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Antipromastigote and cytotoxic activities of some chemical constituents of *Hypericum lanceolatum* Lam. (Guttifereae)

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ABSTRACT

Phytochemical investigation of the ethanol extract of the stem bark of *Hypericum lanceolatum* Lam. (Guttifereae) afforded eight known compounds including 2,2′,5,6′-tetrahydroxybenzophenone (**1**), 5-hydroxy-3-methoxyxanthone (**2**), 3-hydroxy-5-methoxyxanthone (**3**), betulinic acid (**4**), hydroquinone (**5**) 6,7-dihydroxy-1,3-dimethoxyxanthone (**6**), calophyllumin A (**7**), and 1,3,5,6-tetrahydroxy-4-prenylxanthone (**8**). Compound **1** was submitted to acetylation reaction to give 5-acetoxy-2,2′,6′-trihydroxybenzophenone (**9**), a new hemisynthetic derivative. Compounds **5** and **8** were isolated for the first time from this plant. The structures were established by extensive analysis of their mass spectrometry and nuclear magnetic resonance (NMR) spectroscopic data and comparison with those from the literature. The isolated compounds (**1**, **2**, **4**, **5**, and **8**) and the derivative of benzophenone (**9**) were tested for their antipromastigote and cytotoxic activities against visceral leishmaniasis parasite *Leishmania donovani* and macrophage RAW 264.7 cell line, respectively. Compound **9** was the most active with an IC_{50} value of 6.1 µg/ml, while compounds **1**, **2**, **4**, and **8** were moderately active with IC_{50} values ranging from 11.4 to 34.8 µg/ml against *L. donovani* and were not cytotoxic except compound **5** that was very toxic and not active. The findings of the present study suggested that compounds **1**, **2**, **4**, and **8** could be considered as a potential source of therapeutic medicine for the treatment of leishmaniasis.

INTRODUCTION

Leishmaniasis is a noncontagious, infectious-parasitic disease, caused by protist parasites of the genus *Leishmania* and transmitted by the bite of a female phlebotomine sand fly. These neglected diseases are considered as a serious health concern, which is occurring in Africa, Asia, Southern Europe, and Latin America (WHO, 2016). According to the World Health Organization records, around 12 million people are infected with leishmaniasis, and 350 million people are considered at risk of acquiring an infection (WHO, 2016). The disease is spreading at an alarming rate with an estimated 2 million new cases occurring annually (WHO, 2016). Leishmaniasis can be categorized into different forms such as mucocutaneous leishmaniasis (MCL), visceral leishmaniasis, and cutaneous leishmaniasis (CL) (Alves *et al.*, 2003). Reported studies revealed about 0.5 and 1.5 million cases of visceral and CL, respectively (Ejazi and Ali, 2013; Shah *et al.*, 2014). Chemotherapy remains the mainstay for the control of leishmaniasis, as effective vaccines have not been developed (Den Boer *et al.*, 2011; Kedzierski, 2011). Available drugs based

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on pentavalent antimonial as sodium stibugluconate (Pentostam) and meglumine antimoniate (Glucantime) are the current treatment (Rodriguez *et al.*, 2015). However, these limited synthetic drugs require long-term treatments are of high toxicity with severe side effects, high cost, and face severe resistance associated with therapeutic failures (Duthie *et al.*, 2012; Essid *et al.*, 2015). This situation underlines the urgent need to search for new agents for the treatment of leishmaniasis. In recent years, the use of medicinal plants as an alternative for leishmaniasis treatment is quite common in endemic areas (Bahmani *et al.*, 2015).

The genus *Hypericum* belongs to the Guttiferae family and comprises about 400 species throughout the world except the deserts and low-altitude areas of tropical regions (Lewis, 2003; Meseguer and Sanmartin, 2012). In Cameroon, *Hypericum* genus is represented by six species including *Hypericum lanceolatum* Lam., which is a small shrub occurring in mountainous region of West Cameroon (Hutchinson and Dalziel, 1963). In traditional medicine, the leaves of the plant are extracted with palm wine and used for the treatment of skin infections, epilepsies, and tumors, while the roots are boiled in water and used to treat venereal diseases, gastrointestinal disorders, and infertility (Focho *et al.*, 2009). Diverse compounds have been isolated from this genus, including xanthones (Tanaka and Takaishi, 2006), naphtodianthrones, anthraquinones (Yu, 1998), flavonoids (Evangelos *et al.*, 2007; Yu, 1998), phenolics acid (Ming-Jaw *et al.*, 2004), benzophenones (Tanaka *et al.*, 2004), xantholignoids (Iinuma *et al.*, 1996), triterpenes (Seabra *et al.*, 1992), essential oils (Vera *et al.*, 1996), and prenylated derivatives of phloroglucinol (Bruneton, 1999). Previous phytochemical investigations of *H. lanceolatum* revealed the presence of benzophenones, xantholignoids, triterpenes, xanthones, steroids (Wabo *et al.*, 2012), essential oils (Vera *et al.*, 1996), phloroglucinols (Fobofou *et al.*, 2016), anthraquinone derivatives, and flavonoids (Yu, 1998). Moreover, reviews have reported the antimicrobial (Tchakam *et al.*, 2012) and antiplasmodial activities of *H. lanceolatum* (Zofou *et al.*, 2011). In this work, both *in vitro* antileishmanial activity and cytotoxicity effect of some constituents from *H. lanceolatum* leaves were evaluated. Furthermore, the hemisynthetic derivative was also evaluated against *Leishmania donovani* promastigote and macrophage RAW 264.7 cell line.

