SUPPLEMENTARY MATERIAL

Cytotoxic flavonoids and other constituents from the stem bark of *Ochna schweinfurthiana*.

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ABSTRACT

Seven flavonoids, hemerocallone (**1**), 6,7-dimethoxy-3',4'-dimethoxyisoflavone (**2**), amentoflavone (**4**), agathisflavone (**6**), cupressuflavone (**8**), robustaflavone (**9**), and epicatechin (**10**), together with three other compounds, lithospermoside (**3**), β-D-fructofuranosyl-α-D-glucopyranoside (**5**) and 3β-*O*-D-glucopyranosyl-β-stigmasterol (**7**) were isolated from the ethyl acetate extract of the stem bark of *Ochna schweinfurthiana* F. Hoffm. All the compounds were characterized by spectroscopic and mass spectrometric methods, and by comparison with literature data. Cytotoxicity of the extracts and compounds against cervical adenocarcinoma (HeLa) cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Compounds **4** and **6** exhibited good cytotoxic activity, with IC_{50} values of 20.7 μM and 10.0 μM, respectively.

Key words: *Ochna schweinfurthiana*, Ochnaceae, flavonoids, cytotoxic activity

Experimental

General experimental procedures

Thin layer chromatography (TLC) analyses were done using aluminium sheets coated with silica gel 60 F_{254}. Flash column chromatography (FC) was carried out using Brunschwig silica gel 60 Å (32-63 mesh). Commercially available products were used without further purification. ^{1}H- and ^{13}C-NMR spectra were recorded with a FT 300 MHz or FT 360 MHz spectrometer with solvent residual signals used as a reference. Chemical shifts are given in ppm, coupling constants *J* are expressed in Hertz (multiplicity: *s* = singulet, *d* = doublet, *dd* = double of doublet, *t* = triplet, *dt* = double triplet, *q* = quadruplet, *m* = multiplet). IR spectra were recorded with a Fourier transform FTIR spectrometer. High resolution ESI mass spectra were measured with an ion-cyclotron FT/MS (4.7 T) spectrometer.

Plant material

The stem bark of *O. schweinfurthiana* F. Hoffm. were collected in the North of Cameroon in December 2011. The identity of the plant material was confirmed at the Cameroonian National Herbarium in Yaoundé by Victor Nana, where a voucher specimen (HNC 40171) has been deposited.

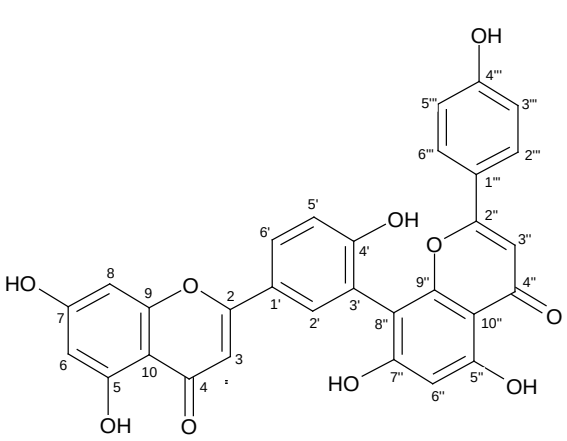
Extraction and isolation

The EtOAc extract (30.6 g), obtained from partition of methanolic crude extract of the stem bark of *O. schweinfurthiana* resuspended in water, was separated using various chromatographic techniques. It was first subjected to normal-phase silica gel column and eluted with CH_2Cl_2/MeOH of increasing polarity (30:1 to 5:1, MeOH) to give seven fractions (Fr.1-Fr.7) on the basis of TLC composition. Fr.2 (2.1 g) was subjected to repeated CC on silica gel eluted with CH_2Cl_2/MeOH (10:1) to yield 6 mg hemerocallone (Zhang & Liang 2011) and 5 mg 6,7-dimethoxy-3',4'-dimethoxyisoflavone (Sree et al. 1986). Fr.3 (2.9 g) was also subjected to silica gel column chromatography, eluted with CH_2Cl_2-MeOH gradient and afforded a precipitate that were washed with CH_2Cl_2 to give 125 mg lithospermoside (Sosa et al. 1977). The mother liquors from Fr.3 was applied to silica gel column chromatography, and eluted with (CH_2Cl_2/MeOH, 10:1) to yield 17 mg amentoflavone (Ndongo et al. 2010; Ying et al. 2010, Geiger et al. 1994). Fr.5 (3.8 g) was purified by CC with CH_2Cl_2/MeOH (10:1, 8:1, and 5:1) to yield 400 mg β-D-fructofuranosyl α-D-glucopyranoside (Fang et al. 2011), 11 mg agathisflavone (Ndongo et al. 2010; Shrestha et al. 2012; Geiger et al. 1994), and 7 mg 3β-O-D-glucopyranosyl-β-stigmasterol (Chaurasia & Wichtl 1987). Fr. 6 was first chromatographed

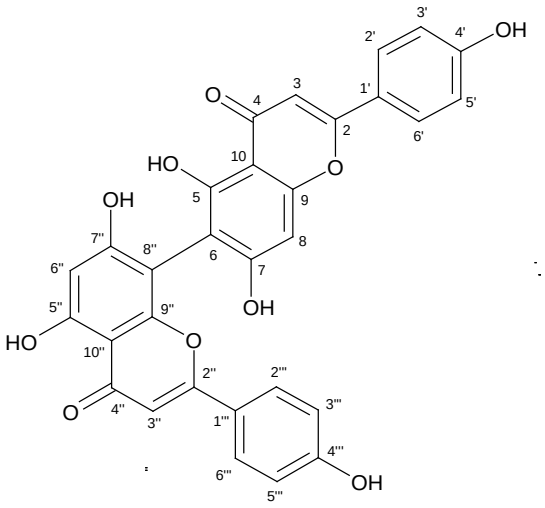
on a silica gel column (CH_2Cl_2/MeOH, 10:1 and 5:1) to give sub-fractions Fr.6A and Fr.6B, which were further purified using the same procedure as before to yield 8 mg cupressuflavone (Lin et al. 1989) and 10 mg robustaflavone (Lin and Chen 1974). Further purification of Fr.1 was conducted by repeated silica gel column chromatography and preparative TLC to give 6 mg epicatechin (Spek et al. 1984). The chemical structure of each isolated compound was elucidated on the basis of spectroscopic methods, including 2D NMR experiments, and confirmed by comparison with the literature.

