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Sapelenins G–J, acyclic triterpenoids with strong anti-inflammatory activities from the bark of the Cameroonian medicinal plant *Entandrophragma cylindricum*

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ABSTRACT

Four acyclic triterpene derivatives named sapelenins G–J (**1**–**4**), along with eight known compounds, sapelenins A–D, ekeberin D2 (**5**), (+)-catechin and epicatechin, and anderolide G, were isolated from the stem bark of the Cameroonian medicinal plant, *Entandrophragma cylindricum* Sprague, on the basis of bioassay-guided fractionation. Their structures were determined by means of high-resolution mass spectrometry and NMR spectroscopic data, as well as by comparison with the literature values of their analogs. The absolute configurations of the compounds (**1**–**4**) were assigned by the modified Mosher's method in conjunction with NOESY experiments and chemical modifications. The anti-inflammatory activities of the sapelenins were evaluated by assessing their ability to suppress or inhibit the secretion of cytokine interleukin-17 (IL-17) by human peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA). The cytotoxicity of these compounds on PMBCs was further assessed for correctly interpreting their anti-inflammatory responses. The tested compounds demonstrated moderate to significant anti-inflammatory activities by suppressing the secretion of IL-17 by PHA-stimulated human PBMCs. One of them, sapelenin G (**1**), showed high potency in suppressing the secretion of IL-17 by PBMCs comparable to reference cyclosporine A, without causing any cytotoxic effects (negligible), and deserves further considerations towards developing an effective anti-inflammatory drug.

1. Introduction

In our search for bioactive metabolites from Cameroonian medicinal plants, we recently encountered a plant in the Dja rainforest, locally known in Cameroon as 'Sapele'. This plant is botanically known as *Entandrophragma cylindricum* Sprague (Meliaceae). The genus *Entandrophragma* contains ten species which are generally recognized as distinct, together with some four varieties that probably do not rank in any species (Adesida and Taylor, 1967). *E. cylindricum* is one of the only five species that have been discovered so far in Cameroon (Letouzey, 1985). It is a very bulky and tall forest tree, up to 45 m in height and native to tropical Africa. The bark of this timber has been extensively exploited by the 'Bantu' and 'Baka' tribes in the eastern region of Cameroon, for the treatment of rheumatism in the traditional medicine subsector. Although phytochemical studies on this plant genus have been reported earlier with the most prominent classes of compounds isolated being complex limonoids (Taylor and Wragg, 1967; Chan et al., 1970; Baxter et al., 1998; Nsiama et al., 2011) and highly oxygenated acyclic triterpenes (Ngnokam et al., 1993, 1995, 2005), little pharmacological investigation has so far been performed (Huang et al., 2009).

Here, we report the isolation and the characterization of four new acyclic triterpene derivatives named sapelenins G–J (**1**–**4**), together with eight known compounds, sapelenins A–D (Ngnokam et al., 1993, 1995), ekeberin D2 (**5**) (Murata et al., 2008), (+)-catechin and epicatechin (Kashiwada et al., 1990a,b), and anderolide G (Tanaka et al., 2011). We also report the anti-inflammatory activities of the novel sapelenins by evaluating their ability to suppress/inhibit secretion of cytokine IL-17 (interleukin-17) by human peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA). We further evaluated the cytotoxicity of the tested compounds (**1**–**4**) on PMBCs in order to correctly interpret their anti-inflammatory responses.

2. Results and discussion

Compound **1**, named sapelenin G (Fig. 1), was isolated as a colorless oil. Its molecular formula ($C_{30}H_{56}O_6$) was determined from

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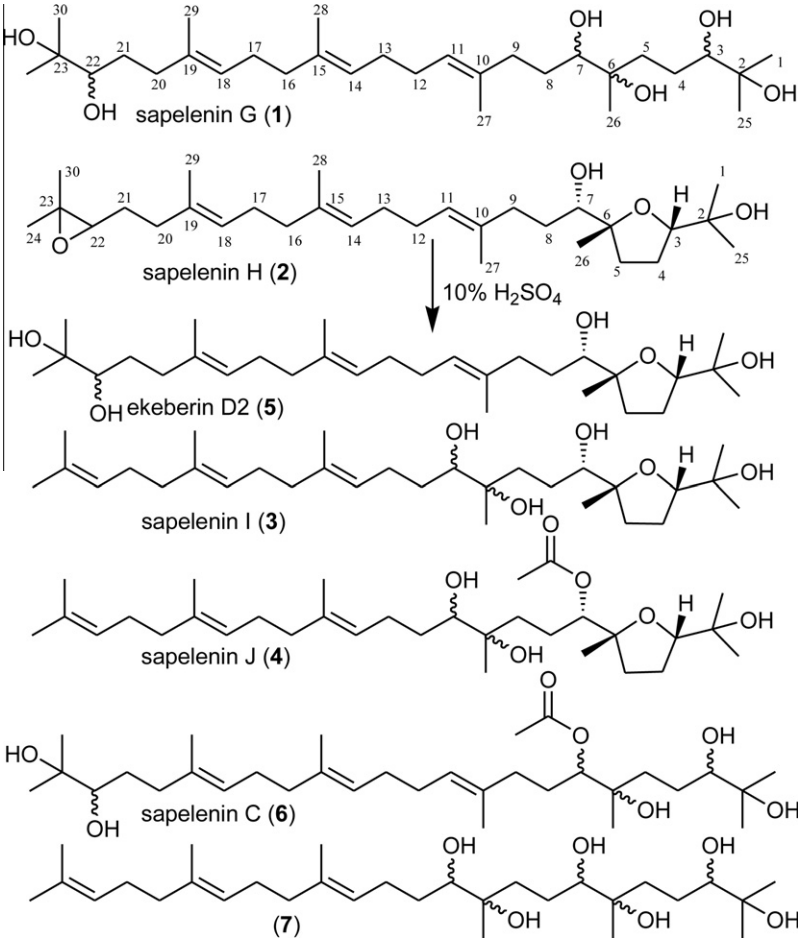

Fig. 1. Structures of sapelenins G–J (**1**–**4**) and compounds **5**–**7**.

the pseudomolecular ion peak at m/z = 513.4152 (calcd. for $C_{30}H_{57}O_6$, 513.4155), obtained by high-resolution mass spectrometry (HR-MS) and is consistent with three degrees of unsaturation. The IR spectrum exhibited bands for a hydroxyl functional group at ν_{max} = 3386 cm^{-1}. The ^{13}C NMR (see Supplementary data, Table S1) displays 30 carbon signals which can be sorted into eight methyls, ten methylenes, three oxymethines, three sp^2 methines and six quaternary carbons including three sp^2 carbons, and three bearing oxygen. The ^1H NMR (see Supplementary data, Table S1) spectrum showed resonances for eight methyl groups including three vinylic ones (δ_H 1.57, 2 × δ_H 1.59), and five attached to sp^3 carbon.

