The antiplasmodial assays of *A. klaineanum* crude extract and its isolated compounds were also reported (Douanla et al., 2015), and recently we purified and characterized eight compounds, allowing the isolation of a new derivative (Fouokeng et al., 2017). Finally, *in vitro* anti-HIV activity has also been reported for plants of Anacardiaceae family (*Rhus parviflora, Rhus succedanea…*) (Lin et al. 1997; Modi et al., 2013).

Plants of the genus *Diospyros* are used in traditional medicine for the treatment of several ailments such as leprosy, skin eruptions, eye infections, cough, fever, diarrhea, dysentery, malaria and skin diseases (Mallavadhani et al., 1998; Pathak et al., 2004; Ganapaty et al., 2006; Maridass, 2008; Sinha and Bansal, 2008; Sutthivaiyakit et al., 2012; Choi et al., 2015). This plant family is likewise used in the treatment of parasitic diseases, more especially those transmitted by protozoa (Freiburghaus et al., 1997; Mallavadhani et al., 1998; Bizimana et al., 2006; Norhayati et al., 2013).

Previous phytochemical studies on the genus *Diospyros* resulted in the isolation of various classes of secondary metabolites including triterpenes, naphthoquinones, coumarins and phenolic glycosides (Maridass, 2008; Sinha and Bansal, 2008). Limited information is known about the chemical constituents of *D. conocarpa*. Only seven compounds have been isolated and characterized from the stem bark of this plant (Feusso et al., 2016). Finally, in modern medicine various metabolites from *Diospyros* species have shown potent inhibitory activity against HIV-I protease, such as ursolic acid, whereas other compounds from this genus, such as beta-amyrin,

betulin or diospyrin, exhibit moderate to potent cytotoxic actions on different carcinoma models (Sinba and Bansal, 2008).

In the present work, we carried out investigations on the antitrypanosomal, antimalarial and anti-HIV activities of the crude extract and isolated compounds from the two plants. In addition, cytotoxicity was evaluated to determine the selectivity/specificity of the plant samples.

2. Materials and methods

2.1. *Plant materials*

The trunk and root bark of *A. klaineanum* were collected on mount Eloumden in the central region of Cameroon in November 2014. The trunk and leaves of *D. conocarpa* were collected at Ntouessong in the central region in April 2013, whereas the roots were collected at Nkoemvone in the south region in January 2015. The plant identification was established by the members of the National Herbarium of Cameroon, where voucher specimens were deposited (No 21247SRF/CAM and 24030/SRF/CAM for *A. klaineanum* and *D. conocarpa*, respectively).

2.2. Extraction and isolation

2.2.1 *Extraction and isolation from D. conocarpa*

The different parts of *D. conocarpa* (leaves, trunk and roots) were air-dried at ambient temperature, then grounded and extracted at room temperature with methanol for 48 h. The extracts were concentrated in a rotary evaporator to afford 210.8, 300.0, and 123.2 g of crude extracts from 1.6, 8.5 and 2.4 kg of leaves, trunk and roots, respectively. The whole amounts of the three extracts were used for further separations and biological tests.

The leaf extract (EDCF) was subjected to chromatography on silica gel eluted with a *n*-Hex, a *n*-Hex/EtOAc gradient of increasing polarity and finally MeOH to yield four fractions: **A** (5.01 g), **B** (94.8 g), **C** (7.4 g) and **D** (35.6 g). Non-polar fraction **A** and polar fractions **C** and **D** have not been studied here. Fraction **B** was further subjected to silica gel column

chromatography eluted with an *n*-Hex/EtOAc gradient of increasing polarity. Elution with an *n*-Hex/EtOAc (7:1) gave **1** (30.0 mg) and with *n*-Hex/EtOAc (7:3) **2** (38.2 mg).

The trunk (EDCTr) and roots (EDCR) extracts were subjected to silica gel column chromatography eluting with a mixture of *n*-Hex/ EtOAc, pure ethyl acetate and a mixture of EtOAc/ MeOH. Elution of EDCTr extract with *n*-Hex/EtOAc (39:1) gave **3** (10.1 mg), and with EtOAc/ MeOH (19:1) afforded **4** (30.2 mg). For EDCR extract, elution with *n*-Hex/EtOAc (37:3) led to the isolation of **5** (9.4 mg) while **6** (12.5 mg) was isolated using *n*-Hex/EtOAc (9:1). Pure ethyl acetate was used to isolate **7** (23.5 mg) and **8** (918.0 mg) was isolated with EtOAc/MeOH (19:1).

2.2.2 *Extraction and isolation from A. klaineanum*

2.2.2.1. Extraction and isolation from trunk bark

The air-dried and powdered trunk bark of *A. klaineanum* (AKET) (8.5 kg) was submitted to extraction twice with methanol at room temperature for 48 h. After evaporation with a rotary evaporator under reduced pressure at 40 °C, 833.4 g of crude extract was obtained and was fractionated using vacuum chromatography into fractions **A** [hexane/ethyl acetate (1:0 and 1:1), 84.5 g], **B** [ethyl acetate, 48.7 g] and **C** [methanol, 638.2 g]. Fractions **B** and **C** have not been studied yet.

Fraction **A** (AKETF1) (84.5 g) was subjected to silica gel column chromatography and eluted with a gradient system of *n*-hexane/EtOAc (1:0 to 0:1); 475 sub-fractions (100 mL each) were collected and pooled on the basis of their TLC profile. Sub-fractions 32-101 obtained by eluting the column with *n*-hexane/EtOAc (97.5:2.5) afforded **9** (236.6 mg) and **10** (452.3 mg). Sub-fractions 102-205 (6.9 g) obtained by eluting the column with *n*-hexane/EtOAc (95:5 and 9:1) were further chromatographed on silica gel column and eluted with a gradient of *n*-hexane/EtOAc (0:1 to 8:2) to give **6** (237 mg). Elution with *n*-hexane/EtOAc (8:2) (232-254 sub-

fractions) gave **11** (63 mg) and **12** (108 mg). Finally, **13** (104 mg) was obtained from sub-fractions 406-460 using elution with *n*-hexane/EtOAc (1:1).