MATERIALS AND METHODS

General experimental procedures

Mass spectral data [Electron impact ionization mass spectroscopy (EIMS)] were recorded on a Finnigan MAT-95 spectrometer (70 eV) with perfluorkerosine as reference substance for high-resolution electrospray ionization time-of flight mass spectrometry (HR-ESI-TOF-MS) (Japan). Melting points of the isolated compounds were determined using an Electrothermal IA9000 Series digital melting-point apparatus (Bibby scientific, Great Britain) and were uncorrected. Ultraviolet and visible spectra were recorded in MeOH at 25°C using a Kontron Uvikon spectrophotometer. The infrared (IR) spectra were measured on a Perkin Elmer 1750 FTIR spectrometer. The nuclear magnetic resonance (NMR) spectra were measured on Bruker 300, 500, and 600 MHz NMR Avance II spectrometers equipped with cryoprobe, with TMS as an internal reference. Chemical shifts were recorded in δ (ppm), and the coupling constants (J) are in hertz (Hz). Silica gel 60 F_{254} (70–230; Merck; Darmstadt, Germany) was used for column chromatography. Precoated silica gel Kieselgel 60 F_{254} plates (0.25 mm thick) were used for thin-layer chromatography (TLC), and spots detected by spraying with 50% sulfuric acid (H_2SO_4) followed by heating at 100°C. All solvents were distilled before use.

Sample collection

The stem bark of *H. lanceolatum* was collected on the Mount Bamboutos flanks (Western Region, Cameroon) in May 2011. The sample identification was confirmed by Mr. Victor Nana, a botanist at the Cameroon National Herbarium in Yaounde, where a voucher specimen was deposited (Voucher No 32356/HNC).

Chemicals and reagents

RPMI-1640 Medium, Phosphate Buffered Saline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and dimethylsulfoxide (DMSO) were obtained from Sigma–Aldrich Co. Fetal bovine serum was purchased from GibcoInvitrogen.

Extraction and isolation

The dried stem barks of *Hypericum lanceolatum* (2.0 Kg) were extracted with ethanol (EtOH) (10 l) for three days at room temperature. The resulting extract was then concentrated with the aid of rotavapor to yield 50 g of crude extract.

This extract was subjected to a silica gel column chromatography and eluted with gradients of *n*-hexane-ethyl acetate (EtOAc) and EtOAc-methanol (MeOH). Forty-five fractions of 400 ml each were collected and combined on the basis of their TLC profiles into four major fractions (F1–F4). Fraction F1 (7 g) was purified on a silica gel column chromatography, eluted with a gradient of *n*-hexane-EtOAc to yield six major sub-fractions (F1A-F1F). Betulinic acid (**4**, 90 mg) crystallized from sub-fraction F1C in the mixture of *n*-hexane/EtOAc (9:1). Fraction F2 was purified on a silica gel column chromatography, eluted with a gradient of *n*-hexane-EtOAc to give hydroquinone (**5**, 10 mg) and 2,2′,5,6′-tetrahydroxybenzophenone (**1**, 50 mg). Fraction F3 was submitted to a silica gel column chromatography, eluted with gradients of dichloromethane (CH_2Cl_2)-EtOAc and EtOAc-MeOH to yield 5-hydroxy-3-methoxyxanthone (**2**, 9 mg). Fraction F4 was also chromatographed on a silica gel column with increasing mixtures of *n*-hexane-EtOAc. Sub-fractions eluted with *n*-hexane-EtOAc (8:2) were further purified on Sephadex LH-20 using CH_2Cl_2-MeOH (1:1) to give 6,7-dihydroxy-1,3-dimethoxyxanthone (**6**, 8.5 mg) and 3-hydroxy-5-methoxyxanthone (**3**, 15 mg). Sub-fractions eluted with *n*-hexane-EtOAc (1:1) were rechromatographed on silica gel with CH_2Cl_2-EtOAc (7:3) as a solvent system to yield 1,3,5,6-tetrahydroxy-4-prenylxanthone (**8**, 3.5 mg) and calophyllumin A (**7**, 4 mg).

In vitro antipromastigote assay

In vitro antileishmanial activity of compounds was investigated against *Leishmania donovani* promastigote using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide/ phenazinemethosulfate (MTS/PMS, Promega) colorimetric assay (Wong *et al.*, 2014). Briefly, stationary-phase promastigotes

were seeded into 96-well flat-bottomed microtiter plates at 1.10^7 parasites per well, in a final volume of 100 µl medium, and were incubated at 28°C with a series of concentrations of compounds, which dissolved in dimethyl sulfoxide and ranging from 12.5 to 200 µg/ml, in triplicate. After 72 hours of incubation, the plates were examined under an inverted microscope to assure the growth of the controls and sterile conditions and 10 µl of MTS/PMS was added to each well of the microtiter plate. The plate was then incubated at 28°C for color development. After 4 hours of incubation, the optical density (OD) values were read at 490 nm using an automated microtiter plate reader (TECAN Infinite M200 Pro Plate Reader, Austria). Amphotericin B was used as a positive control, while DMSO was used as a negative control. The experiments were conducted in triplicate. The percent growth inhibition was calculated from the absorbance relative to the negative control, and 50% cell cytotoxicity (IC_{50}) values were determined.