From the above evidence and by comparing these data with those described in the literature (Ngnokam et al., 1993, 1995; Miller and Tinto, 1995; Murata et al., 2008), sapelenin G (**1**) was confirmed as a derivative of squalene with a structure similar to that of sapelenin C (**6**) (Ngnokam et al., 1993). The presence of three low field methyl groups around ~δ_H 1.5–1.8 and the lack of any correlation peaks from two of these olefinic methyl groups in the HMBC spectrum indicated clearly the absence of the two terminal vinylic methyl groups and consequently, suggested the oxidation of the terminal double bonds of the squalene skeleton assigned to compound **1**. In the HMBC experiment (Fig. 3a$_1$), the proton signal at δ_H 3.28 (H-22) showed correlation peaks with carbon signals at δ_C 23.4 (CH_3-24), 26.7 (CH_3-30) and 37.4 (CH_2-20), and cross peaks were also observed between the proton signal at δ_H 3.32 (H-3) and the carbon signals at δ_C 23.4 (CH_3-1), 26.7 (CH_3-25) and 33.4 (CH_2-5), respectively. Furthermore, the methyl signal at δ_H 1.10 (H-26) showed cross peaks with two carbon signals at δ_C 33.4 (C-5) and 78.9 (C-7). In the COSY spectrum, connectivity was observed between the protons at δ_H 3.32 (H-3), 1.64 (H-4a), 1.36 (H-4b), and 1.62 (H-5), respectively, indicating the oxidation of two contiguous double bonds in **1**. The relative configurations of C-3, C-6, C-7 and C-22 could not be assigned. The compound was assigned the structure (10*E*,14*E*,18*E*)-2,6,10,15,19,23-hexamethyltetracosa-10,14,18-triene-2,3,6,7,22,23-hexaol and named sapelenin G (**1**). Its isomer (**7**) has been described as one of the constituents of the anti-inflammatory and anti-allergy extract of nettle (Alberte et al., 2010).

Compound **2** (Fig. 1) was obtained as a colorless oil. Its molecular formula was determined as $C_{30}H_{52}O_4$ by HR-MS (m/z 477.3945 $[M+H]^+$). IR absorption bands were observed at 3430 nm (OH) and 1660 nm (C=C). The ^{13}C NMR spectroscopic data of **2** (see Supplementary data, Table S1) exhibited 30 typical resonances for an acyclic triterpene skeleton including eight methyl and ten methylene groups (Ngnokam et al., 1993, 1995; Murata et al., 2008). A 2-hydroxypropanyl-2-methyltetrahydrofuranyl sub-structure was established from spectral data evidence. An oxymethine (δ_C 87.6), an oxygenated tertiary carbon (δ_C 86.1), two methylene carbons (δ_C 26.6 and 31.4) and two methyl groups (δ_C 23.8 and 27.7) were observed in the ^{13}C NMR spectrum (see Supplementary data, Table S1). The HMBC experiment (Fig. 3a$_2$) showed cross peaks with proton signal at δ_H 3.77 (oxymethine) and carbon signals at δ_C 86.1, 23.8 and 27.7. The long-range correlation between the methyl protons at δ_H 1.15 (δ_C 23.8) and the carbon signals at δ_C 76.2 (oxymethine), δ_C 87.1 and δ_C 31.4 established that the dihydrofuran moiety resided next to an oxymethine group (δ_H 3.51). Furthermore, in the $^1H/^{13}C$ NMR spectrum, we could distinguish distinctive resonances for an epoxy function ($\delta_{H/C}$ 2.71/

64.2, CH and δ_C 58.3, qC). In the HMBC experiment, important cross peaks from H-30 (δ_H 1.27) to C-24 (δ_c 24.9); from H-30 (δ_H 1.27) to C-22 (δ_c 64.2); H-24 (δ_H 1.31) to C-30 (δ_c 27.4) and H-24 (δ_H 1.31) to C-22 (δ_c 64.2) were observed, indicating that the epoxy group was located at C-22, C-23. This suggests that the structure of **2** may be a precursor of ekeberin D2 (**5**), isolated for the first time from this plant (Murata et al., 2008). Compound **2**, on hydrolysis using aqueous acid (10% H_2SO_4) yielded ekeberin D2 (**5**), which was confirmed by comparison of the spectral data with that reported (Murata et al., 2008). This finding was strongly supported by the ^1H–^1H COSY and HMBC results. The presence of a secondary alcohol at C-7 of **2** allowed us to determine its absolute stereochemistry by utilizing the modified Mosher's method (Ohtani et al., 1991; Lamshöft et al., 2003). (*R*)- and (*S*)-MTPA esters of sapelenin H (**2**) were prepared by treatment with (*R*)- and (*S*)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) chloride in DMAP followed by drops of pyridine. Compound **2** was converted to the corresponding MTPA esters *S* and *R*, respectively. Significant $\Delta\delta$ values ($\Delta\delta = \delta_{S\text{-MTPA-ester}} - \delta_{R\text{-MTPA-ester}}$) were observed for the protons near the chiral center C-7 as shown in Fig. 2. According to Mosher's rule, the absolute configuration at C-7 was determined as *S*. As a consequence, the absolute configurations of the other two stereogenic centers in the dihydrofuran portion of **2** were deduced to be 3*S*, 6*R*, on the basis of the established relative stereochemistry from the significant NOE effect between H-3, CH_3-6 and H-7 (Fig. 3b). The absolute configuration at C-22 could not be determined. Thus, compound **2** is accordingly (1*S*,4*E*,8*E*,12*E*)-15-(3,3-dimethyloxiran-2-yl)-1-((2*R*,5*S*)-5-(2-hydroxypropan-2-yl)-2-methyltetrahydrofuran-2-yl)-4,9,13-trimethylpentadeca-4,8,12-trien-1-ol.