2.2.2.2. Extraction and isolation from root bark

The air-dried and powdered root bark of *A. klaineanum* (AKER) (7.5 kg) was subjected twice to extraction with methanol at room temperature for 48 h. After evaporation with a rotary evaporator under reduced pressure at 40 °C, 435.5 g of crude extract was obtained and was fractionated using vacuum chromatography into fractions **A** [hexane/ethyl acetate (1:0 and 1:1), 20.40 g], **B** [ethyl acetate, 170.35 g] and **C** [methanol, 180.25 g]. Only the fraction **A** (AKERF1) has been tested here.

2.3. Compound identification

The isolated compounds were characterized using various spectroscopic and spectrometric techniques such as 1D/2D-Nuclear Magnetic Resonance spectroscopy (NMR) and mass spectrometry (MS). The optical rotations were measured with a Perking-Elmer polarimeter (model 241) at the sodium D line (λ = 589 nm). Melting points were determined on a Melter FP61 melting point apparatus. The IR spectra were recorded on a FT/IR-4100 Jasco spectrometer. UV/Vis spectra were obtained on a Jasco V-650 spectrophotometer. The NMR spectra were recorded on a Varian Inova-600 NMR spectrometer at 600 MHz (^{1}H NMR) or 150 MHz (^{13}C NMR). Chemical shifts were given in δ values (ppm), and coupling constants are reported in [Hz]. HR-ESI mass spectra were obtained on a Bruker TOF LC-MS spectrometer. Open column chromatography was performed with silica gel (70-230 mesh). Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 F254 plates (Merck), and the TLC spots were viewed at 254 nm and visualized by heating the plates at 80 °C for 10 minutes after spraying with 50% aqueous sulfuric acid.

2.4. Physico-chemical properties of isolated compounds

Mangiferolic acid (**1**): white powder, ESI-MS: $[M-H]^-$ 455.4. ^{13}C NMR: 32.0 C-1; 30.4 C-2; 77.4 C-3; 40.4 C-4; 47.1 C-5; 21.1 C-6; 26.0 C-7; 47.8 C-8; 19.7 C-9; 26.1 C-10; 26.3 C-11; 32.1 C-12; 45.3 C-13; 48.8 C-14; 35.4 C-15; 26.1 C-16; 52.6 C-17; 18.2 C-18; 29.7 C-19; 36.1 C-20; 19.4 C-21; 34.8 C-22; 25.9 C-23; 125.8 C-24; 146.1 C-25; 169.6 C-26; 12.6 C-27; 18.2 C-28; 14.5 C-29; 19.3 C-30. ^1H NMR (300 MHz; $CDCl_3$): 1.28 (m) H-1; 1.77 (m) H-2; 3.28 (m) H-3; 1.32 (m) H-5; 1.61 (m) H-6; 1.30 (m) H-7; H-8; 1.20 (m) H-11; 1.63 (m) H-12; 1.35 (m) H-15; 1.10 (m) H-16; 1.64 (m) H-17; 0.98 (s) H-18; 0.32 (d, J = 4.2); 0.56 (d, J = 4.2) H-19; 1.30 (m) H-20; 0.94 (d, J = 7.2) H-21; 1.60 (m) H-22; 1.37 (m) H-23; 6.89 (t, J = 6.6; 1.2) H-24; 1.81 (s) H-27; 0.63 (s) H-28; 0.83 (s) H-29; 0.98 (s) H-30.

3β,22(R)-dihydroxycycloart-24E-en-26-oic acid (**2**): white powder, ESI-MS: $[M-H]^-$ 471.4. ^{13}C NMR: 33.4 C-1; 31.3 C-2; 78.0 C-3; 41.1 C-4; 47.5 C-5; 21.5 C-6; 28.6 C-7; 48.1 C-8; 20.1 C-9; 26.2 C-10; 26.6 C-11; 32.9 C-12; 45.8 C-13; 49.2 C-14; 35.8 C-15; 26.3 C-16; 53.3 C-17; 19.6 C-18; 29.9 C-19; 44.1 C-20; 14.9 C-21; 65.8 C-22; 26.8 C-23; 127.3 C-24; 146.6 C-25; 170.8 C-26; 13.2 C-27; 18.4 C-28; 26.6 C-29; 26.2 C-30. ^1H NMR (300 MHz; $CDCl_3$): 1.28(m) H-1; 1.76 (m) H-2; 3.56 (m) H-3; 1.31 (m) H-5; 1.61 (m) H-6; 1.30 (m) H-7; 1.53 (m) H-8; 1.20 (m) H-11; 1.63 (m) H-12; 1.35 (m) H-15; 1.10 (m) H-16; 1.64 (m) H-17; 1.06 (s) H-18; 0.32 (d, J = 4.1); 0.58 (d, J = 4.1) H-19; 1.30 (m) H-20; 0.91 (d, J = 7.2) H-21; 4.98 (t, J = 3.7) H-22; 1.35 (m) H-23; 7.51 (d, J = 6.7) H-24; 2.08 (s) H-27; 1.11 (s) H-28; 0.98 (s) H-29; 1.04 (s) H-30.

Lupeol (**3**): white fiber, ESI-MS: $[M+Na]^+$ 449.4. ^{13}C NMR: 38.7 C-1; 27.4 C-2; 79.0 C-3; 38.8 C-4; 55.3 C-5; 18.3 C-6; 34.3 C-7; 40.8 C-8; 50.4 C-9; 37.1 C-10; 20.9 C-11; 25.1 C-12; 38.0 C-13; 42.8 C-14; 27.4 C-15; 35.6 C-16; 43.0 C-17; 48.0 C-18; 48.3 C-19; 151.6 C-20; 29.7 C-21; 40.0 C-22; 28.0 C-23; 15.3 C-24; 15.9 C-25; 16.1 C-26; 14.5 C-27; 18.0 C-28; 109.3 C-29; 19.3 C-30.