Cytotoxicity assay

A toxicological assessment was carried out against RAW cell lines using the resazurin assay and has been used to determine the selectivity indices of active compounds as described by Süzgeç-Selçuk *et al.* (2011). From sub-confluent cultures in 75 cm² culture flasks, they were trypsinized, counted, suspended in respective medium, and then seeded into triplicate wells of a 96-well plate (100 µl per well) at concentrations of 1.10^5 cells per ml and incubated. Cells were allowed to attach overnight and then treated in triplicate with 10 µl per well of 5-fold serially diluted individual compound (3.2–2,000 µg/ml) in the culture medium and incubated for 48 hours. After incubation, 10 µl of 2.5 mM of resazurin solution was added to each well and then incubated for 4 hours at 37°C. Fluorescence signal was measured using the microplate reader at the excitation and emission wavelengths of 530 nm and 590 nm, respectively. Curcumin was used as a positive control. Experiments were conducted in triplicate. The percent growth inhibition was calculated from the absorbance relative to the negative control, and the concentration of extract that inhibited 50% of cells (CC_{50} values) was determined. The selectivity index (SI) ratio (CC_{50} for macrophages/IC_{50} for amastigotes) was used to compare the toxicity of the compounds against the macrophages and their activity against the parasites.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). The IC_{50} and CC_{50} values were calculated fitting the data as a non-linear regression using a dose-response inhibitory model, in the GraphPad Prism 7.0 program.

RESULTS

Fractionation and isolation of compounds

Eight compounds were isolated from crude EtOH extract of stem bark of *H. lanceolatum* and identified as 2,2′,5,6′-tetrahydroxybenzophenone (**1**) (Wabo *et al.*, 2012), 5-hydroxy-3-methoxyxanthone (**2**) (Wabo *et al.*, 2012), 3-hydroxy-5-methoxyxanthone (**3**) (Wabo *et al.*, 2012), betulinic acid (**4**) (Wabo *et al.*, 2012), hydroquinone (**5**) (Muhammad *et al.*, 2017), 6,7-dihydroxy-1,3-dimethoxyxanthone (**6**) (Wabo *et al.*, 2012), calophyllumin A (**7**) (Iinuma *et al.*, 1996), and 1,3,5,6-tetrahydroxy-

4-prenylxanthone (**8**) (Wu *et al.*, 1998) (Fig. 1). Compound **9**, namely, 5-acetoxy-2,2′,6′-trihydroxybenzophenone, was obtained from the acetylation of 2,2′,5,6′-tetrahydroxybenzophenone (**1**), and its structure was deduced from different mass and NMR techniques. Among these compounds, compound **9** was considered as a new compound, additionally, compound **5** was isolated from the stem bark of *H. lanceolatum* for the first time. The spectral data of eight compounds were described in detail below, and the structures were shown in Figure 1.

2,2′5,6′-tetrahydroxybenzophenone (1)

Yellow needles from the mixture of hexane-AcOEt (90:10); m.p 201°C–202°C; IR (KBr), v_{max} (cm⁻¹): 3,367, 3,265, 1,637 (C=O), 1,527, 1,479; ¹H NMR (600 MHz, acetone-d_6), δ_H (ppm): 12.70 (1H, 2′-OH), 9.00 (1H, 2-OH), 7.68 (1H, t, J = 8.3 Hz, H-4′); 7.58 (1H, d, J = 3.0 Hz, H-6) ; 7.50 (1H, d, J = 9.0 Hz, H-4); 7.41 (1H, dd, J = 3.0, 9.0 Hz, H-4); 6.97 (1H, dd, J = 8.2, 1.0 Hz, H-5′), 6.75 (1H, dd, J = 8.2, 1.0 Hz, H-7); ¹³C NMR (150 MHz, acetone-d_6), $_C$ (ppm): 182.9 (C=O); 162.7 (C-2′); 154.9 (C-2); 157.3 (C-6′); 151.0 (C-5); 137.7 (C-4′); 126.1 (C-4), 120.1 (C-3); 110.5 (C-3′); 109.1(C-6); 109.1 (C-1′); 107.0 (C-5′). EIMS: m/z 228.0413 ([M-H₂O]⁺ (calcd for $C_{13}H_8O_4$ 228.0423). According to the literature Wabo *et al.* (2012).