Compound **3** (Fig. 1) was isolated as an optically active colorless oil, $[\alpha]_D^{20} = +194^\circ$ [*c* 0.01, CH_3OH]. Its HR-MS mass spectrum showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* = 495.4054 corresponding to the molecular formula $C_{30}H_{55}O_5$. The IR spectrum contained hydroxyl group absorption at 3430 cm^{-1}. Important fragments were obtained at *m/z* 477 $[[M+H]^+ - H_2O]^+$, 459 $[[M+H]^+ - 2 \times H_2O]^+$ and 441 $[[M+H]^+ - 3 \times H_2O]^+$ in its mass spectrum, indicating three hydroxyl groups in the structure of **3**. Its $^1H/^{13}C$ NMR spectra showed resonances at $\delta_{H/C}$ 3.79/88.1, 3.56/77.7 and 3.44/78.9, corresponding to three oxymethine groups (see Supplementary data, Table S1). Furthermore, the ^1H NMR spectrum indicated resonances for eight methyl groups [δ_H 1.17, 1.22, 1.65, 1.69 (each 3H), 1.15 (6H), 1.61 (6H)] and resonances of three olefinic protons at δ_H 5.18 (*J* = 6.9), 5.11 (*J* = 6.5), 5.12 (*J* = 6.8). In the ^{13}C NMR spectrum, a total of 30 resonances were observed. Compound **3** is, thus, an acyclic triterpene containing at least three hydroxyl groups. In the ^{13}C NMR spectrum, one can distinguish resonances for a 2-hydroxypropanyl-2-methyl-tetrahydrofuran-2-yl moiety at δ_C 86.6, 31.5, 27.0, 88.1, 71.1, 23.9, and 27.1. Resonances at $\delta_{H/C}$ 5.11/124.7 (olefinic carbon), 2.09/27.0 (methylene protons), 1.61/18.0 and 1.69/25.7 (two methyl groups) in the $^1H/^{13}C$ NMR spectra were typical for those of a prenyl group (Kouam et al., 2007). However, in the HMBC experiment (Fig. 3a$_3$), we observed correlations of proton resonances at δ_H 1.61 (CH_3-30) and 1.69 (CH_3-24) with the carbon signal at δ_C 124.7 (C-22) which in turn showed a cross peak with a proton signal at δ_H 2.01 (H-20). In addition, a cross peak was observed between signal at δ_H 2.01 and a carbon resonating at δ_H 1.61 (CH_3-29) suggesting a geranyl moiety in the structure of **3**. The remaining two hydroxyl groups were located at C-10 and C-11, based on the careful examination of the HMBC data. In fact, the methylene protons (δ_H 1.42 and 1.58 (CH_2-8)) indicated correlations with two quaternary oxygenated carbons (δ_C 74.6 (C-10), and 86.6 (C-6)) and the methyl group at δ_H 1.15 (CH_3-27) showed cross peaks with carbon signals at δ_C 78.9 (C-11), 74.6 (C-10) and 33.6 (C-9). Sapelenin I (**3**) is, thus, an isomer of ekeberin D2 (**5**) (Murata et al., 2008). Because two secondary hydroxyl groups are present in the structure of sapelenin I (**3**), the determinations of their absolute configurations is difficult. However, treatment of **3** with 2,2-dimethoxypropane and *p*-toluene sulfonic acid in acetone afforded the corresponding acetonide (**3a**). The absolute configurations of the dihydrofuran moiety were elucidated by both NOESY and Mosher's experiments (Figs. 2 and 3b). NOESY correlations were observed between 1-CH_3, H-3 and 26-CH_3, and H-7 and 26-CH_3. The modified Mosher's method (Fig. 2) was applied to determine the absolute configuration of C-7 to be the same as in **2** (7*S*) and consequently, those of C-3 and C-6 were found to be respectively, 3*S* and 6*R*. However, it was not possible to determine the absolute configurations at C-10 and C-11. On the basis of the above evidence, the structure of compound **3** was assigned to be (1*S*, 8*E*,12*E*)-1-((2*S*,5*R*)-5-(2-hydroxypropan-2-yl)-2-methyltetrahydrofuran-2-yl)-4,9,13,17-tetramethyl octadeca-8,12,16-triene-1,4,5-triol.

Compound **4** (Fig. 1) was obtained as an optically active colorless oil {$[\alpha]_D^{20} + 36$ (*c* 0.01, CH_3OH)} with the molecular formula $C_{32}H_{56}O_6$, established by HR-MS in conjunction with the NMR spectra. The IR spectrum of **4** indicated the presence of hydroxyl (3430 and 3360 cm^{-1}) and ester carbonyl (1730 cm^{-1}) groups. The chemical shift values of the ^1H and ^{13}C NMR spectra of **4** in $CDCl_3$ (see Supplementary data, Table S1) were almost identical with those of compound **3**. However, in the ^1H NMR spectrum of compound **4**, a resonance was observed for an acetyl group at δ_H 2.09 and the signal at δ_H 3.56 (H-7) in **3** shifted downfield to δ_H 4.95, confirming the presence of an acetyl group. This was corroborated by the resonances at δ_C 171.1 in the ^{13}C NMR spectrum. From this evidence, it was clear that the hydroxy moiety was replaced in **3** by an acetoxyl group. Based on the formation of the sapelenins G–I (**1**–**3**) in the same plant extract, we speculate that they possess the same biogenetic origin. In addition to the consis-

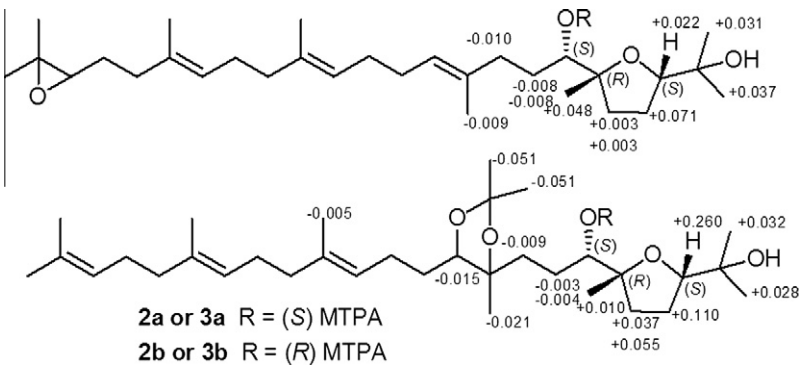

Fig. 2. $\Delta\delta(\delta_S - \delta_R)$ values (in ppm) for the MTPA esters of **2** and **3**.