Aridanin (**4**): white powder, ESI-MS: $[M-H]^-$ 658.5. ^{13}C NMR: 38.6 C-1; 26.4 C-2; 89.2 C-3; 39.3 C-4; 55.8 C-5; 18.6 C-6; 33.2 C-7; 39.8 C-8; 48.0 C-9; 37.0 C-10; 23.7 C-11; 122.6 C-12; 144.8 C-13; 42.2 C-14; 28.3 C-15; 23.8 C-16; 46.7 C-17; 42.0 C-18; 46.5 C-19; 31.0 C-20; 34.3 C-21; 33.3 C-22; 28.2 C-23; 17.0 C-24; 15.4 C-25; 17.4 C-26; 26.2 C-27; 180.1 C-28; 33.3 C-29; 23.7 C-30; 104.8 C-1'; 58.2 C-2'; 76.1 C-3'; 72.8 C-4'; 78.2 C-5'; 63.1 C-6'. ^1H NMR (300 MHz; pyridin-d_5): 1.20 (m) ; 0.82 (t) H-1; 2.09 (m) ; 1.72 (m) H-2; 3.23 (m) H-3; 0.76 (s) H-5; 1.25 (m) ; 1.15 (m) H-6; 1.15 (m) ; 1.24 (m) H-7; 1.62 (t) H-9; 1.83 (d) ; 1.87 (d) H-11; 5.50 (sl) H-12; 2.07 (m) H-15; 2.04 (t) H-16; 3.23 (m) H-18; 1.15 (m); 1.82 (m) H-19; 1.12 (d); 1.24 (d) H-21; 1.82 (m); 2.00 (t) H-22; 1.11 (s) H-23; 0.98 (s) H-24; 0.76 (s) H-25; 0.97 (s) H-26; 1.15 (s) H-27; 0.95 (s) H-29; 1.01 (s) H-30; 5.04 (d, J = 8.2) H-1'; 4.26 (m) H-2'; 4.22 (t ; J = 9) H-3'; 4.08 (t ; J = 9Hz) H-4'; 3.95 (m) H-5'; 4.18 (m) ; 4.38 (m) H-6'; 2.07 (s) OCH_3; 8.82 (d, J = 9) $NHCOOCH_3$.

Betulin (**5**): white powder, ESI-MS: $[M+H]^+$ 443.4. ^{13}C NMR: 38.8 C-1; 27.4 C-2; 79.0 C-3; 38.9 C-4; 55.3 C-5; 18.3 C-6; 34.3 C-7; 41.0 C-8; 50.4 C-9; 37.4 C-10; 20.9 C-11; 25.3 C-12; 37.2 C-13; 42.8 C-14; 27.1 C-15; 29.2 C-16; 47.8 C-17; 47.8 C-18; 48.8 C-19; 150.5 C-20; 29.8 C-21; 34.0 C-22; 28.0 C-23; 16.1 C-24; 16.3 C-25; 16.3 C-26; 16.1 C-27; 60.6 C-28; 109.7 C-29; 19.1 C-30.

Betulinic acid (**6**): white powder, ESI-MS: $[M+Na]^+$ 479.4. ^{13}C NMR: 39.0 C-1; 27.6 C-2; 78.2 C-3; 39.0 C-4; 55.5 C-5; 18.4 C-6; 34.5 C-7; 40.8 C-8; 50.7 C-9; 37.3 C-10; 21.0 C-11; 25.6 C-12; 38.2 C-13; 42.5 C-14; 30.4 C-15; 32.6 C-16; 56.3 C-17; 47.1 C-18; 49.4 C-19; 150.0 C-20; 29.9 C-21; 37.3 C-22; 27.9 C-23; 15.4 C-24; 16.2 C-25; 16.3 C-26; 14.6 C-27; 180.6 C-28; 108.8 C-29; 19.6 C-30.

Bergenin (**7**): colorless powder, ESI-MS: $[M-H]^-$ 327.4. ^{13}C NMR: 164.3 C-2; 119.5 C-3; 111.1 C-4; 152.7 C-5; 141.9 C-6; 149.3 C-7; 116.6 C-8; 73.9 C-9; 83.5 C-11; 72.1 C-12; 75.5 C-13; 81.3 C-14; 60.2 C-15; 62.6 C-16. ^1H NMR (300 MHz; pyridin-d_5): 7.75 (s) H-4; 5.12 (d, J =

10 Hz) H-9; 4.12 (s) H-11; 4.43 (t, *J* = 9.5; 10 Hz) H-12; 4.60 (t, *J* = 10.7; 2.5 Hz) H-13; 4.70 (dd, *J* = 9.5; 8.5 Hz) H-14; 3.96 (s) H-15; 4.09 (m) H-16.

D-quercitol (**8**): colorless powder, ESI-MS: $[M-H]^-$ 163.4. ^{13}C NMR: 69.0 C-1; 74.6 C-2; 71.1 C-3; 72.3 C-4; 68.6 C-5; 33.4 C-6. ^1H NMR (300 MHz; D_2O): 3.82 (m) H-1; 3.61 (t, *J* = 9.4Hz) H-2; 3.82 (m) H-3; 3.98 (t, *J* = 3.1Hz) H-4; 4.03 (t, *J* = 3.1Hz) H-5; 1.88 (ddd; *J* = 3.0; 0.7; 13); 2.03 (dt, *J* = 2.6; 10.3) H-6.