5-hydroxy-3-methoxyxanthone (2)

Yellow needles from the mixture of hexane-AcOEt (90:10); m.p 166°C–168°C; IR (KBr), v_{max} (cm⁻¹): 3,326; 1,641 (C=O); 1,579 (C=C); 1,120; 864; ¹H NMR (600 MHz, DMSO-d_6), δ_H (ppm): 10.45 (1H, 5-OH), 7.65 (1H, d, J = 8.4 Hz, H-1); 7.60 (1H,dd, J = 6.4, 1.5 Hz, H-8) ; 7.55 (1H, d, J = 3.0 Hz, H-4); 7.46 (1H, dd, J = 8.4, 3.0 Hz, H-2); 7.32 (1H, dd, J = 6.4, 1.5 Hz, H-6), 7.25 (1H, t, J = 6.4 Hz, H-7); 3.95 (3H, s, 3-OMe); ¹³C NMR (150 MHz, DMSO-d_6), δ_C (ppm): 175.3 (C-9); 155.5 (C-3); 150 (C-4a); 146.5 (C-5); 145 (C-10a); 124.4 (C-2), 123.7 (C-7); 121.5 (C-8a); 121.2 (C-9a); 119.8 (C-1); 119.8 (C-6); 115 (C-8); 105.6 (C-4); 56.6 (3H, s, 3-OCH₃). EIMS: m/z (rel int.): 242 [M]⁺ (100); 241 (61); 227 (52); 228 (15); 213 (30); 212 (34); 199 (20); 171 (42); 115 (28). HREIMS: m/z 242.0413 ([M]⁺ (calcd for $C_{14}H_{10}O_4$ 242.0579). According to the literature Wabo *et al.* (2012).

3-hydroxy-5-methoxyxanthone (3)

Yellow needles from the acetone; m.p 284°C–285°C; IR (KBr), $_{max}$ (cm⁻¹): 3,218; 1,623; 1,593; 1,500; 864; ¹H NMR (600 MHz, DMSO-d_6), δ_H (ppm): 7.71 (1H, dd, J = 8.1, 5.0 Hz, H-8); 7.60 (1H,d, J = 9.1 Hz, H-1) ; 7.49 (1H, brd, J = 8.2 Hz, H-6); 7.47 (1H, d, J = 3.0 Hz, H-4); 7.37 (1H, t, J = 8.0 Hz, H-7); 7.32 (1H, dd, J = 9.0, 2.9 Hz, H-2); 3.99 (3H, s, 5-OMe); ¹³C NMR (150 MHz, DMSO-d_6), δ_C (ppm): 175.8 (C-9); 154.0 (C-3); 149.0 (C-5); 148.4 (C-4a); 145.8 (C-10a); 124.5 (C-2), 123.5 (C-7); 121.5 (C-8a); 121.1 (C-9a); 119.6 (C-1); 116.3 (C-8); 115.8 (C-6); 108.3 (C-4); 56.1 (3H, s, 5-OCH₃). EIMS: m/z (rel int.): 242 [M]⁺ (100), 241 (61), 227 (52). HREIMS: m/z 243.0650 ([M+H]⁺ (alcd for $C_{14}H_{10}O_4$ 243.6519). According to the literature Wabo *et al.* (2012).

Betulinic acid (4)

White powder from the mixture of hexene-EtOAc (90:10); m.p. 319°C–320°C; IR (KBr), v_{max} (cm⁻¹): 3,456 (OH);
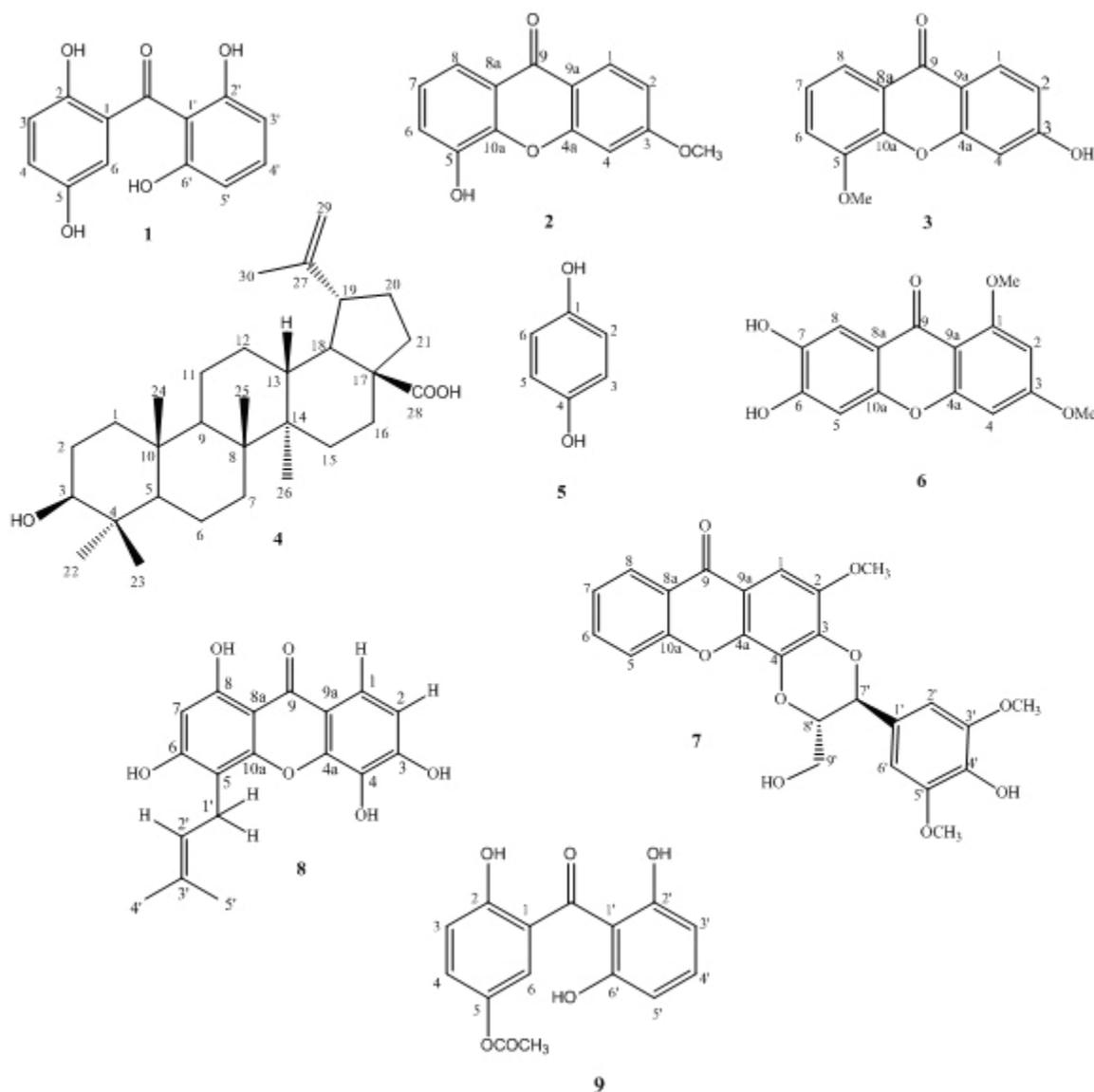
Figure 1. Chemical structure of the isolated compounds (**1–9**) from *H. lanceolatum*.