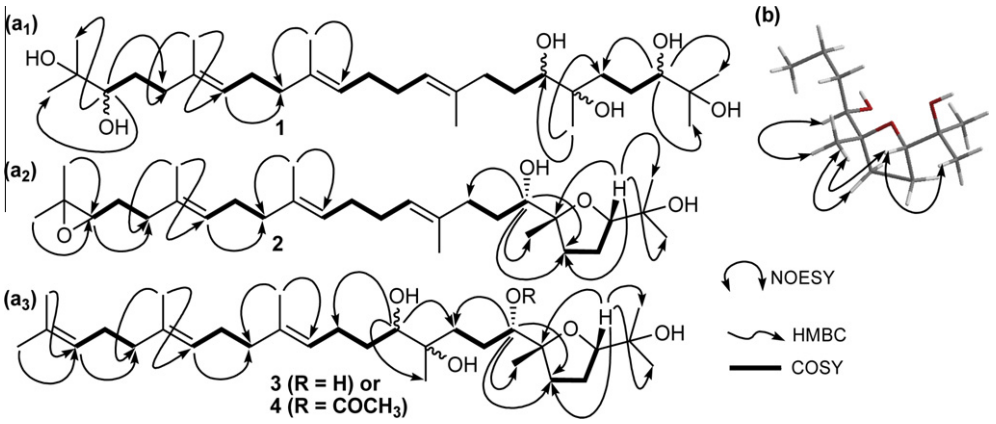

Fig. 3. (a) ^{1}H–^{1}H COSY (bold bonds) and selected HMBC (curved arrows) correlations for **1**–**4**. (b) Key NOE correlation (curved arrows) observed for the dihydrofuran portion for **2**–**5** NOESY spectrum (in $CDCl_3$, 500 MHz).

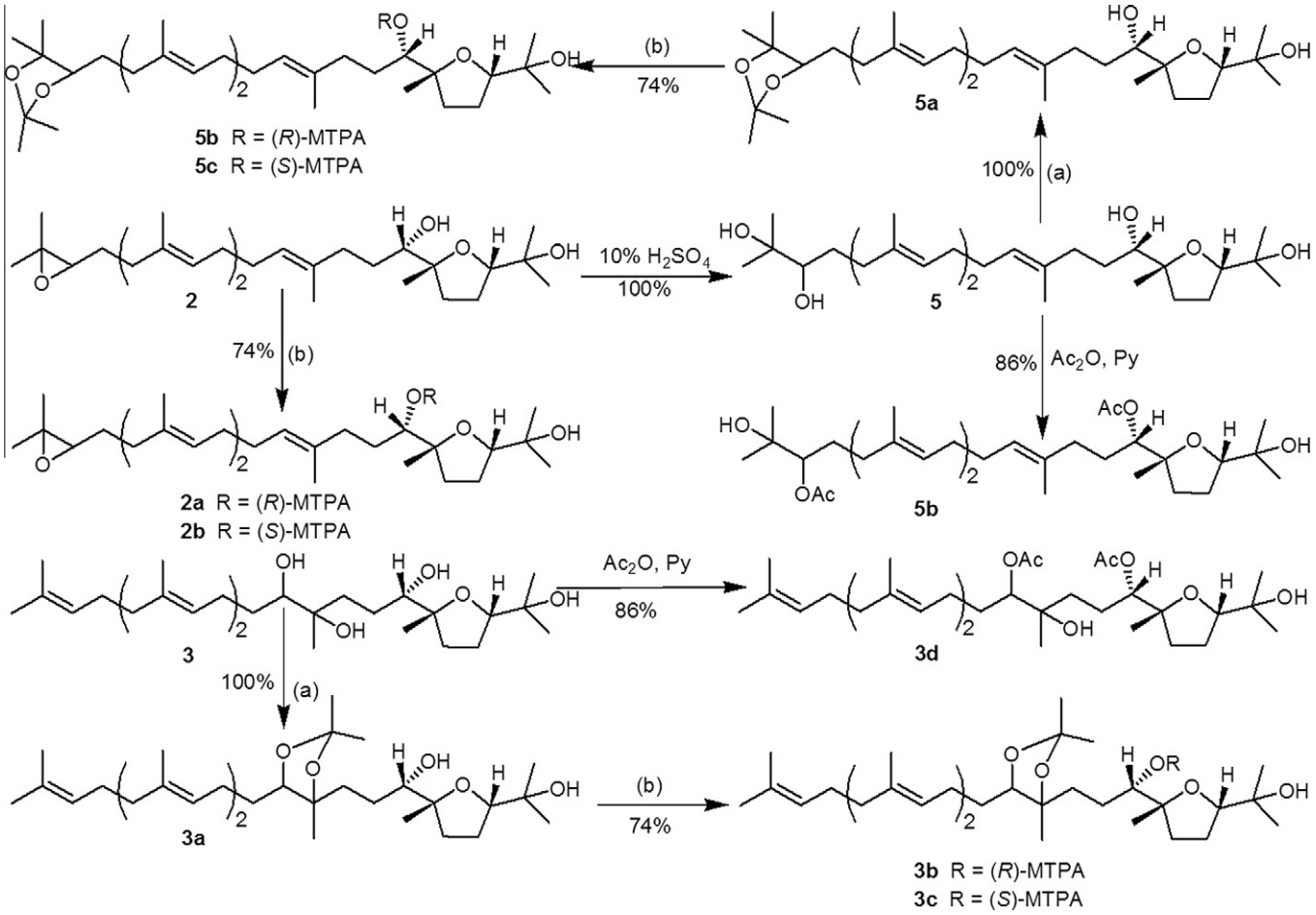

Fig. 4. Schematic representation of the chemical conversion of sapelenin derivatives. (a) 2,2-dimethoxypropane, *p*-toluenesulfonic acid, acetone at rt, 30 mn, 98%; (b) (+) or (−)- MTPA chloride, DMAP, at RT, overnight, 74%.

