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Abstract

Within an ongoing research program on saponins with potential antileishmanial activity, four previously undescribed saponins were isolated from *Maesa argentea* leaves and identified by LC-MS/MS, GC-MS and 1D and 2D NMR spectroscopy as 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-butanoyloxy-13β,28-oxidoolean-16α,28α-diol (**1**), 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β, 22α-angeloyloxy-13β,28-oxidoolean-16α,28α-diol (**2**), 3β-*O*-{([-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galacto-pyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-(*E*)-cinnamoyloxy-13β,28-oxidoolean-16α,28α−diol (**3**) and 3β-*O*-{([α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-(*E)*-cinnamoyloxy-13β,28-oxidoolean-16α,28α-diol (**4**). Leaf material was obtained from a germinated seed that was clonally propagated using *in vitro* tissue culturing. Compounds **1** - **4** showed structural similarity with maesasaponins and maesabalides reported before from other *Maesa* spp. All four compounds showed *in vitro* activity against *Plasmodium falciparum* K1 and *Leishmania infantum* at micromolar concentrations. However, the observed inhibitory action must be considered non-specific since they were also cytotoxic in the same concentration range.

Key words

Maesa argentea, Myrsinaceae, saponins, antiprotozoal activity, maesabalides, maesasaponins

Introduction

M. argentea (Wallich) A. de Candolle is a shrub or rarely a small tree up to 5 m tall. The plants, belonging to the family of the Myrsinaceae, grow in broad-leaved forests, hilly areas, valleys, stream banks, damp places, between 1500–2900 m height, and can be found in China, India, Myanmar and Nepal [1]. According to ethnobotanical information the leaves of this plant are known for their piscicidal activity and use in fish catching [2]. Previous studies on a methanol extract of the leaves of *M. argentea* revealed *in vitro* inhibition of *Leishmania infantum*. Although LC-MS analysis had disclosed the presence of several known maesasaponins, this initial mass spectrometric analysis also revealed the presence of several other compounds, possibly saponins as well, which might contribute to the high *in vitro* antileishmanial activity (IC_{50}: < 0.125 µg/mL) [3,4]. Since plants are still important sources of new lead compounds, these additional potentially active constituents were isolated and identified in the present study.

Results and discussion

The crude methanol (80 %) extract of the leaves of *M. argentea* was partitioned using petroleum ether to remove lipids and flash chromatography was performed on the remaining defatted extract. Fractions were monitored by TLC and combined according to their chromatographic pattern. Semi-preparative HPLC of a selected combined fraction resulted in the isolation of 4 saponins (**1 – 4**), whose *m/z* values of the sodiated molecules $[M + Na]^+$ and the specific optical rotation are shown in Table 1. Information on the structure of the compounds was obtained by analysing the MS^n data of the crude extract. The mass fragmentation pattern of compound **1** and **2** with $[M - H]^-$ ions at *m/z* 1465 and 1477 was closely related to respectively maesasaponin V.3 and VI.2, but the glycan part contained one additional hexose residue [4]. The *m/z* 1363 MS^2 product ion spectrum (compound **4**) revealed peaks at *m/z* 1201 and 1183, corresponding to Y and Z type ions formed by the loss of an hexose residue (162 u) and a hexose (180 u), respectively, and an ion at *m/z* 1217, corresponding to the Y ion formed by the loss of a pentose residue. Both hexose and pentose were found in a terminal

position [5]. Other product ions generated from *m/z* 1363 included *m/z* 1055 $[M - H - 308]^-$, *m/z* 893 $[M - H - 470]^-$ and *m/z* 717 $[M - H - 646]^-$. The formation of product ion at *m/z* 1055 $[M - H - 308]^-$ could be attributed to the loss of a pentose and hexose residue, product ion at *m/z* 893 $[M - H - 470]^-$ to the loss of one pentose residues and two hexose residues, respectively, while product ion at *m/z* 717 $[M - H - 646]^-$ was due to the loss of two pentose residues, one hexose residue and one hexuronic acid. The signals at *m/z* 1215 $[M - H - 148]^-$ and m/z 1263 $[M - H - 100]^-$ in the *m/z* 1363 MS^2 product ion spectrum could be explained by the loss of a cinnamic acid residue and an angelic acid residue, respectively.