2,966; 1,685 (C=O); 1,627; 1,512; 1,260; 1,175; 759; ^{1}H NMR (500 MHz, CDCl$_3$), δ_H (ppm): 0.60 (3H, s, H-24); 0.68 (3H, s, H-25); 0.80 (3H, s, H-26); 0.81 (3H, s, H-23); 0.85 (3H, s, H-27); 1.55 (3H, s, H-30); 3.00 (1H, m, H-3); 3.80 (1H, sl, H-29a); 4.60 (1H, sl, H-29b); ^{13}C NMR (100 MHz, CDCl$_3$), δ_C (ppm): 14.6 (C-27); 15.3 (C-24); 15.9 (C-26), 16.1 (C-25); 18.0 (C-6), 19.3 (C-30), 20.6 (C-11); 25.3 (C-2); 26.7 (C-12); 28.0 (C-23); 29.4 (C-15); 30.3 (C-21); 32.0 (C-16); 34.1 (C-7); 36.9 (C-10); 37.0 (C-22); 38.1 (C-4); 38.4 (C-1); 38.5 (C-13); 40.4 (C-14); 42.1 (C-8); 46.8 (C-19); 49.1 (C-18); 50.3 (C-9); 55.2 (C-5); 56.0 (C-17); 78.6 (C-3); 109.7 (C-29); 150.3 (C-20); 179.5 (C-28). EIMS: *m/z* 456 ([M]$^+$ for C$_{30}$H$_{48}$O$_3$. According to the literature Wabo *et al.* (2012).

Hydroquinone (5)

Light brown cristal; m.p. 171°C–172°C; IR (KBr), ν$_{max}$ cm^{-1}: 3,595; 2,921; 1,590; 1,465; .^{1}H NMR (500 MHz, CD$_3$OD), $_H$ (ppm): 6.74 (2H, dd, *J* = 6.0, 2.0 Hz, H-2 et H-8); ^{13}C NMR

(125 MHz, CD$_3$OD), δ_C (ppm): 146.3 (C-1 & C-4); 120.9 (C-2 & C-6); 116.4 (C-3 & C-5). HREIMS: *m/z* 110.1102 ([M]$^+$ (calcd for C$_6$H$_6$O$_2$ 110.1106). According to the literature Muhammad *et al.* (2017).

6,7-dihydroxy-1,3-diméthoxyxanthone (6)

Yellow powder in the acetone; IR (KBr), ν$_{max}$ cm^{-1}: 3,330; 1,633 (C=O); 1,581 (C=C); ^{1}H NMR (400 MHz, Pyridine-d_5), δ_H (ppm): 10.82 (1H, s, 7-OH); 10.50 (1H, s, 6-OH); 7.36 (1H, s, H-8) 6.79 (1H, s, H-5); 6.36 (1H, s, H-2); 6.31 (1H, s, H-4); 3.80 (3H, s, 1-OCH$_3$); 3,61 (3H, s, 3-OH). ^{13}C NMR (100 MHz, Pyridine-d_5), δ_C (ppm): 175.3 (C-9); 155.5 (C-3); 150 (C-4a); 146.5 (C-5); 145 (C-10a); 124.4 (C-2), 123.7 (C-7); 121.5 (C-8a); 121.2 (C-9a); 119.8 (C-1); 119.8 (C-6); 115 (C-8); 105.6 (C-4); 56.6 (3H, s, 3-OCH$_3$). HREIMS: *m/z* 288.0635 ([M]$^+$ (calcd for C$_{15}$H$_{12}$O$_6$ 288.0634). According to the literature Wabo *et al.* (2012).