tent chemical correlations (Fig. 4), we assume identical absolute configurations of the three stereogenic centers C-3, C-6 and C-7 of the derivatives presented in Fig. 4. The absolute configurations at C-10 and C-11 could not be determined. The structure of **4** was, thus, determined as a (1*S*,8*E*,12*E*)-4,5-dihydroxy-1-((2*S*,5*R*)-5-(2-hydroxypropan-2-yl)-2-methyltetrahydrofuran-2-yl)-4,9,13,17-tetramethyloctadeca-8,12,16-trienyl acetate.

The IL-17 (or IL-17A), originally called cytotoxic T-lymphocyte antigen-8, is the signature cytokine of the recently identified T helper 17 (Th17) cell subset of CD_4^+ T cells, and is associated with several immune regulatory functions, including the inflammatory process during infection and in autoimmune diseases (Iwakura et al., 2011; Angkasekwinai and Dong, 2011). In fact, the IL-17 family of cytokines is involved in the development of inflammation and host defense against infection by inducing the expression of genes encoding proinflammatory cytokines (such as TNF, IL-1, IL-6, G-CSF, and GM-CSF), chemokines (such as CXCL1,CXCL5, IL-8, CCL2, and CCL7), antimicrobial peptides (such as defensins and

S100 proteins), and matrix metalloproteinases (such as MMP1, MMP3, and MMP13) from fibroblasts, endothelial cells, and epithelial cells (Kolls and Linden, 2004; Iwakura et al., 2008, 2011; Reynolds et al., 2010). Furthermore, IL-17 also promotes SCF- and G-CSF-mediated granulopoiesis and recruits neutrophils to the inflammatory sites (Iwakura et al., 2011). Therefore, we employed the 96 well-plate format of measurement of IL-17 secretion by human PBMCs stimulated with PHA using the human IL-17 ELISA kit (Ray Biotech, Inc.). Sapelenins G–J (**1**–**4**) demonstrated moderate to significant anti-inflammatory activities by suppressing the secretion of IL-17 by human PBMCs stimulated with PHA (Fig. 5). We used cyclosporine A (CsA) as an additional control (reference). Sapelenins G (**1**), I (**3**), and J (**4**) significantly suppressed IL-17 secretion at the concentration of 11.1 μM, which became pronounced in a concentration-dependent manner. Their activity was comparable to that of the reference (CsA) at higher concentrations. Sapelenin H (**2**) was not potent in suppressing the secretion of IL-17 even at the highest concentration tested (100.0 μM). However, to confirm that the data obtained for IL-17 secretion was not affected by the cytotoxicity of the sapelenins towards PBMCs, we evaluated the viability of the PBMCs treated with the respective compounds by a cell viability assay (CellTiter-Blue®, Promega) in parallel (Fig. 6).

The cytotoxicity assay (Fig. 6) revealed that sapelenin G (**1**) had only negligible cytotoxic effects against the PBMCs even at higher concentrations. Sapelenin H (**2**) did not show any cytotoxic effect at any tested concentration. Sapelenins I (**3**) and J (**4**) started demonstrating cytotoxicity against the PBMCs at 33.3 μM and 11.1 μM, respectively, that increased in a concentration-dependent exponential manner at higher concentrations; that is, at 100 μM for both the sapelenins. This assay, thus, allowed us to discriminate the real anti-inflammatory responses of the tested compounds from the false positives.

Taken together, the anti-inflammatory and cytotoxicity assays revealed that sapelenin G (**1**) is highly potent in suppressing the secretion of IL-17 by PBMCs without causing any cytotoxic effects, and deserves further consideration for development as an anti-inflammatory drug. Sapelenin H (**2**) neither possessed the potency to suppress IL-17 secretion by the PBMCs nor had any cytotoxic activity towards them. However, since sapelenins I (**3**) and J (**4**) showed a concentration-dependent cytotoxic effect on the PBMCs, their IL-17 suppression data were considered invalid at the higher concentrations.

3. Experimental

3.1. General experimental procedures

3.1.1. NMR

The NMR spectra were recorded on a Bruker DRX-500 NMR. Chemical shifts (δ) were quoted in parts per million (ppm) from internal standard tetramethylsilane (TMS). The $^3J_{C,H}$ couplings were measured by means of pulsed field gradient HMBC spectra recorded by varying the *J*-refocusing time between $t = 0.04$ and 0.14 s.

3.1.2. Column chromatography and preparative HPLC

Flash column chromatography was performed using silica-gel 60 (Merck, 0.040–0.063 mm). Preparative reversed-phase HPLC was carried out with a Gilson system consisting of pump 322 with a UV detector 152 ($\lambda = 205$ nm) using a Nucleodur Gravity column from Macherey–Nagel (Düren, Germany) (250 × 16 mm, 5 μm particle size). Separation was achieved by using a H_2O (A)-MeOH (B) gradient program as follows (flow rate 4 mL min^{-1}): 70% A linear to 75% B for 15 min, linear gradient to 20% A over 13 min, linear gradient to 0% A over 2 min, after 100% B isocratic for 7 min. Afterwards, the system returned to its initial condition (70% A) within 1 min, and was equilibrated for 5 min.

3.2. Plant material

The stem bark of *E. cylindricum* was collected from the Dja rainforest, east region, Cameroon, in June 2010. The botanical identification was made at the National Herbarium of Cameroon in Yaoundé by Mr. Victor Nana (botanist) and a voucher specimen (No. 54965/SFR/CAM) was deposited.