Amentoflavone (**4**), Amorphous yellow powder, m.p. 231-232°, $C_{30}H_{18}O_{10}$, ESI-MS: *m/z* 539.1 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6) : δ (ppm) :13.03 (1H, s, OH-5), 6.70 (1H, s, H-3), 6.25 (1H, d, *J* = 2.0 Hz, H-6), 6.32 (1H, d, *J* = 2.0 Hz, H-8), 6.81 (1H, d, *J* = 2.2 Hz, H-2'), 6.68 (1H, d, *J* = 8.7 Hz, H-5'), 7.61 (1H, d, *J* = 8.7; 2.2 Hz, H-6'), 13.36 (1H, s, OH-5"), . 6.67 (1H, s, H-3"), 6.44 (1H, s, H-6"), 6.94 (2H, d, *J* = 8.7 Hz, H-2'"/6'"), 6.68 (2H, d, *J* = 8.7 Hz, H-3'"/5'"). ^{13}C-NMR (125 MHz, DMSO-d_6): δ (ppm) : 164.1 (C-2), 102.0 (C-3), 181.7 (C-4), 161.1 (C-5), 98.6 (C-6), 163.6 (C-7), 93.7 (C-8), 157.1 (C-9), 103.4 (C-10), 118.3 (C-1'), 131.1 (C-2'), 122.0 (C-3'), 162.7 (C-4'), 117.8 (C-5'), 126.6 (C-6'), 162.8 (C-2"), 102.2 (C-3"), 181.2 (C-4"), 160.2 (C-5"), 100.3 (C-6"), 161.4 (C-7"), 103.6 (C-8"), 154.4 (C-9"), 102.3 (C-10"), 121.5 (C-1'"), 127.9 (C-2'"/6'"), 115.3 (C-3'"/5'"), 160.4 (C-4'").

Agathisflavone (**6**), Amorphous yellow powder, $C_{30}H_{18}O_{10,}$ ESI-MS: *m/z* 539.2 $[M+H]^+$ ^{1}HNMR (500 MHz, CD_3OD): δ (ppm): 6.81 (1H, s, H-3), 6.68 (1H, s, H-8), 7.52 (2H, d, *J* = 8.8 Hz, H-2'/6'), 6.72 (2H, d, *J* = 8.8 Hz, H-3'/5'), 6.63 (1H, s, H-3"), 6.35 (1H, s, H-6"), 7.87 (2H, d, *J* = 8.7 Hz, H-2'"/6'"), 6.92 (2H, d, *J* = 8.7 Hz, H-3'"/5'"). ^{13}C-NMR (125 MHz, CD_3OD): δ (ppm) : 165.9 (C-2), 103.3 (C-3), 183.7 (C-4), 162.4 (C-5), 100.0 (C-6), 105.4 (C-7), 94.8 (C-8), 156.9 (C-9), 103.8 (C-10), 123.2 (C-1'), 129.1 (C-2'/6'), 116.8 (C-3'/5'), 164.4 (C-4'), 166.0 (C-2"), 105.1 (C-3"), 184.1 (C-4"), 162.3 (C-5"), 100.5 (C-6"), 164.3 (C-7"), 104.8 (C-8"), 158.9 (C-9"), 103.6 (C-10"), 123.3 (C-1'"), 129.4 (C-2'"/6'"), 116.9 (C-3'"/5'"), 164.8 (C-4'").



4



6

Cell lines

The human cervical cancer cell line HeLa was obtained from the American Type Culture Collection (ATCC). HeLa cells were cultured in RPMI 1640 culture medium supplemented with 10 % fetal bovine serum (Life Technologies, Switzerland), 100 μg mL^{-1} penicillin, 250 μg mL^{-1} streptomycin (Life Technologies). The growth medium was changed every 2 or 3 days and the cells were maintained in a humidified atmosphere supplemented with 5 % CO_2 at 37°C.

Cell viability assay

Cell viability was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann 1983). Briefly, HeLa cells were seeded in 96-well plates at 5,000 cells/well in 200 μL culture medium. The cells were allowed to adhere for 24 hours under standard conditions then treated with increasing concentrations of compounds for 72 hours. After the incubation, the cells received 20 μL of MTT solution for 2 hours. The media and MTT mixture were then aspirated and the formazan-containing cells were solubilised in 100 μL of DMSO. Optical density of formazan was measured at 550 nm on a Biotek plate reader. The percentage of cell viability was calculated as the absorbance of each test well divided by the average absorbance of the vehicle control wells then multiplied by 100. The concentration that resulted in a 50% reduction in viability (IC_{50}) was calculated from the log10 concentration versus normalized response curves using the following equation:

$y = 100/(1 + 10^{(LogIC50-x)*hill\ slope})$, where y is the measured absorbance at 550 nm and x is the drug concentration.

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