Entilin C (**9**): White powder, HRESI-MS: $[M+Na]^+$ 511.2 (calculated for $C_{27}H_{36}O_8$, 488.24102). ^1H NMR (300 MHz, $CDCl_3$): 3.76 (1H; d;1.7) H-3; 5.28 (1H, t, 1.6) H-5; 1,87; 1,97 (2H; t) H-11; 1.48 (2H, m) H-12; 2.03 (1H, t, 3.3) H-14; 1,91; 2.00 (2H, t) H-15; 5.50 (H, s) H-17 0.79 (3H, s) H-18; 1.83 (3H, s, 1.5) H-19; 7.33 (1H, s) H-21; 6.33 (1H, d, 1.8) H-22; 7.40 (1H, d, 1.70) H-23; 1.07 (3H, s) H-28; 1.16 (3H, s) H-29; 4.60 (1H, s) H-30; 1.91 (1H, m) H-2'; 1.01 (3H, d, 7.0) H-3'; 1.03 (3H, d; 7.0) H-4'; 3.59 (1H, s) 1-OH; 2.93 (1H, s) 2-OH; 3.42 (3H, s) 9-OMe.

Entilin A (**10**): White powder, HRESI-MS: $[M+Na]^+$ 488,24102 (calculated for $C_{28}H_{44}O_8$, 414.33932) ^{13}C NMR (75 MHz, $CDCl_3$): 98.8 C-1; 76.7 C-2; 81.0 C-3; 38.1 C-4; 133.8 C-5; 79.7 C-8; 97.6 C-9; 127.6 C-10; 30.9 C-11; 32.2 C-12; 35.4 C-13; 38.3 C-14; 22.6 C-15; 72.7 C-17; 24.5 C-18; 16.5 C-19; 124.4 C-20; 139.5 C-21; 10.6 C-22; 143.5 C-23; 27.8 C-28; 27.9 C-29; 30.9 C-30; 38.8 C-1'; 16.9 C-2'; 17.0 C-4'. ^1H NMR (300 MHz, $CDCl_3$): 3.76 (1H; d;1.7) H-3; 5,23 (1H; t; 1,6) H-5; 1,87; 1,97 (2H; t) H-11; 1.48 (2H, m) H-12; 2.03 (1H, t, 3.3) H-14; 1,91; 2.00 (2H, t) H-15; 0.79 (3H, s) H-18; 1.83 (3H, s, 1,5) H-19; 7.28 (1H, s) H-21; 6.29 (1H, dd, 1.8) H-22; 7.35 (1H, d, 1.71) H-23; 1.07 (3H, s) H-28; 1.16 (3H, s) H-29; 4.60 (1H, s) H-30; 1.91 (1H, m) H-2'; 1.01 (3H, d, 7,0) H-3'; 1.03 (3H, d; 7.0) H-4'; 3.59 (1H, s) 1-OH; 2.93 (1H, s) 2-OH; 3.28 (1H, s) 9-OH.

Antrocarine A (**11**): White powder, ESI-MS: $[M+Na]^+$ 451.4 (calculated for $C_{29}H_{48}O_2$) ^{13}C NMR (75 MHz, $CDCl_3$): 37.1 C-1; 31.4 C-2; 42.1 C-3; 123.9 C-4; 146.3 C-5; 65.4 C-6; 71.4 C-7; 37.6 C-8; 42.3 C-9; 37.4 C-10; 20.8 C-11; 39.2 C-12; 42.2 C-13; 49.5 C-14; 24.3 C-15; 28.3 C-16; 55.7 C-17; 11.7 C-18; 18.3 C-19; 35.8 C-20; 18.8 C-21; 24.8 C-22; 30.9 C-23; 156.9 C-24; 33.8 C-25; 22.0 C-26; 21.9 C-27; 106.6 C-28. ^1H NMR (300 MHz, $CDCl_3$) (σ (ppm), m, *J*(Hz)): 1.51, 1.91 (2H, t) H-1; 1.85, 1.93 (2H q) H-2; 2.32, 2.42(2H, m) H-3; 5.65, (1H, dd, (8, 5.4)) H-4; 3.91 (s, br) H-6; 3.91 (1H, d) H-6; 3.62 (1H, m) H-7 1.17 (1H, t) H-8; 1.35 (1H, t) H-9; 1.54; 1.61 (2H, q) H-11; 2.02; 2.1 (2H, t) H-12; 1.50 (1H, t) H-14; 1.74; 1.83 (2H, q) H-15; 1.38, 1.97 (2H, t) H-16; 1.26 (1H, t) H-17; 0.73 (3H, s) H-18; 1.05 (3H, s) H-19; 1.58 (1H, q) H-20; 0.99 (3H, d, 6.6) H-21; 1.65, 2.24) (2H, q) H-22; 2.13, 2.21(1H, d) H-23; 2.31(1H, m) H-25; 1.07 (3H, d, 6.6) H-26; 1.05 (3H, d, 6.6) H-27; 4.71; 4.78 (2H, d, 1.5) H-28.

7α,20(*S*)-dihydroxy-4,24(28)-ergostadien-3-one (**12**): white powder, ESI-MS: $[M+Na+2H]^+$ *m/z* = 437.3 (calculated for $C_{28}H_{44}O_2$ (413.3407)) ^{13}C NMR (75 MHz, $CDCl_3$): 35.3 C-1; 33.8C-2; 199.0 C-3; 127.8 C-4; 168.1 C-5; 41.0 C-6; 68.5 C-7; 39.0 C-8; 44.9 C-9; 38.4 C-10; 20.6 C-11; 39.5 C-12; 42.6 C-13; 50.5.5 C-14; 23.0 C-15; 22.3C-16; 57.7 C-17; 13.4 C-18; 19.9C-19; 75.0 C-20; 26.2 C-21; 42.4 C-22; 23.9 C-23; 156.1C-24; 33.9 C-25; 21.9 C-26; 21.9 C-27; 106.2 C-28. ^1H NMR (300 MHz, $CDCl_3$) (σ (ppm), m, *J*(Hz)): 1.75; 2.02, (2H, t), H-1; (2.36 (2H, q) H-2; 5.77, (1H, s) H-4; 2.40, 2,60 (1H, m) H-6; 3.95 (1H, q, 3.1) H-7; 1.55 (1H, m) H-8; 1.44 (1H, m) H-9; 1.44; 1.55(2H, m) H-11; 2.00 (2H, m) H-12; 1.36 (1H, m) H-14; 1.69 (2H, m) H-15; 1.90; 1.30 (2H, m) H-16; 1.118 (1H, m) H-17; 0.69 (3H,s) H-18; 1.27 (3H, s) H-19; 1.40 (1H, m) H-20; 0.93 (3H, d, 6.2) H-21; 1.15, 1.52(2H, m) H-22; 2.06, 1.87 (2H, m) H-23; 2.20 (1H, m) H-25; 1.00 (3H, d, 6.9) H-26; 0.99 (3H, d, 6.9) H-27; 4.69 4.78 (2H, d, 1.5) H-28.