Both MS^2/MS^3 data was obtained for compound **3**. The *m/z* 1525 MS^2 product ion spectrum revealed ions at *m/z* 1363 and 1345, corresponding to Y and Z type ions formed by the loss of a terminal hexose residue (162 u) and a hexose (180 u), respectively [5]. Compared to compound **4**, the *m/z* 1525 MS^2 product ion spectrum of compound **3** indicated that there was no deoxyhexose in a terminal position, which was further supported by the absence of a product ion at *m/z* 1379 $[M - H - 146]^-$. The presence of a cinnamic acid residue and an angelic acid residue was confirmed by the signals at *m/z* 1377 $[M - H - 148]^-$ and m/z 1424 $[M - H - 100]^-$ in the *m/z* 1525 MS^2 product ion spectrum. The *m/z* 1525 → *m/z* 1363 MS3 product ion spectrum showed ions at *m/z* 1201 $[M - H - 324]^-$, *m/z* 1217 $[M - H - 308]^-$ and *m/z* 893 $[M - H - 633]^-$, *m/z* 717 $[M - H - 808]^-$, which could be assigned to the combined loss of two hexose residues, one hexose residue and one deoxyhexose residue, three hexose residues and one deoxyhexose residue, and three hexose residues one deoxyhexose residue and one hexuronic acid.

The identification of all monosaccharides, obtained after acid hydrolysis of the individual compounds was performed by GC-MS analysis. Comparison of the retention times obtained from trimethylsilylated reference sugars with the results obtained from the GC-MS analysis of the trimethylsilylated monosaccharides of the samples, revealed the presence of galactose, rhamnose, glucose and glucuronic acid in the hydrolysed samples of compounds **1-3**, while the hydrolysed sample of compound **4** contained galactose, rhamnose, and glucuronic acid (table 2) .

Thorough investigation of the 1D and 2D NMR spectra of compounds **1 – 4** was performed and the structures of the glycosidic moieties as well as the aglycons were elucidated by detailed analysis of the DQF-COSY, HSQC and HMBC correlations and by comparison with spectral data found in the literature for related compounds [6-8]. All ^{1}H- and ^{13}C-NMR signals are listed in Table 3.

1D and 2D NMR spectra of compound **1** (maesargentoside I) revealed that the aglycon of the saponin was identical to that of maesasaponin V_3 (Fig. 1). The presence of an angeloyl substitution of the aglycon, indicated by mass spectrometric analysis, could be confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Signals characteristic of the angeloyl group occurred at δ_H 6.05 (H-3), 1.86 (H-4), 1.75 (H-5) in ^{1}H-NMR and at δ_C 166.5 (C-1), 127.7 (C-2), 137.1 (C-3), 15.3 (C-4), 20.2 (C-5) in ^{13}C-NMR. The position of esterification (C-21) was resolved by the long-range C-H correlation between the carbonyl group of angelic acid (δ_C 166.5) and H-21 (δ_H 5.89, d, *J* = 9.6 Hz) observed in an HMBC experiment. A direct C-H correlation of H-21 with δ_C 78.1 (C-21) was observed in the HSQC experiment. H-21 and H-22 (δ_H 5.39, d, 10.1) were correlated in the ^{1}H-^{1}H DQF-COSY spectra, while the HMBC experiment also revealed a long-range C-H correlation between C-22 (δ_C 71.6) and H-21. The long-range C-H correlation observed in the HMBC experiment between the carbonyl group of butanoic acid (δ_C 166.5) and H-22 showed the presence of an *n*-butanoyl group at position 22 of the aglycon. Other signals characteristic of the *n*-butanoyl group were present at δ_H 2.02 (H-2a), 2.09 (H-2b), 1.42 (H-3a and b), 0.78 (H-4) in ^{1}H NMR and at δ_C 171.1 (C-1), 35.7 (C-2), 18.0 (C-3), 13.3 (C-4) in ^{13}C-NMR. Based on the previously reported ^{1}H- and ^{13}C-NMR data and coupling constants of maesasapoinin V_3 and all DQF-COSY, HSQC and HMBC correlations, the aglycon could unambiguously be elucidated as 21β-angeloyloxy-22α-butanoyloxy-13β,28-oxidoolean-16α,28α-diol. Whereas the aglycon was previously reported, the glycan part displayed some differences with known saponins, since NMR data in accordance with MS data, indicated the presence of five anomeric carbons.