3.3. Extraction and isolation

The air-dried stem bark of *E. cylindricum* (7 kg) was powdered and extracted twice with MeOH at room temperature for 48 and 8 h, respectively. The organic solvent was concentrated to afford a crude extract (890 g) which was re-extracted with ethyl acetate/methanol (90/10) to obtain a 390 g extract, which was further subjected to flash silica gel column chromatography, using a gradient of ethyl acetate in hexane, to give seven fractions named fr_0 (pure hexane); fr_1 (hexane/ethyl acetate, 9/1); fr_2 (hexane/ethyl acetate, 4/1); fr_3 (hexane/ethyl acetate, 7/3); fr_4 (hexane/ethyl acetate, 1/1); fr_5 (pure ethyl acetate) and fr_6 (ethyl acetate/methanol, 4/1). Fr_4 and fr_5 were combined based on their LC-MS profiles. Fraction fr_0 was found to contain mainly mixtures of hydrocarbons and phytosterols. Fractions fr_1 (38 g), fr_2 (41 g), fr_3 (36 g), fr_4 (20 g) and fr_5 (30 g) which showed strong anti-inflammatory activities, were again subjected separately to flash silica gel column chromatography, using the same gradient of solvent, to afford four series of six (A_1–A_6); eight (B_1–B_8), seven (C_1–C_7) and seven (D_1–D_7) subfractions. Each subfraction was submitted to the semipreparative HPLC at wavelength 205 nm with the solvent system H_2O (B)-MeOH (A) with gradient program as described above. Fractions A_6 and B_4 gave, respectively, sapelenins H (**2**, 22 mg) and J (**4**, 7 mg); fractions C_4 and C_6 yielded ekeberin D_2 (**5**, 76 mg) and sapelenins B (80 mg), D (49 mg) and I (**3**, 50 mg); Fractions D_5 gave sapelenins A (37 mg) and C (92 mg); and Fraction D_6 afforded (+)-catechin (68 mg), epicatechin (20 mg) and sapelenin G (**1**, 21 mg).

3.4. Accurate mass measurement

The high-resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 200–1000) with nominal mass resolving power of 60 000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; Bis(2-ethylhexyl)phthalate: *m/z* = 391.284286. The spectrometer was attached with an Agilent 1200 HPLC system (Santa Clara, USA) consisting of LC-pump, PDA detector ($\lambda = 205$ nm), auto sampler (injection volume 10 μl) and column oven (30 °C). MS/MS experiments were performed by CID (collision induced decay, 35 eV) mode. Following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, tube lens 70 V. Nitrogen was used as sheath gas (50 arbitrary units) and auxiliary gas (five arbitrary units). Helium served as the collision gas. The separations were performed by using a Nucleodur Gravity column (50 × 2 mm, 1.8 μm particle size) from Macherey–Nagel (Düren, Germany) with a H_2O (+0.1% HCOOH, +10 mM NH_4Ac) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 300 μl min^{-1}). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min, linear gradient to 100% B over 13 min, after 100% B