β-sitosterol glucoside (**13**): Beige powder, Tf: 257-258°C; ESI-MS: $[M+Na+2H]^+$ *m/z* = 599.4 (calculated for $C_{35}H_{60}O_6Na$ (599.4255) ^{13}C NMR (75 MHz, $CDCl_3$): 38. 5 C-1; 33.5 C-2; 77.1 C-3; 37.0 C-4; 140.5 C-5; 121.3 C-6; 31.5 C-7; 31.6 C-8; 49.8 C-9; 36.4 C-10; 22.7 C-11; 41.9 C-

12; 42.0 C-13; 56.2 C-14; 25.0 C-15; 28.8 C-16; 55.5 C-17; 11.8 C-18; 19.3 C-19; 35.6 C-20; 18.8 C-21; 138.1 C-22; 129.0 C-23; 50.7 C-24; 24.0 C-25; 12.0 C-26; 29.4 C-27; 20.7 C-28 ; 100.9 C-1'; 73.5 C-2'; 77.1 C-3'; 70.2 C-4'; 76.9 C-5'; 61.2 C-6'. ^{1}H NMR (300 MHz, $CDCl_3$) (σ (ppm), m, *J*(Hz)): 1.24 (2H, m) H-1; (1.39 (2H, m) H-2; 1.93 (2H, dd) H-4; 5.32 (1H, dd) H-6; 1.80 (2H, m) H-7;) 1.50 (1H, m) H-8; 1.50 (1H, m) H-9; 1.50(2H, m) H-11; 1.50 (2H, m) H-12; 1.15 (1H, m) H-14; 1.80 (2H, m) H-15; 1.80 (2H, m) H-16; 1.24(1H) H-17; 0.82 (3H,s) H-18; 0.95 (3H, s) H-19; 2.12 (1H, dd) H-20; 0.90 (3H, d) H-21; 5.16 (1H, d) H-22; 5.03 (1H, m) H-23; 1.15 (2H, m) H-25; 1.50 (3H, H-26; 1.63 (1H, m) H-27; 0.76 (3H, dd) H-28; 0.99 (3H, d) H-29; 4.22 (d) H-1'; 2.90 (m) H-2'; 3.12 (m) H-3'; 3.06 (dd) H-4'; 3.02 (dd) H-5'; 3.64 (dd) H-6' 4.86 (t) 2'-OH; 4.89 (d) 3'-OH; 4.86 (t) 4'-OH; 4.42 (t) 6'-OH.

2.5. *Antiplasmodial activity*

Activity against *Plasmodium falciparum* chloroquine-sensitive 3D7 strain was assessed following a procedure already described (Desjardins et al., 1979). Malaria parasites were maintained in RPMI 1640 medium containing 2 mM L-glutamine and 25 mM Hepes (Lonza). The medium was further supplemented with 5% Albumax II, 20 mM glucose, 0.65 mM hypoxanthine, 60 µg/mL gentamycin and 2-4% hematocrit human red blood cells. The parasites were cultured at 37^{o}C under an atmosphere of 5% CO_2, 5% O_2, 90% N_2 in sealed T25 or T75 culture flasks. For screening samples against malaria parasites, a compound or natural extract was added to parasite cultures in 96-well plates and incubated for 48 h in a 37 ^{o}C CO_2 incubator. After 48 h, the culture plates were taken out from the incubator. Then, 20 µL of culture were removed from each well and mixed in a fresh 96-well plate with 125 µL of a mixture of Malstat solution and NBT/PES solution. The parasite lactate dehydrogenase (pLDH) activity was measured in these solutions. A purple product was formed when pLDH was present, and this product could be quantified by absorbance at 620 nm (Abs_{620}). The Abs_{620} reading in each well was thus an indication of the pLDH activity and thereby of the number of parasites in the well.

2.6. Antitrypanosomal activity

To assess antitrypanocidal activity, *in vitro* cultures of *Trypanosoma brucei brucei* in 96-well plates were carried out. After an incubation period of 48 h, the number of parasites surviving drug exposure was determined by adding a resazurin-based reagent. Resazurin is reduced to resorufin by living cells. Resorufin is a fluorophore ($Excitation_{560}/Emission_{590}$) and can be quantified in a multi-well fluorescence plate reader.

2.7. HIV-1 integrase strand transfer reaction assay

The HIV-1 subtype C integrase (CIN) strand transfer inhibition assay was adapted from a previously described method (Grobler et al., 2002). Briefly, 20 nM double-stranded biotinylated donor DNA (5'-5 Biotin TEG/ACCCTTTTAGTCAGTGTGGAAAATCTCTAGCA-3' annealed to 5'-ACTGCTAGAGATTTTCCACACTGACTAAAAG-3') was immobilized in streptavidin coated 96-well microtiter plates (R&D Systems, USA). Following incubation at room temperature for 40 minutes and, as stringent wash step, 5 µg/mL purified recombinant HIV-1 CIN in buffer 1 (50 mm NaCl, 25 mM Hepes, 25 mM $MnCl_2$, 5 mM β-mercaptoethanol, 50 µg/ml BSA, pH 7.5) was added to individual wells. Recombinant HIV-1 CIN was assembled onto the pre-processed donor DNA through incubation for 45 minutes at room temperature. Strand transfer reaction was initiated through the addition of 10 nM (final concentration) double-stranded FITC-labelled target DNA (5'-TGACCAAGGGCTAATTCACT/36-FAM/-3' annealed to 5'-AGTGAATTAGCCCTTGGTCA-/36-FAM/-3') in integrase buffer 2 (same as buffer 1, except 25 mm $MnCl_2$ replaced with 2.5 mm $MgCl_2$). After an incubation period of 60 minutes at 37°C, the plates were washed using PBS containing 0.05% Tween 20 and 0.01% BSA, followed by the addition of peroxidase-conjugated sheep anti-FITC antibody (Thermo Scientific, USA) diluted 1:1000 in the same PBS buffer. Finally, the plates were washed and peroxidase substrate (Sure Blue Reserve™, KPL, USA) was added to allow for detection at 620 nm using a Synergy

MX (BioTek®) plate reader. Absorbance values were converted to percentage of enzyme activity relative to the readings obtained from control wells (enzyme without inhibitor).