The glycan part of the molecule was elucidated as ([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl), which in fact was the glycan part of maesasaponin V3 with one additional

β–glucopyranosyl residue linked to the α-rhamnopyranosyl residue. The glycan part was linked in position C-3 of the aglycon, based on a long-range correlation observed between C-3 of the aglycon (δ_C 88.9) and H-1 of the β–glucuronopyranosyl moiety (δ_H 4.44, d, *J* = 6.6 Hz). The position of further glycosyl substitutions was evident from several long-range C-H correlations. C-1 (δ_C 102.4) of the terminal β–galactopyranosyl unit displayed a correlation with H-2 (δ_H 3.57, m) of the β–glucuronopyranosyl unit, while the anomeric carbon (δ_C 99.4) of the second β-galactopyranosyl unit showed a correlation with the H-3 (δ_H 3.81, m) of the β–glucuronopyranosyl unit. The chemicals shifts for this 2,3-glycosidic derivative of 3-β-glucuronopyranosyl were in agreement with those previously described for this substitution pattern [8]. The anomeric carbon (δ_C 99.4) of the α–rhamnopyranosyl moiety showed a correlation with H-2 (δ_H 3.53, m) of the second β–galactopyranosyl unit, while a long-range C-H correlation of the anomeric carbon (δ_C 105.6) of the terminal β–glucopyranosyl unit with H-2 (δ_H 3.76, m) of the α–rhamnosyl moiety was observed. The chemical shifts of all carbons of β–galactopyranose, β–glucopyranose and α-rhamnopyranose units were in agreement with those reported before for related compounds [6-8]. Hence, compound **1** (maesargentoside I) could unambiguously be identified as 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-butanoyloxy-13β,28-oxidoolean-16α,28α-diol.

1D and 2D NMR spectra of compound **2** (maesargentoside II) revealed, as previously considered by LC-MS analysis, that the aglycon of the saponin was identical to that of maesasaponin VI_2 [4]. A difference between the aglycon of compound **1** and **2** could be observed at position 22, where an additional angeloyl substituent was present instead of an *n*-butanoyl group. The long-range C-H correlation observed in the HMBC experiment between the carbonyl group of angelic acid (δ_C 165.7) and H-22 showed the presence of an angeloyl group at position 22 of the aglycon. Other signals characteristic of the angeloyl group could be observed at δ_H 5.88 (H-3), 1.76 (H-4), 1.72 (H-5) in ^{1}H-NMR and at δ_C 165.7 (C-1), 128.3 (C-2), 134.9 (C-3), 15.0 (C-4), 20.2 (C-5) in ^{13}C-NMR. The glycan part was identical to that of compound **1**, i.e. ([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-

D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl). Hence, compound **2** (maesargentoside II) could be identified as 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β, 22α-angeloyloxy-13β,28-oxidoolean-16α,28α-diol.

Although the structure of compounds **1** and **2** was related to maesasaponins V3 and VI2, originally isolated from *M. lanceolata*, compounds **3** and **4** more closely resembled known maesabalides, originally obtained from *Maesa balansae*. The aglycon of both compounds **3** and **4** was identical to that of maesabalide IV [6]. 1D and 2D NMR spectra of compound **3** and **4** revealed the same position (C-21) of esterification with angelic acid, but the long-range C-H correlation between the carbonyl group of a cinnamic acid moiety (δ_C 164.8) and H-22 showed the presence of a cinnamoyl group at position 22 of the aglycon. Other signals characteristic of this acyl group were observed at δ_H 6.41 (H-2, d, *J*=16.1), 7.52 (H-3, d, *J*=15.6), 7.36 (H-5 and H-9), 7.57 (H-6 and H-8), 7.40 (H-7) in ^{1}H NMR and at δ_C 164.8 (C-1), 118.5 (C-2), 143.9 (C-3), 134.1 (C-4), 130.4 (C-5 and C-9), 128.1 (C-6 and C-8), 129.7 (C-7) in ^{13}C NMR. Based on the coupling constant (16 Hz) between the olefinic protons H-2 and H-3, the (*E*)-configuration of the cinnamoyl group was established. Compounds **1-3** contain the same glycan moiety and differed from maesabalide IV [6]. Compound **3** (maesargentoside III) could be identified as 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-(*E*)-cinnamoyloxy-13β,28-oxidoolean-16α,28α-diol.

While compound **4** was identical to compound **3** in terms of the aglycon, the 1D and 2D NMR spectra revealed only 4 anomeric carbons. The terminal β-glucopyranosyl unit observed in compounds **1-3** was not present in compound **4**. The absence of the terminal β–glucopyranosyl unit was confirmed by the chemical shift of the C-2 of the α-rhamnosyl unit, being δ_C 70.5 instead of δ_C 81.0 (C-2) as seen in compound **1-3** and the correlation of H-2 (δ_H 3.67 m) with H-1 (δ_H 5.02, d) and H-3 (δ_H 3.50, m) in the COSY spectrum. Hence, compound **4** (maesargentoside IV) was elucidated as 3β-*O*-{([α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-

glucuronopyranosyl)}-21β-angeloyloxy-22α-(*E*)-cinnamoyloxy-13β,28-oxidoolean-16α,28α-diol. All ^{1}H-and ^{13}C-NMR signals could be assigned by means of the correlations observed in the 2D NMR spectra, and were in agreement with the proposed structure.