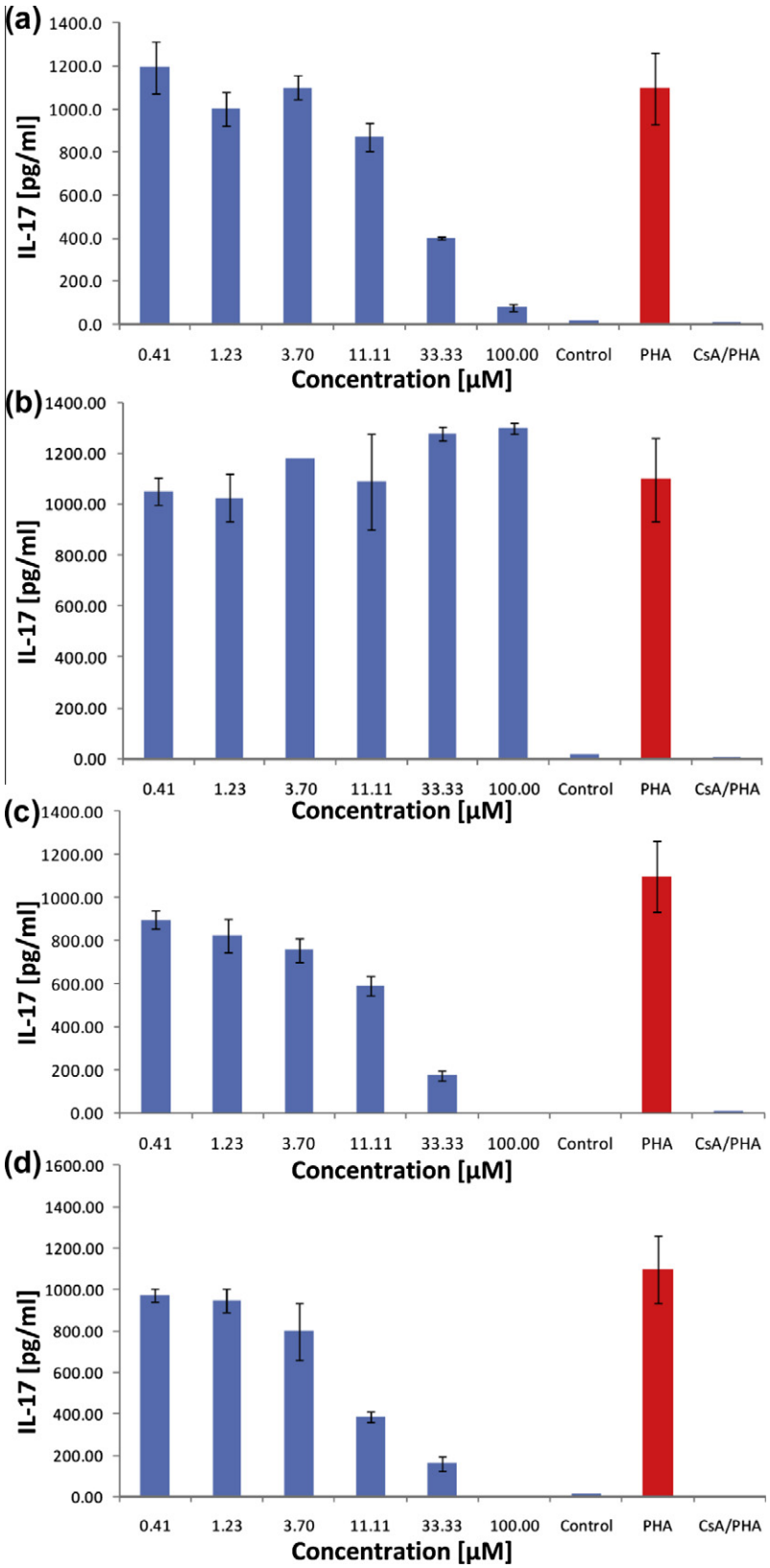

Fig. 5. Effect of the tested compounds on the secretion of IL-17 by PHA-stimulated PBMCs. (a) Sapelenin G (**1**); (b) Sapelenin H (**2**); (c) Sapelenin I (**3**); (d) Sapelenin J (**4**). As negative control, IL-17 secretion in unstimulated PBMCs was determined. As positive control, IL-17 secretion in PHA-stimulated PBMCs without adding any tested compound was determined. An additional control (reference) of CsA was used to determine the comparative efficacy of the tested drugs. (Concentrations used: 0.41, 1.23, 3.70, 11.11, 33.33, and 100 µM).

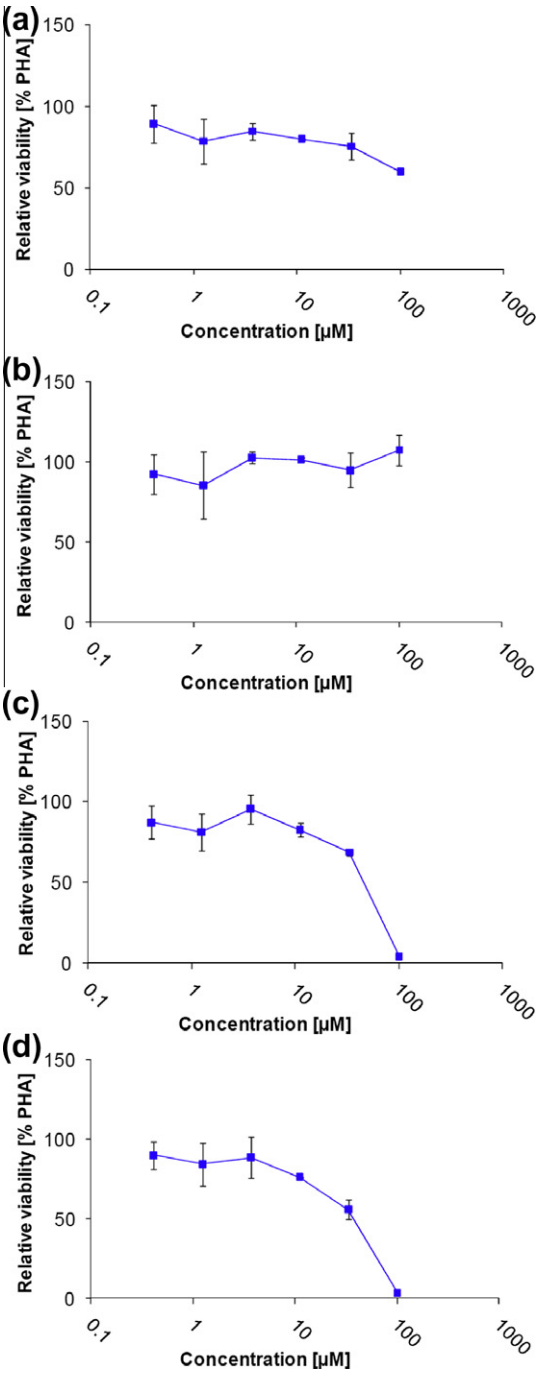

Fig. 6. Effect of the tested compounds on the viability of PBMCs. (a) Sapelenin G (**1**); (b) Sapelenin H (**2**); (c) Sapelenin I (**3**); (d) Sapelenin J (**4**). (Concentrations used: 0.41, 1.23, 3.70, 11.11, 33.33, and 100 µM).

isocratic for 5 min, the system returned to its initial condition (90% A) within 0.5 min, and was equilibrated for 4.5 min.

3.5. Physico-chemical constants of **1–4**

Sapelenin G (**1**): oil, $[\alpha]_D^{20}$ + 260 (c 0.01, MeOH); IR (KBr); ν_{max} 3386 (OH), 1660 (C=C), 1447, 1373, 1160, 1069, 1008 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) are shown in Table S1 (Supplementary data); HR-MS $[M+H]^+m/z$ 513.4152 (calcd. for $C_{30}H_{57}O_6$, 513.4155).

Sapelenin H (**2**): oil, $[\alpha]_D^{20}$ + 58 (c 0.01, MeOH); IR (KBr); ν_{max} 3430 (OH), 1660 (C=C), 1447, 1378, 1173, 1078, 1026 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) are shown in Table S1 (Supplementary data); HR-MS $[M+H]^+m/z$ 477.3945 (calcd. for $C_{30}H_{53}O_4$, 477.3944).

Sapelenin I (**3**): oil, $[\alpha]_D^{20}$ + 194 (c 0.01, MeOH); IR (KBr); ν_{max} 3430 (OH), 1660 (C=C), 1447, 1378, 1173, 1078, 1026 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) are shown in Table S1 (Supplementary data); HR-MS $[M+H]^+m/z$ 495.4054 (calcd. for $C_{30}H_{55}O_5$, 495.4049).

Sapelenin J (**4**): oil, $[\alpha]_D^{20}$ + 36 (c 0.01, MeOH); IR (KBr); ν_{max} 3430 (OH), 3360 (OH), 1730 (C=O), 1660 (C=C), 1443, 1373, 1243, 1082, 1021 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) are shown in Table S1 (Supplementary data); HR-MS $[M+H]^+m/z$ 537.4150 (calcd. for $C_{32}H_{57}O_6$, 537.4155).