2.8. Cytotoxic activity

To assess the overt cytotoxicity, plant samples were incubated for 24 h, at concentration of 20 µM (for pure compounds) or 25 µg/mL (for extracts) in 96-well plates containing HeLa cells (human cervix adenocarcinoma), maintained in a culture medium made of Dulbecco's Modified Eagle's Medium (DMEM) with 5 mM L-glutamine (Lonza), supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin/fungizone - PSF). The number of cells surviving drug exposure was evaluated using the resazurin based reagent and resorufin fluorescence quantified ($Excitation_{560}/Emission_{590}$) in a multi-well plate reader.

2.9. Single concentration screening

The percent of parasitemia, or cell viability, was calculated at a fixed concentration of 20 µM for isolated compounds or 25 µg/mL for natural plant extracts. Experiments were performed in triplicate wells and a standard deviation (SD) was derived. For comparative purposes, chloroquine (an anti-malarial drug), emetine (which induced cell apoptosis) or pentamidine (an existing drug used in the treatment of trypanosomiasis) were used as positive control drug standards at a concentration of 10 µM for the two first drug or of 1 µM in case of pentamidine. Compounds were tested for HIV-1 integrase at a concentration of 20 µM (pure compounds) and 25 µg/mL (crudes extracts) and chicoric acid was used as positive control for HIV-1 integrase at a concentration of 20 µM.

2.10. Dose response

The IC_{50} (50% inhibitory concentration) of tested extract/compound exhibiting a low percentage viability was determined from the resulting dose-response curve by non-linear regression using Prism 5 program (Version 5.02, graph Pad Software, Inc). Chloroquine and

pentamidine were used as drug standards according to the type of test performed. IC_{50} values for cytotoxicity were not determined due to the low inhibition observed by the preliminary single concentration screening (see results). Extracts and isolated compounds were tested in a concentration range from 100 to 0.045 µg/mL or µM respectively using 3-fold serial dilutions for antiplasmodial tests and from 100 to 0.00001 µg/mL or µM respectively, with 10-fold serial dilutions for antitrypanosomal assays.

3. Results and discussion

3.1.Phytochemistry study

The different crude extracts of *D. conocarpa* (EDCR, EDCF and EDCTr) were extracted by silica gel column chromatography and preparative thin-layer chromatography, resulting in the isolation and characterization of eight pure compounds: mangiferolic acid (**1**), 3β,22(R)-dihydroxycycloart-24E-en-26-oic acid (**2**) (Anjaneyulu et al., 1999), lupeol (**3**) (Prachayasittikul et al., 2010), aridanin (**4**) (Adesina and Reisch, 1985), betulin (**5**) (Prachayasittikul et al., 2010), betulinic acid (**6**) (Yili et al., 2009), bergenin (**7**) (Nunomura et al., 2009) and D-quercitol (**8**) (Venkateswara Rao et al., 2014) (Figure 1).

The n-hexane/ethyl acetate (1:1) fraction (AKETF1), obtained by chromatography on the crude extract of *A. klaineanum* trunk bark, was separated by chromatography on silica gel column and thin-layer plates. Six pure compounds were isolated and identified, namely entilin C (**9**) (Daniewski et al., 1994), entilin A (**10**) (Tchouankeu et al., 1990), betulinic acid (**6**) (Yili et al., 2009), antrocarine A (**11**) (Douanla et al., 2015), 7α,20(*S*)-dihydroxy-4,24(28)-ergostadien-3-one (**12**) (Tchouankeu et al., 1996) and β-sitosterol glucoside (**13**) (Yili et al., 2009) (Figure 2).

3.2. Antiplasmodial activity

The two crude extracts of *A. klaineanum* [AKET (crude extract of trunk bark of *A. klaineanum*) and AKER (crude extract of stem bark of *A. klaineanum*)] did not reduce

significantly the viability of the parasite whereas AKERF1 [hexane/EtOAc (1:1) fraction of crude extract of root bark of *A. klaineanum*] and AKETF1 [hexane/EtOAc (1:1) fraction of crude extract of trunk bark of *A. klaineanum*] fractions showed the best antiplasmodial activities with IC_{50} values of 0.44 and 4.43 µg/mL, respectively, compared with chloroquine here used as a drug reference (IC_{50} 0.009 µM). No pure compounds significantly reduced the viability of the parasite (Table 1). The low activity of *A. klaineanum* crude extracts could be due to the presence of compounds having antagonistic effects, while the antiplasmodial activity of fractions AKERF1 and AKETF1 suggests that the compounds contained in these fractions might be acting in synergy (Chithambo et al., 2017). In another study, the crude extract of *A. klaineanum* stem bark also showed moderate antiplasmodial activity with an IC_{50} value of 16.7 µg/mL on the 3D7 *P. falciparum* strains (Douanla et al., 2015).