Compounds **1** – **4** displayed a non-selective antiprotozoal activity (Table 4). IC_{50} values down to 0.8 µM were observed against *Plasmodium falciparum* strain K1 (*Pf* K1), but cytotoxicity against MRC-5 lung fibroblasts was in the same dose range. Related 13,28-epoxy-oleanane triterpene saponins, i.e. the maesabalides, were previously reported to exhibit a potent and selective activity against *Leishmania* which was explained by their amphiphilic nature, inducing the formation of micelles that are selectively taken up by phagocytic macrophage host cells. Hence, saponins tend be more cytotoxic *in vitro* to peritoneal mouse macrophages (PMM) than to other cells . Although compound **3** and **4** showed a similar structure to the maesabalides, selectivity could not be observed for the present compounds. The immunomodulating effect of saponins may also play a role in the elimination of infection, endorsing the concept that some saponins could still move forward in antileishmanial drug discovery [3,9].

Materials and Methods

Plant material

Authentic *Maesa argentea* seeds (no. 61-2068) (Myrsinaceae) were provided by the internationally recognised National Botanical garden (Meise, Belgium). Plant material derived from a single authentic seed that was clonally micropropagated following the *in vitro* propagation protocol previously described [10]. Shoot cuttings were cultivated for three months and harvested leaf material was subsequently air dried.

Extraction and isolation

Dried and powdered leaves (55 g) were macerated with methanol 80% (MeOH) until exhaustion. After concentration under reduced pressure, the methanolic extract was subjected to liquid–liquid

partitioning with petroleum ether (14 g). The residual methanolic layer was evaporated under reduced pressure to dryness. An amount of 2.58 g of the dried methanolic extract was mixed with silica 60 GF_{254} (3.46 g) and subjected to flash chromatography using a Reveleris iES Flash chromatography system and a 80 g Reveleris silica column (Grace Vydac). Gradient elution was applied: solvent A: dichloromethane; solvent B: ethyl acetate; solvent C: methanol; gradient: from 100 % A to 100% B in 8.33 min, 100% B during 1.67 min, from 100% B to 45% C in 60 min; flow rate: 60 mL/min. Fractions were collected in volumes of 24 mL and were monitored by normal phase–TLC (NP-TLC) (20 x 20 cm, silica gel 60 F254). Subsequently the plate was developed with the organic phase of *n*-butanol-acetic acid-water 4:1:5, sprayed with anisaldehyde-sulphuric acid reagent and heated to 105 °C. Based on the observed TLC pattern, similar fractions were combined, yielding 7 fractions.

Fractions 3 till 7 were separated by repeated semi-preparative HPLC by the aid of an autopurification system with Quattro micro mass detector (Waters) on a Luna C18 Column (Phenomenex) (250 x 10 mm, 5 μm). Gradient elution was applied: solvent A: 0.05% formic acid; solvent B: acetonitrile + 0.05% formic acid, gradient: 0 min - 42% B, 3 min – 42% B, 22 min - 54% B, 24 min – 42% B, 30 min - 42% B; flow rate: 4.75 mL/min, injection volume: fraction 3 - 1000 μL, fraction 4 till 7 – 300 μL, concentration fractions: 20 mg/mL. A make-up flow was used containing methanol 80% + 0.1% Formic acid. Detection was performed at positive ion mode under following conditions: capillary voltage, 3.50 KV; Cone voltage, 51 V; extractor voltage, 3 V; source temp., 120 °C; desolvation temp., 400 °C; desolvation gas flow, 800 L/hr; cone gas flow, 50 L/hr. Four compounds were isolated (**1 - 4**) (yield: 17.9 mg, 14.4 mg, 16.6 mg, 18.2 mg respectively).

Structure elucidation

LC/MS analysis of the crude extract of *M.* argentea was performed according to the HPLC method of Theunis et al. [11] employing a Surveyor LC system (Thermo Fisher) and a silica-based 300 monomeric C18 column (Grace Vydac) (250 x 3.2 mm, 5 μm). The flow rate was 0.5 mL/min, the

gradient program was as follows: solvent A: 0.05% acetic acid; solvent B: acetonitrile + 0.05% acetic acid; gradient: 10 min 25% B – from 25 to 60% B in 30 min – from 60 to 90% B in 7 min – from 90 to 25% B in 3 min – 5 min 25%B. The injection volume was 20 µL. The LC system was coupled to an LXQ linear ion trap (Thermo Fisher). The experimental conditions for operation of the instrument in the (–)ESI mode were optimized by direct infusion of a solution of maesasaponins (100 µg/mL). The optimal conditions were as follows: sheat gas flow, 36 arbitrary units; auxiliary gas flow, 14 arbitrary units; sweep gas flow, 3 arbitrary unit; spray voltage, + 4.0 kV; ion transfer tube temperature, 350 °C; and capillary voltage, –3 V. MS^n Mass spectral data were recorded using data-dependant scanning of a parent mass list containing the m/z $[M-H]^-$ value of all compounds of interest. For MS^n experiments an isolation width of 2 Da was used and a normalized collision energy of 35% was applied. All data were recorded and processed using Xcalibur software, version 2.0 (Thermo Fisher).