Calophyllumin A (7)

Yellow amorphous powder from the mixture CH_2Cl_2-MeOH (98:2); IR (KBr), ν_{max} (cm^{-1}): 3,161; 2,956; 1,728; 1,647, 1,566; 1,487; 1,103; 1,110; 918; ^{1}H NMR (600 MHz, Pyridine-d_5), δ_H (ppm): 13.01 (1H, s, 1-OH); 11.05 (1H, s, 3-OH); 8.59 (1H, s, 4'-OH), 7.11 (1H,s, H-8); 6.77 (2H, s, H-2' et H-6'); 6.38 (1H, d, J = 2,0 Hz, H-4); 6.19 (1H, d, J = 2.0 Hz, H-2); 5.08 (1H, t, J = 7.8 Hz, 9'-OH); 5.04 (1H, d, J = 7.8 Hz, H-7'); 4.41 (1H, m, H-8'); 3.83 (3H, s, 7-OCH$_3$); 3.75 (6H, s, 3'-OCH$_3$, 5'-OCH$_3$); 3.70 (1H, dl, J = 12.7 Hz, H-9'a); 3.43 (1H, dd, J = 12.7 et 4.4 Hz, H-9'b); ^{13}C NMR (125 MHz, Pyridine-d_5), δ_C (ppm): 180.6 (C-9); 167.3 (C-3); 164.7 (C-1); 158.9 (C-4a); 149.9 (C-3'); 149.8 (C-5'); 147.2 (C-7); 142.0 (C-10a); 141.2 (C-6); 136.4 (C-4'); 134.0 (C-5); 126.7 (C-1); 114.0 (C-8a); 106.6 (C-2'); 103.5 (C-6'); 99.7 (C-9a); 97.2 (C-2); 95.3 (C-8); 95.3 (C-4); 80.1 (C-8'); 78.5 (C-7'); 61.4 (C-9'); 56.1 (7-OCH$_3$); 56.7/56.6 (3'-OCH$_3$). HRFABMS: m/z 497.1106 ([M+H]$^+$ (calcd for $C_{25}H_{22}O_{11}$ 497.1084). According to the literature Iinuma *et al.* (1996).

1,3,5,6-tetrahydroxy-4-prenylxanthone (8)

Yellow powder from a mixture of hexane-AcOEt (9:1); m.p 197°C–198°C; ν_{max} (cm^{-1}): 3,420 (OH); 1,652 (C=O); 1,600; 1,575 (C=C); 1,529; 1,465; 1,346; 1,280; 1,182; 1,083; 811; ^{1}H NMR (600 MHz, MeOD), δ_H (ppm): 7,54 (1H, d, J = 8.7 Hz, H-8); 6.84 (1H, d, J = 8.7 Hz, H-7); 6.18 (1H, s, H-2); 5.32 (1H, t, J = 6,9 Hz, H-2'); 3.52 (2H, d, J = 6.9 Hz, H-1'); 1.83 (3H, s, 4'-Me); 1.63 (3H, s, 5'-Me); ^{13}C NMR (150 MHz, MeOD), δ_C (ppm): 180.7 (C-9); 162.8 (C-3); 160.8 (C-1); 154.7 (C-4a); 151.6 (C-10a); 146.7 (C-6); 132.5 (C-8a); 130.7 (C-3'); 122.2 (C-2'); 115.9 (C-8); 113.3 (C-5); 111.8 (C-7); 106.8 (C-4); 101.6 (C-9a); 96.8 (C-2), 24.5 (3H, s, 4'-Me); 20.9 (2H, s, H-1'); 16.6 (3H, s, 5'-Me). EIMS: m/z 328 ([M]$^+$ for $C_{18}H_{16}O$ According to the literature Wu *et al.* (1998).

Chemical modification of compound 1

2,2',5,6'-tetrahydroxybenzophenone (**1**) (20 mg) was dissolved in pyridine (3 ml) and acetic anhydride (3 ml) and stirred at room temperature for 24 hours. Ten milliliters of water were added to the mixture and stirred for 30 minutes. Extraction with CH_2Cl_2 and purification over a silica gel column with *n*-hexane-AcOEt (7:3) as solvent gave 5-acetoxy-2,2',6'-trihydroxybenzophenone (**9**) (5 mg) (Kopa *et al.*, 2014). Compound **9** was obtained as yellow crystals having a melting point of 246°C–247°C. Its IR spectrum exhibited strong absorptions at 1,610 cm^{-1} (OH) and 1,757 cm^{-1} corresponding to typical vibration bands of a conjugated carbonyl and an ester carbonyl, respectively. Its molecular formula was deduced as $C_{32}H_{38}O_8$ from the rigorous analysis of its 1D and 2D NMR data jointly with its EIMS spectra, which did not show the peak of the molecular ion but that of a fragment ion at m/z 270 [M-H$_2$O]$^+$ corresponding to the loss of a molecule of H$_2$O. Another important fragment ion was observed at m/z 228 [M-COMe-H$_2$O+H]$^+$. The comparison of the molecular weight of this compound with that of 2,2',5,6'-tetrahydroxybenzophenone (**1**) thus reveals a difference in mass of m/z 43 corresponding to the molar mass of an acetyl unit. 1D and 2D NMR data of compound **9** are almost identical to those of its precursor 2,2',5,6'-tetrahydroxybenzophenone (**1**). The main