For crude extracts, IC_{50} values below 100 µg/mL were proposed as endpoint criteria in anti-infective assays (Cos et al., 2006), although most promising antimalarial extracts exhibit IC_{50} values under 10 µg/mL (Soh and Benoit-Vical, 2007; Krettli, 2009). Hence, as the hexane/EtOAc (1:1) fraction AKERF1 exhibited an IC_{50} value lower than 1 µg/mL against *P. falciparum*, this fraction represents undoubtedly a good candidate for further bioassay-guided fractionation. Finally, taking all together the results support the use of the bark of *A. klaineanum* in traditional medicines for treating malaria (Matig et al., 2006; Betti and Lejoly, 2010; Douanla et al., 2015).

The crude extracts of *D. conocarpa* roots and trunk [EDCR (crude extract of roots of *Diospyros conocarpa*) and EDCTr (crude extract of trunk of *Diospyros conocarpa*)], as well as the isolated compounds, showed low activities again *P. falciparum* strains with viability percentages (PVs) consistently greater than 90%. This is in contrast with the ethyl acetate extract of *Diospyros hispida* roots which displayed growth inhibitory activity with an IC_{50} value of 1 µg/mL against *P. falciparum* (Albernaz et al., 2010). According to the literature, the roots, barks,

heartwood and stems of some *Diospyros* species are used in traditional medicine to treat malaria (Kantamreddi and Wright, 2008; Mohamed et al., 2009; Prachayasittikul et al., 2010) but no antimalarial activity has been yet reported for *D. conocarpa*. Due to the low level of activity, its traditional use as antimalarial might not be ascribed to the extracts tested in our study.

3.2. Antitrypanosomal activity

According to the traditional pharmacopoeia, the trunk bark of *A. klaineanum* has antiparasitic activity. Besides the antiplasmodial activity described in the previous section, we proposed to assay the antitrypanosomal activity of this plant, in particular as some related species, such as *Magnifera indica* and *Spondias mombim*, exhibit antitrypanosomal effects (Nwodo et al., 2015).

The results showed that the three crude extracts, AKER, AKET and EDCTr, as well as the fraction AKERF1, exhibited moderate activities, with PVs of 26.75, 43.96, 52.42 and 4.48, respectively (Table 1). Fraction AKETF1 displayed an antitrypanosomal activity with an IC_{50} value between 10-1 µg/mL. Aridanin (**4**) and antrocarine A (**11**) exhibited trypanocidal activities on the *T. b. brucei* strain with IC_{50} values of 10-1 and 8.9 µM, respectively.

The two crude extracts, AKER and AKET, were less active than their related fractions AKERF1 and AKETF1. This could be due to the presence of compounds having antagonistic effects (Zhu et al., 1997). However, the strong activity of the antrocarine A (**11**), which is the major compound isolated from the fraction AKETF1, sustains the trypanocidal effect of this latter fraction (Table 1). The antitrypanosomal activity of aridanin (**4**) (PV= 0.59 ± 0.08) was comparable to that of the extract EDCR (PV= 1.04 ± 0.18), from which the compound was extracted (Table 2). These results confirm the use of the plant extracts of *Diospyros* genus in the treatment of parasitic diseases, more especially those transmitted by protozoa (Freiburghaus et al., 1997; Mallavadhani et al., 1998; Bizimana et al., 2006; Norhayati et al., 2013).

3.5. Cytotoxic activity

The crude extracts, some fractions and the pure compounds have been tested against HeLa cells. As the percentages of cell viability remained high (> 48%) for all tested samples, the IC_{50} values were not assessed. Hence, all the samples were non-cytotoxic against HeLa cervical cancer cells.

3.6. HIV-1 Integrase inhibitory activity

The results showed that all crude extracts except EDCR exhibited moderate HIV-1 IN inhibitory activity with IC_{50} values of 24.04, 17.39 and 1.96 µg/mL for EDCTr, AKET and AKER, respectively (Table 1). The fractions AKERF1 and AKETF1 also displayed a moderate HIV-1 IN inhibitory action with IC_{50} values of 35.08 and 3.60 µg/mL, respectively, compared with chicoric acid taken as a reference drug (IC_{50} 0.008 µM). Among the pure compounds tested, only aridanin (**4**) presented a moderate activity with an IC_{50} of 18.32 µM (Table1).

According to phytochemical screening studies, the *A. klaineanum* methanol extract contains steroids, flavonoids, tannins and phenolic compounds (Sima et *al.*, 2015; Fongang et *al.*, 2017). Some *Anarcadiaceae* species, such as *Spondias speciosa, Mangifera indica* and *Rhuspar viflora*, exhibited anti-HIV-1 effects (Guha et *al.*, 1996; Sahar et *al.*, 2009; Modi et *al.*, 2013).

The HIV-1 inhibitory activity observed for aridanin (**4**) (IC_{50} 18.32 µM) corroborated the effects of the EDCTr extract (IC_{50} 24.04 µg/mL). However, the crude extract of *D. Conocarpa* roots (EDCR) exhibited no significant activity with a PV of 105.64. The same trend was observed for lupeol (**3**), betulin (**5**) and betulinic acid (**6**) with PV of 96.95, 95.58 and 85.52, respectively. These later compounds are pentacyclic triterpenes belonging to the lupane serie. According to the literature, betulinic acid and its derivatives have been reported to inhibit HIV-1 entry and replication, HIV protease or the HIV reverse transcriptase (Fujioka and Kashiwanda, 1994; Mayaux et al., 1994; Pengsuparp et al., 1994; Xu et al., 1996; Dzubak et al., 2006). HIV-1

IN inhibitory activities were reported for the wood of *D. decandra*, the bark of *D. rhodocalyx* Kurz, the leaves of *D. chloroxylon* Roxb. and *D. Montana* Roxb. (Sahar et *al.*, 2009; Bunluepuech and Tewtrakul, 2009; 2011). Such activity was found here for the first time in plant extracts of *D. conocarpa* and *A. klaineanum*.

4. Conclusion

The *in vitro* antimalarial, antitrypanosomal and HIV-1 inhibitory integrase activities were assessed for different extracts of two Cameroonian plants. Interestingly, the performed extractions led to the isolation and the identification of 13 different chemical compounds that were additionally tested for their *in vitro* activities. Although all these compounds have already been isolated from other plants, we clearly established here their presence in the different extracts of *A. klaineanum* and *D. conocarpa*.