Besides mass measurements using a linear ion trap, accurate mass measurements were carried out using a QTOF 6530 mass spectrometer (Agilent technologies) equipped with an electrospray ionisation (ESI) source operated in the positive ion mode at 20000 resolution. Accurate mass measurements were obtained using external calibration. MS spectra of the isolated compounds (**1 - 4**) were obtained using direct infusion. All data were acquired and processed using MassHunter software, version B.06.

For GC-MS analysis compounds **1-4** (4 mg) were hydrolysed by trifluoroacetic acid (1.5 mL, 2 M) at a temperature of 120 °C for 5 h. 150 µL of the reaction mixture was dried and the residue was dissolved in pyridine (20 µL) and treated with 80 µL N,O-Bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane (BSTFA + TMCS). The sample was sonicated for 1 h. The reference monosaccharides (D-ribose, L-rhamnose monohydrate, L-arabinose, L-xylose, D-glucuronic acid, D-glucose, D-fructose, L-fucose, D-galactose, D-mannose, D-galacturonic acid (100 µg)) were also treated with pyridine and the trimethylsilylation reagent. All samples were analysed according to Medeiros et al. [12], employing a Voyager GC-MS with Trace 2000 GC (Thermo Finnigan) and a Alltech Heliflex AT-5ms capillary column with a length of 30 m, internal diameter of 0.25 mm and film

thickness of 0.25µm (Alltech Associates, Inc). The carrier gas He was used at a constant flow rate of 1.3 mL/min, the injector and MS source temperature were maintained at 200 °C and 230 °C respectively. The temperature program was as follows: start at 65 °C – hold on 65 °C for 2 min. – temperature increase of 6 °C/min till 300 °C – hold on 300 °C for 15 min. The MS was operated in the electron impact mode with ionisation energy of 70 eV. Chromatograms were recorded using selected ion monitoring (SIM), looking for prominent mass peaks at *m/z* 204 and 217. 1 µL of every sample was analysed with a split ratio of 8. All data were recorded and processed using Xcalibur software, version 1.0 (Thermo Fisher). The monosaccharide units present in the hydrolysed sample were elucidated by means of the retention time of the reference sugars.

NMR Spectra were recorded in DMSO-d_6 on a Bruker DRX-400 instrument, operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, using standard Bruker software. Chemical shifts are expressed in ppm and coupling constants (*J*) in Hz.

Specific rotation was determined on a Jasco P-2000 polarimeter.

Isolated compounds

Compound 1 (maesargentoside I): 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-butanoyloxy-13β,28-oxidoolean-16α,28α-diol, white powder, amorphous, $[\alpha]^{20}$ and HR-MS see table 1, ^{1}H- and ^{13}C-NMR see table 3.

Compound 2 (maesargentoside II): 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β, 22α-angeloyloxy-13β,28-oxidoolean-16α,28α-diol, white powder, amorphous, $[\alpha]^{20}$ and HR-MS see table 1, ^{1}H- and ^{13}C-NMR see table 3.

Compound 3 (maesargentoside III): 3β-*O*-{([β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-

angeloyloxy-22α-(*E*)-cinnamoyloxy-13β,28-oxidoolean-16α,28α–diol, white powder, amorphous, $[\alpha]^{20}$ and HR-MS see table 1, ^{1}H- and ^{13}C-NMR see table 3.

Compound 4 (maesargentoside IV): 3β-*O*-{([α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→3)]-[β-D-galactopyranosyl-(1→2)]-β-D-glucuronopyranosyl)}-21β-angeloyloxy-22α-(*E*)-cinnamoyloxy-13β,28-oxidoolean-16α,28α-diol, white powder, amorphous, $[\alpha]^{20}$ and HR-MS see table 1, ^{1}H- and ^{13}C-NMR see table 3.

Antiprotozoal activity

The antiprotozoal activity against *L. infantum* and *P. falciparum* strain K1 and cytotoxicity of the isolated components was evaluated as described before [13,14]. The used reference drugs miltefosine, chloroquine and tamoxifen were pure (>95%) compounds and were obtained from the fine chemical supplier Sigma Chemical Co. Testing for antiprotozoal activity was performed in 96-well plates (Greiner), each plate containing 16 samples at 4-fold dilutions in a dose-titration range of 64 µg/mL to 0.25 µg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). Each assay was triplicated in an independent screening experiment. IC_{50}-values were calculated from the individual dose-response curves using GraphPad Prism software. The mean IC_{50}-values of the replicate assays are presented in Table 4.