differences are observed on the ^{1}H NMR spectrum of compound **9** by the disappearance of the signal of a hydroxyl group in favor of a characteristic methyl signal of an acetyl group at δ_H 2.36 (3H, s). This is confirmed by the appearance on the ^{13}C NMR spectrum of compound **9** of two additional signals at δ_C 181.7 and δ_C 21.0, respectively, corresponding to carbonyl and methyl of an acetyl group. Moreover, when comparing the data from the ^{13}C NMR spectra of the two compounds, we can clearly see that the chemical shifts of carbons C-5 and C-4 have undergone a decrease of δ_C − 5.2 and an increase of δ_C + 4.9, respectively, and the chemical shift of the H-4 proton evolved toward the weak fields (δ_H + 0.63). This can only be explained by the presence of the C-5 acetate group, and the fact that the H-4 proton is strongly deshielded by the proximity of the carbonyl group of the acetate moiety. This position of MeO-group at C-5 instead of C-2 or C-2' is confirmed by the HMBC correlation of the methyl protons of the acetyl group with the carbon at C-5 (δ_H 146.7).

According to the literature, these different observations allowed us to identify this compound as 5-acetoxy-2,2'6'-trihydroxybenzophenone (**9**), which is a semisynthetic derivative characterized here for the first time.

5-acetoxy-2,2',6'-trihydroxybenzophenone (9)

Yellow needles; mp 246°C–247°C; IR (KBr): ν_{max} cm^{-1}: 3,064, 1,757, 1,610, 1,577, 1,485; ^{1}H NMR (500 MHz, CDCl$_3$): δ_H 12.70 (1H, s, 2'-OH), 9.00 (1H, s, 2-OH), 7.98 (1H, s, H-6), 7.61 (1H, t, J = 8.3 Hz, H-4'), 7.50 (2H, J = 8.3 Hz, H-3 and H-4), 6.83 (1H, d, J = 8.3 Hz, H-3'), 6.81 (1H, d, J = 8.3 Hz, H-5'), 2.36 (3H, s, H$_3$COCO-5); ^{13}C NMR (125 MHz, CDCl$_3$): δ_C 181.7 (C=O), 169.3 (H$_3$COCO), 161.9 (C-6'), 156.3 (C-2'), 153.7 (C-2), 146.7 (C-5), 110.7 (C-4'), 129.7 (C-4), 121.1 (C-1), 107.0 (C-3), 118.0 (C-6), 137.0 (C-5'), 108.7 (C-1'), 119.2 (C-3'), 21.0 (OCOCH$_3$). EIMS: m/z = 270 ([M-H$_2$O]$^+$, 228 ([M − COMe-H$_2$O + H]$^+$, 100), 288 [M]$^+$.

DISCUSSION

Eight compounds were isolated from the EtOH extract of the stem barks of *H. lanceolatum*, and some of them were evaluated *in vitro* for their antileishmanial activity against *L. donovani* promastigotes using Amphotericin B as a standard drug (Table 1). 2,2',5,6'-tetrahydroxybenzophenone (**1**), 5-hydroxy-3-methoxyxanthone (**2**), betulinic acid (**4**), and 1,3,5,6-tetrahydroxy-4-prenylxanthone (**8**) exhibited moderated activity (IC$_{50}$ 11.36–34.81 μg/ml) and moderate selectivity (SI 2.46–10.47).

Based on the molecular framework of isolated xanthones, the relationship between the *in vitro* antileishmanial activity and the chemical structure were examined with respect to different functional group therein. Structural comparison between 5-hydroxy-3-methoxyxanthone (**2**) and 1,3,5,6-tetrahydroxy-4-prenylxanthone (**8**) revealed the presence of three hydroxyl groups and one prenyl in compound **8**, which are absent in compound **2** that contains only one hydroxyl group and one methoxy group, which are absent in compound **8**. Azebaze *et al.* (2008) showed that the prenyl group at position 4 could increase the activity of xanthones. However, the presence of the hydroxyl group in both molecules had an important effect on their antileishmanial activity of compound **8**.

Betulinic acid (**4**), a naturally occurring lupane-type triterpene found in many plant species, which has been reported to

Table 1. Antipromastigote and cytotoxic activities of isolated compounds from the ethanol extract of the stem bark of *H. lanceolatum*.