The *A. klaineanum* hexane/EtOAc (1:1) fractions of root and trunk barks, AKERF1 and AKETF1, showed the best antiplasmodial activities whereas on the contrary the corresponding crude extracts did not significantly decrease the viability of the parasite *P. falciparum*. The presence of chemicals with antagonistic effects in crude extracts could explain these results. In marked contrast, it was found that *D. conocarpa* extracts of roots and trunk, as well as all isolated compounds, had only little effects against *P. falciparum* growth. Therefore, the observed activities of AKERF1 and AKETF1 could result from synergetic action of chemicals or phytocomplexes present in these fractions. Furthermore, according our results the traditional use *D. conocarpa* as antimalarial would not be assigned to the two plant parts tested, but more likely to other parts of the plant.

The crude extract EDCR of *D. conocarpa*, fractions AKERF1 and AKETF1, as well as isolated compounds aridanin (**4**) and antrocarine A (**11**), all presented moderate trypanocidal activity, confirming thereby the antiprotozoal potential of *Antrocaryon* and *Diospyros* genus. In addition, the comparison between the parasiticidal and cytotoxicity effects suggests that the

decrease in viability of parasites may not be caused by a general cytotoxicity of the tested extracts. Concerning the anti-HIV potential of the plants, it is worthy of note that the AKER and AKETF1 fractions of *A. klaineanum* exhibited attractive inhibitory activity against HIV integrase. Finally, further studies are therefore necessary to determine putative new antiplasmodial, antitrypanosomal and/or anti-HIV agents for drug preparation.

Competing interest

The authors declare no conflict of interest.

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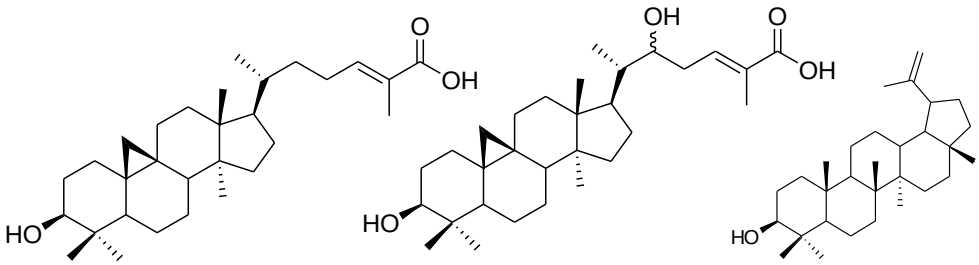
1 Figure 1 Chemical structures of compounds isolated from leaves, stems, and roots of D.
2 conocarpa



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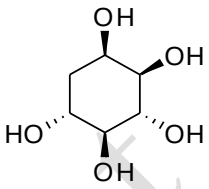
2 3b, 22(R ou S)-dihydroxycycloart-24E-en-26-oic acid

3 Lupeol



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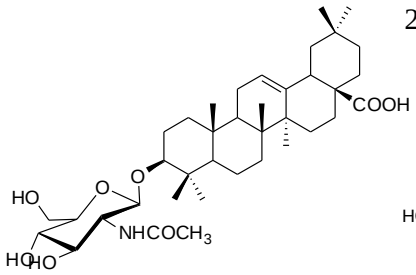

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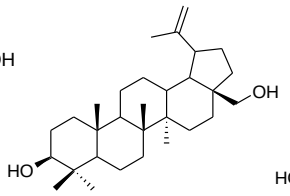

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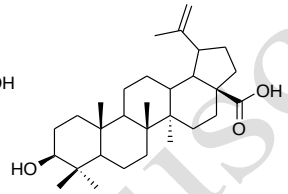

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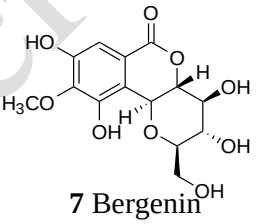

7 Bergenin

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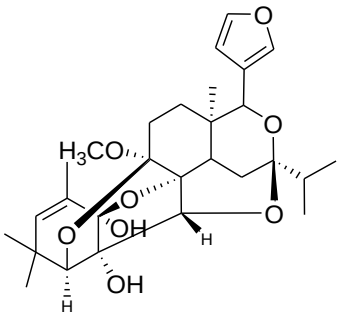
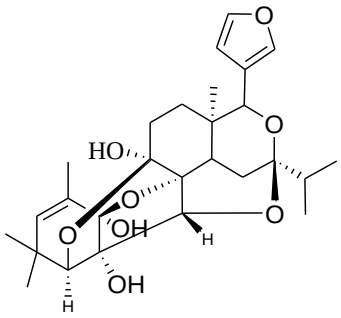
1 Figure 2. Chemical structures of compounds isolated from trunk bark of A. klaineanum



9 Entilin C



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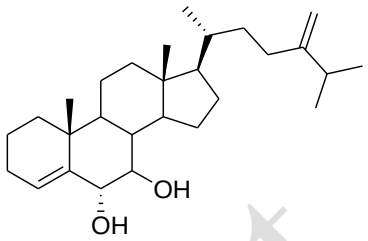
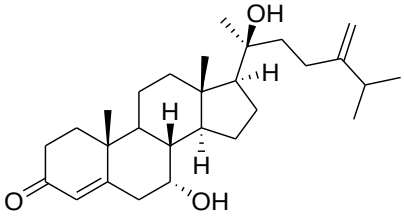


11 Antrocarine A



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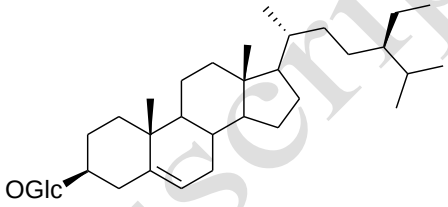

13 Stigmasterol-3-O-β-D-glucoside

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