Antileishmanial assay: *Leishmania infantum* MHOM/MA(BE)/67 was obtained from the Institute of Tropical Medicine Antwerp (ITMA) and is maintained in the golden hamster and spleen amastigotes are collected for infection. Primary peritoneal mouse macrophages (PMM) are used as host cells and collected 2 days after peritoneal stimulation with a 2% potato starch suspension. Assays are performed in 96-well microtiter plates, each well containing 10 µL of the compound dilutions to which 190 µL of macrophage-parasite inoculum (3.10^5 cells + 3.10^6 parasites/well // RPMI-1640 + 5% FCSi) was added. After 5 days of incubation, cells were dried, fixed with methanol and stained with

20% Giemsa to assess total intracellular amastigote burdens through microscopic reading. The results are expressed as percentage reduction of amastigote burden compared to untreated control cultures and IC_{50}-values were calculated. Miltefosine was included as a reference compound [13,14].

Antiplasmodial assay: Chloroquine-resistant *P. falciparum* 2/K 1-strain was obtained from the London School of Hygiene and Tropical Medicine and is cultured in human erythrocytes O^+ at 37°C under micro-aerophilic atmosphere (3% O_2, 4% CO_2, and 93% N_2) in RPMI-1640 supplemented with 10% human serum. Two hundred µL of infected RBC (1% parasitaemia, 2% haematocrit) was added in each well of a 96 well plate containing pre-diluted extract. The test plates were kept in the modular incubator chamber for 72 h at 37°C and subsequently put at -20°C to lyse the red cells upon thawing. Next, 100 µL of Malstat TM reagent were put in new microtiter plate to which 20 µL of haemolysed parasite suspension was added. After 15 minutes incubation at room temperature, 20 µl of NBT/PES solution was added. The plate was incubated in the dark for another two hours at room temperature and spectrophotometrically read at 655nm. Chloroquine was included as a reference compound [13,14].

Cytotoxicity assay: MRC-5 SV2 cells were purchased from Sigma-Aldrich and are cultivated in MEM medium, supplemented with L-glutamine (20 mM), 16.5 mM $NaHCO_3$ and 5% FCS at 37°C and 5% CO_2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the pre-diluted samples and incubated at 37°C and 5% CO_2 for 3 days. Cells viability were determined fluorimetrically after addition of resazurin. The results are expressed as % reduction in cell growth/viability compared to untreated control wells and a CC_{50} is determined [13,14]. The mean and standard deviation (SD) of at least 3 experiments were calculated. Tamoxifen was included as a reference compound [13,14].

Supporting information

1D and 2D NMR spectra of **compound 1-4** are available as Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

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Tables

Table 1: Chemical formulas and accurate mass measurements of the sodiated molecules $[M + Na]^+$, isolated from *Maesa argentea* and the optical rotation of the compounds.

Compound	Chemical formula $[M + Na]^+$	$[M + Na]^+$ calculated	$[M + Na]^+$ experimental	$[\alpha]^{20}$
1	$C_{70}H_{110}O_{33}Na$	1489.6822	1489.6941	-27 (c 0.61, DMSO)
2	$C_{69}H_{110}O_{33}Na$	1501.6821	1501.6920	-25 (c 0.50, DMSO)
3	$C_{74}H_{110}O_{33}Na$	1549.6821	1549.6852	-31 (c 0.48, DMSO)
4	$C_{68}H_{100}O_{28}Na$	1387.6293	1387.6336	-34 (c 0.61, DMSO)

Table 2: Retention times of the trimethylsilylated reference monosaccharides and trimethylsilylated monosaccharides of the hydrolysed samples analysed by GC-MS.

Reference compound	Retention time (min; α–, β–)	Compound **1** Retention time (min; α–, β–)	Compound **2** Retention time (min; α–, β–)	Compound **3** Retention time (min; α–, β–)	Compound **4** Retention time (min; α–, β–)
L-arabinose	19.29, 19.86				
D-fructose	22.76, 22.91				
L-fucose	20.24, 21.01				
D-galactose	23.69, 24.46	23.69, 24.44	23.67, 24.42	23.69, 24.44	23.69, 24.45
D-galacturonic acid	25.29, 26.32				
D-glucose	24.29, 25.82	24.25, 25.78	24.23, 25.78	24.26, 25.78	
D-glucuronic acid	25.63, 26.45	25.63, 26.46	25.62, 26.44	25.64, 26.47	25.64, 26.48
D-mannose	22.81, 24.47				
L-rhamnose	19.49, 20.69	19.49, 20.70	19.47, 20.68	19.50, 20.70	19.50, 20.70
L-xylose	21.09, 22.04				

Table 3: ^{1}H- and ^{13}C-NMR assignments for compounds **1 - 4** [δ (ppm), *J* (Hz), DMSO-d6 (400 and 100 MHz)].