Compounds	IC_{50} on *Leishmania donovani* promastigotes (µg/ml), Mean ± SD	CC_{50} on RAW cell lines macrophages (µg/ml), Mean ± SD	SI = CC_{50}/CI_{50}
2,2′,5,6′-tetrahydroxybenzophenone (1)	16.2 ± 1.2	>200	>12.33
5-hydroxy-3-methoxyxanthone (2)	34.8 ± 1.5	194.2 ± 0.2	5.6
Betulinic acid (4)	11.4 ± 1.1	119 ± 4.14	10.5
Hydroquinone (5)	107.9 ± 2.0	0.1 ± 1.93	0.001
1,3,5,6-tetrahydroxy-4-prenylxanthone (7)	16.4 ± 1.2	40.6 ± 6.8	2.5
5-acetoxy-2,2′,6′-trihydroxybenzophenone (9)	6.1 ± 0.8	130.5 ± 1.9	21.4
Curcumin	nd	16.8 ± 0.02	nd
Amphotericin B	3.10 ± 0.13	Nd	nd

IC_{50}: 50% Inhibitory Concentration; CC_{50}: 50% Cytotoxic Concentration; SI (Selective Index) of bioactive compounds was determined as a measure of their toxicity against RAW cells lines macrophages. SI = CC_{50} against macrophages/IC_{50} against promastigotes; SD = Standard deviation. Nd = Not determined.

exhibit anti-HIV-1, antibacterial, antifungal, antiplasmodial, and anti-inflammatory activities (Yogeeswari and Sriram 2005), and has also been reported to inhibit growth of cancer cells, without affecting normal cells (Einzhammer and Xu 2004). Chan-Bacab *et al.* (2003) have been reported that betulinic acid (4) has the leishmanicidal activity. These results, therefore, confirm the antileishmanial potential of this molecule.

Hydroquinone (5) was previously isolated from the ingredient of many plant-derived products, and an important metabolite of benzene is found to possess strong antiproliferative activity against promastigote forms on *Leishmania major* and *Leishmania tropica in vitro*.

2,2′5,6′-tetrahydroxybenzophenone (1) was previously isolated from the stem bark of *H. lanceolatum* and was not showed any activity against *Plasmodium falciparum*. However, it was not cytotoxic on LLC-MK2 monkey kidney epithelial cells as with on macrophage RAW 264.7 cells line. Acetylation reaction of this compounds gave 5-acetoxy-2,2′,6′-trihydroxybenzophenone (9). The structural comparison of compound (1) and compound (9) revealed only the replacement of the hydroxyl group by acetyl group at position C-5. Although the change had an important effect on antileishmanial activity (IC_{50} 16.2 to 6.1 µg/ml), our results showed that the product of acetylation, 5-acetoxy-2,2′,6′-trihydroxybenzophenone (9) was about 10 times more active than its precursor 1. The acetyl group presents at position C-5 is likely to be responsible for most potential antileishmanial activity. Moreover, we also noted that the cytotoxicity increases. The observed differences in bioactivity of the two compounds may only due to the corresponding change in cytotoxicity (CC_{50} > 200–130 µg/ml). To the best of authors' knowledge, antileishmanial activities of all tested compound have never been reported except for betulinic acid (4).

The cytotoxicity of all these compounds was also assessed (Table 1) on macrophages Raw 264.7 cells line in culture. The results of this assay indicated the selective toxicity by most of the compounds. However, 2,2′,5,6′-tetrahydroxybenzophenone (1) showed no signs of cytotoxicity on macrophages (CC_{50} value of >200 µg/ml), while compound 5-acetoxy-2,2′,6′-trihydroxybenzophenone (9) was slightly cytotoxic with a SI of 21.4. Additionally, the less active compound, hydroquinone (5) was cytotoxic at substantially lower concentration (CC_{50} 0.1132 µg/ml) than its antileishmanial concentration (IC_{50} 107.9 µg/ml). All the other compounds showed higher CC_{50} values compared to their IC_{50} values.

CONCLUSION

The present work showed that the ethanol extract contains compounds with different levels of antileishmanial activity. The results derived from this analysis showed that all the evaluated compounds exhibited promising activities with IC_{50} ranging from 6.1 to 34.8 µg/ml. The new semisynthetic derivative 5-acetoxy-2,2′,6′-trihydroxybenzophenone (9) produced the most potent antileishmanial activity (IC_{50} = 6.1 µg/ml) followed by betulinic acid (4) (IC_{50} = 11.4 µg/ml), 2,2′,5,6′-tetrahydroxybenzophenone (1) (IC_{50} = 16.2 µg/ml), 1,3,5,6-tetrahydroxy-4-prenylxanthone (7) (IC_{50} = 16.4 µg/ml), and 5-hydroxy-3-methoxyxanthone (2) (IC_{50} = 34.8 µg/ml). However, hydroquinone (5) was weakly active against *L. donovani* promastigotes. There was no significant ($p < 0.05$) difference between the activity of 2,2′,5,6′-tetrahydroxybenzophenone (1) and the one of 1,3,5,6-tetrahydroxy-4-prenylxanthone (7). Interestingly, the hemisynthetic derivative 5-acetoxy-2,2′,6′-trihydroxybenzophenone (9) exhibited significant activity with an IC_{50} value of 6.1 µg/ml compared to its precursor 2,2′,5,6′-tetrahydroxybenzophenone (1) with an IC_{50} of 16.2 µg/ml. These findings, therefore, demonstrated the antileishmanial activity of compounds derived from *Hypericum lanceolatum*. This study constitutes the first report on the antileishmanial activity of chemical constituents from *H. lanceolatum*. It opens perspectives to develop new antileishmanial drug classes from natural compounds, having activity against parasites causing MCL associated to the social stigmatization and disfigurement. This preliminary evaluation using promastigotes must be complemented with an evaluation using intracellular amastigotes in macrophages.

CONFLICT OF INTERESTS

Authors declare that they do not have any competing interests.

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