3.6. Chemical conversion of sapelenin derivatives

3.6.1. Acetonidation of sapelenin I (**3**)

To a solution of sapelenin I (**3**) (20.0 mg, 0.040 mmol) in acetone (5 ml) was added 1 ml of 2,2-dimethoxypropane and 4 mg of *p*-toluene sulfonic acid. After 30 min at 20 °C, the solution was quenched with water and the reaction mixture was extracted with hexane and washed with $NaHCO_3$, and then dried over Na_2CO_3. The solvent was evaporated at reduced pressure and the extract was subjected to the HPLC to afford the acetonide (**3a**) (21.4 mg, 99%) as a colorless oil. HR-MS $[M+Na]^+$ *m/z* 557.4166 (calcd. for $C_{33}H_{58}O_5Na$, 557.4182).

3.6.2. Acetonidation of ekeberin D2 (**5**)

The same reaction was performed with **5** (7.0 mg, 0.014 mmol) to afford the expected acetonide (**5a**) (7.5 mg, 99.1%) as a colorless oil. HR-MS $[M+Na]^+$ *m/z* 557.4171 (calcd. for $C_{33}H_{58}O_5Na$, 557.4182).

3.6.3. Acetylation of sapelenin I (**3**)

Dry pyridine (0.5 ml) and Ac_2O (1.0 ml) were added to sapelenin I (**3**, 10 mg, 0.020 mmol) and stirred for 12 h. After the usual work-up, the extract was subjected to the HPLC to afford sapelenin I diacetate (**3d**) (5.7 mg, 48.7%) as a major colorless oil; diacetylsapelenin I, $[\alpha]_D^{20} = +192^\circ$ (c 0.005, MeOH); ^1H NMR (500 MHz, $CDCl_3$): 5.11 (1H, t, *J* = 5.5, H-22), 5.12 (2H, t, *J* = 6.5, H-14,18), 4.85 (1H, dd, *J* = 2.8, 9.7), 4.72 (1H, dd, *J* = 2.1, 10.2), 3.78 (1H, t, *J* = 5.7), 2.23 (1H, m, H-13), 2.12 (1H, m, H-5), 2.11 (3H, s, H_3CCO), 2.09 (3H, s, H_3CCO), 2.07 (4H, m, H-17,H-19), 2.00 (m, H-20), 1.85 (2H, m, H-4), 1.69 (3H, s, CH_3-24), 1.65 (2H, m, H-9), 1.61 (3H, s, CH_3-30), 1.58 (6H, s, CH_3-28,29), 1.54 (1H, m,), 1.53 (3H, m), 1.38 (1H, m), 1.23 (3H, s, CH_3-25), 1.17 (3H, s, CH_3-26), 1.15 (3H, s, CH_3-1); HR-MS $[M+Na]^+$ *m/z* 601.4073 (calcd. for $C_{34}H_{58}O_7Na$, 601.4080).

3.6.4. Acetylation of ekeberin D2 (**5**)

The same reaction was performed with **5** (10 mg, 0.020 mmol) to afford the expected ekeberin D2 diacetate (**5b**) (5.7 mg, 48.7%) of ekeberin D2 (**5**) as a major colorless oil; ekeberin D2 diacetate (**5b**), $[\alpha]_D^{20} = +10^\circ$ (*c* 0.005, MeOH); ^1H NMR (500 MHz, $CDCl_3$): 5.06 (m, 3H), 4.85 (dd, *J* = 2.2, 10.2), 4.72 (dd, *J* = 2.6, 10.2), 3.63 (t, *J* = 8.0), 2.03 (s, 3H), 1.99 (s, 3H), 1.52 (m, 9H), 1.13 (s, 3H), 1.12 (s, 3H), 1.11 (s, 6H), 1.03 (s, 3H); HR-MS $[M+Na]^+$ *m/z* 601.4073 (calcd. for $C_{34}H_{58}O_7Na$, 601.4080).

3.6.5. Hydrolysis of sapelenin H (**2**)

To a solution of sapelenin H (**2**, 10 mg, 0.021 mmol), 1 N H_2SO_4 (5 ml) was added in one portion. The mixture was stirred at room temperature until the HPLC analysis revealed the disappearance of the starting epoxide (10 h). The reaction medium was then ex-

tracted with dichloromethane (5 × 3 ml), and the organic solution was decanted. The solvent was evaporated at reduced pressure and the extract subjected to the preparative HPLC to afford the natural product ekeberin D_2 (**5**, 10.1 mg, 97.3%).

*3.6.6. Esterification of sapelenin H (**2**) with (R) and (S)-MTPA chloride*

Two portions of sapelenin H (**2**, 7 mg, 0.014 mmol each) were treated with (*R*) and (*S*)-MTPA chloride in 4-(dimethylamino)pyridine (DMAP), stirring at room temperature. The two reactions were conducted in parallel and the progress was monitored by the analytical HPLC. After complete consumption of the sapelenin H (**2**), the solvent was evaporated and the reaction mixture submitted to the preparative HPLC to afford the (*S*) and (*R*)-MTPA ester of **2** (7.5 mg each, 74%).

*3.6.7. Esterification of compounds **3a** and **5a** with MTPA chloride*

The same reaction of **3a** (5 mg, 0.009 mmol) and **5a** (10 mg, 0.019 mmol) with (*R*) and (*S*)-MTPA chloride afforded the (*S*) and (*R*)-MTPA ester of **3a** (5.2 mg, 74%) and **5a** (10.2 mg, 74%) respectively.

3.7. Biological activities

3.7.1. Anti-inflammatory assay

The suppression of anti-inflammatory response assay (IL-17 ELISA) was performed according to Zvetkova et al. (2001). The detailed assay parameters are elaborated in the Supplementary data (Table S2 for assay conditions, instrumentations and parameters; Fig. S1 represents the general plate layout for the assay; Fig. S2 shows the viability of PBMC controls).

3.7.2. Viability of PBMCs assay (cytotoxicity assay)

The cytotoxicity of the tested compounds on PBMCs was performed using the Resazurin-based CellTiter-Blue® cell viability assay kit (Promega) strictly following the manufacturer's guidelines (Anonymous, 2009). The detailed assay parameters are elaborated in the Supplementary data (Table S3 for assay conditions, instrumentations and parameters).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.06.004.

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