Carbon no.	**1**		**2**		**3**		**4**	
Aglycon	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	38.6	0.82, m 1.60, m	38.6	0.83, m 1.64, m	38.7	0.80, m 1.57, m	38.7	0.81, m 1.60, m
2	25.6	1.53, m 1.70, m	25.6	1.54, m 1.71, m	25.7	1.49, m 1.80, m	25.7	1.53, m 1.77, m
3	88.9	2.98, m	88.9	2.99, m	88.9	2.91,m	89.1	2.97, m
4	38.7		38.7		38.9		38.9	
5	54.8	0.60, d, 10.6	54.8	0.61, d, 10.8	54.9	0.57, d, 8.7	54.9	0.60, m, 10.1
6	17.1	1.31, m 1.39, m	17.2	1.32, m 1.40, m	17.2	1.27, m 1.38, m	17.2	1.31, m 1.38, m
7	33.5	1.11, m 1.40, m	33.5	1.11, m 1.43, m	33.6	1.06, m 1.40, m	33.5	1.10, m 1.41, m
8	41.8		41.9		41.8		41.9	
9	49.3	1.10, m	49.3	1.11, m	49.3	1.08, m	49.3	1.10, m
10	36.1		36.1		36.1		36.1	

11	18.3	1.37, m	18.3	1.41, m	18.2	1.41, m	18.3	1.42, m
		1.09, m		1.11, m		1.07, m		1.08, m
12	32.3	1.26, m	32.5	1.25, m	32.4	1.26, m	32.3	1.28, m
		1.88, m		1.92, m		1.89		1.90,
13	86.2		86.9		86.3		86.3	
14	42.6		42.7		42.7		42.8	
15	35.1	1.16, m	35.1	1.18, m	35.2	1.15, m	35.2	1.17, m
		1.76, m		1.79, m		1.79, m		1.80, m
16	67.5	4.09, br s	67.8	4.15, br s	67.6	4.18, br s	67.6	4.20, m
17	52.7		52..8		52.8		52.8	
18	44.9	1.73, m	45.7	1.76, m	45.0	1.75, m	45.1	1.76, m
19	37.2	1.23, m	37.1	1.24, m	37.2	1.22, m	37.3	1.25, m
		2.52, m		2.51, m		2.50, m		2.52, m
20	36.5		36.5		36.6		36.6	
21	78.1	5.89, d,	77.9	5.94, d,	78.1	5.86, d,	78.3	5.90, d,
		9.6		9.7		*		10.1
22	71.6	5.39, d,	71.1	5.50, d,	72.2	5.49, m,	72.2	5.53, d,
		10.1		10.2		*		10.1
23	27.4	0.95, s	27.4	0.95,s	27.4	0.91	27.4	0.95,

24	15.8	0.72, s	15.8	0.75, s	15.9	0.69, s	15.9	0.73, m
25	15.9	0.78, s	16.0	0.79, s	16.0	0.75, s	16.0	0.78, s
26	18.0	1.03, s	18.0	1.07,s	18.1	1.02,s	18.1	1.05, s
27	18.9	1.16, s	18.9	1.17, s	19.0	1.15, s	19.0	1.18, s
28	95.7	4.50, s	95.8	4.52, s	95.7	4.50, s	95.7	4.53, s
29	29.3	0.80, s	29.3	0.82, s	29.4	0.80, s	29.4	0.82, s
30	20.2	0.94, s	20.0	0.94, s	20.2	0.93, s	20.2	0.95, s
GlcA								
1	103.6	4.44, d, 6.8	103.6	4.44, d, 7.1	103.7	4.26, d, 7.7	103.8	4.35, d, 7.3
2	78.4	3.57, m	78.5	3.59, m	78.8	3.52, m	78.5	3.58, m
3	80.3	3.81, m	80.3	3.82, m	80.0	3.75, m	80.1	3.79, m
4	69.6	3.39, m	69.6	3.41, m	70.0	3.23, m	69.9	3.33, m
5	75.0	3.66, m	75.1	3.69, m	75.4	3.46, m	75.0	3.57, m
6	170.1		170.1		171.4		170.8	
GalI								
1	102.4	4.54, d, 7.0	102.3	4.55, d, 7.3	102.5	4.52, d, *	102.0	4.62, d, *
2	71.1	3.25, m	71.1	3.26, m	71.3	3.24, m	71.2	3.26, m
3	73.4	3.22, m	73.4	3.22, m	73.4	3.18, m	73.5	3.21, m
4	67.8	3.65, m	67.8	3.66, m	67.8	3.61, m	67.9	3.65, m

5	74.5	3.40, m	74.6	3.43, m	74.5	3.40, m	74.2	3.52, m
6	60.1	3.45, m	60.1	3.44, m	60.1	3.40, m	60.3	3.49, m
GalII								
1	99.4	4.98, d, 7.6	99.4	4.99, d, 6.1	99.2	5.05, d, *	99.5	5.05, d, 6.1
2	73.9	3.53, m	73.9	3.50, m	74.0	3.46, m	74.2	3.55, m
3	75.2	3.49, m	75.3	3.50, m	75.1	3.46, m	75.2	3.36, m
4	69.1	3.57, m	69.1	3.58, m	69.1	3.54, m	74.7	3.38, m
5	75.2	3.38, m	75.2	3.38, m	74.7	3.32, m	74.1	3.52, m
6	60.4	3.50, m	60.4	3.49	60.3	3.46, m	60.5	3.53, m
Rha								
1	99.4	5.31, d, *	99.4	5.31, d, *	99.5	5.25, d, *	100.4	5.02,d, *
2	81.0	3.76, m	81.0	3.75, m	81.2	3.72, m	70.5	3.67, m
3	70.7	3.55, m	70.8	3.55, m	70.8	3.51, m	71.0	3.50, m
4	72.4	3.18, m	72.4	3.19, m	72.4	3.13, m	72.0	3.19, m
5	67.9	3.85, m	67.9	3.85, m	68.0	3.83, m	68.2	3.86, m
6	17.6	1.12, d, *	17.6	1.12, d, 5.7	17.7	1.063, d, *	17.8	1.09, d, 5.9
Glc								
1	105.6	4.27, d, 7.4	105.6	4.28, d, 8.0	105.7	4.23, d, *		
2	74.0	3.01	74.0	3.02	74.0	2.96		

3	76.4	3.14	76.4	3.12	76.4	3.10		
4	69.9	3.05	69.9	3.05	69.9	3.03		
5	76.8	3.05	76.8	3.06	76.8	3.04		
6	61.0	3.44	61.1	3.43	61.0	3.40		
		3.64		3.65		3.61		
R1								
1	166.5		166.5		166.7		166.8	
2	127.7		127.8		127.9		127.9	
3	137.1	6.05, q, 8.2	136.7	5.99, q, *	136.3	5.87, m	136.3	5.90, m
4	15.3	1.86, m	15.2	1.83, m	15.2	1.72, m	15.2	1.73, m
5	20.2	1.75, m	20.2	1.76, m	20.3	1.72, m	20.3	1.73, m
R2								
1	171.1		165.7		164.8		164.8	
2	35.7	2.02, m	128.2		118.5	6.41, d,	118.5	6.41, d,
		2.09,m				16.1		16.0
3	18.0	1.42, m	134.9	5.88, q, *	143.9	7.52, d,	143.9	7.52, d,
		1.42, m				15.6		15.8
4	13.3	0.78, t	15.0	1.76, m	134.1		134.1	
5			20.2	1.72, m	130.4	7.36, d, *	130.4	7.39, d, *

6	128.1	7.57, m	128.1	7.6, m
7	129.7	7.40, m	129.0	7.40, m
8	128.1	7.61, m	128.1	7.61, m
9	130.4	7.40, d, *	130.4	7.39, d, *

* No multiplicity and/or J value could be observed due to extensive overlap

Table 4: Antiprotozoal and cytotoxic activity of the different maesargentosides from *M. argentea* and the reference compounds. Activities are expressed as IC_{50} ± SD (µM).

Compounds	MRC-5	PMM cytotoxicity	*L. inf.*	*Pf*-K1
1	1.47 ± 0.37	<0.14	< 0.10	2.60 ± 1.31
2	3.35 ± 2.38	<0.41	< 0.11	5.23 ± 3.05
3	1.69 ± 0.24	<0.17	< 0.09	0.76 ± 0.54
4	1.44 ± 0.24	<0.14	< 0.09	1.50 ± 0.32
Reference compound	Tamoxifen		Miltefosine	Chloroquine
	10.63 ± 0.13		6.09 ± 2.53	0.21 ± 0.16

Each assay was triplicated in an independent repeat assay

Legend for Figures

Fig. **1**: Chemical structures of compounds **1-4**