Melanin Production Inhibitors from the West African *Cassipourea congoensis*

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ABSTRACT

Cassipourea congoensis (syn. *Cassipourea malosana*) is used in African countries as a skin-lightening agent. Two previously unreported cycloartane triterpenoids, 26-hydroxy-3-keto-24-methylenecycloartan-30-oic acid **1** and 24-methylenecycloartan-3β,26,30-triol **2** along with the known mahuannin B **3**, 7-methoxymahuannin B **4**, 7-methoxygeranin A **5**, methyl-3-(4-hydroxy-3-methoxyphenyl)-2*E*-propenoate, glycerol-1-alkanoate, (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal **6**, (-)-syringaresinol **7**, and stigmast-5-en-3-O-β-D-glucoside, were isolated from the roots of *C. congoensis*. The crude extract and compounds **1** and **5** were found to inhibit the production of melanin at 10 µM with low cytotoxicity validating the ethnomedicinal use of this plant.

Introduction

Several topical agents are available to treat hyperpigmentation and act at different levels of the melanogenesis pathway [1, 2]. The most commonly used compounds are *p*-hydroquinone, corticosteroids, and mercurials [3]. The long-term use of these products may cause undesirable cutaneous or systemic side effects [3–5]. *p*-Hydroquinone was one of the earliest treatments for hyperpigmentation and remains the standard against which other treatments are measured [2, 6]. The misuse of this compound as a skin-lightening agent, despite its sale being prohibited in many countries, is significant, although its use is associated with serious side effects, including irritant contact dermatitis and exogenous ochronosis [3, 5, 7]. We have previously reported the nontoxic melanin production inhibitors from the South African medicinal plant, *Garcinia livingstonei* T.Anderson (Clusiaceae), which is used ethnomedicinally for skin-lightening purposes [8] and we now report the investigation of a second species used for its skin-lightening properties, *Cassipourea congoensis* R Br. ex DC (Rhizophoraceae). *p*-Hydroqui-

none inhibits tyrosinase, preventing the conversion of tyrosine to dihydroxyphenylalanine, a precursor to melanin. Thus, tyrosinase is a target in the search for a medically acceptable, natural, skin-lightening agent for medical or cosmetic purposes.

C. congoensis is a plant endemic to Africa, growing as a shrub but sometimes a small tree of 3–5 m high on forest river banks. It is distributed across Senegal to Nigeria and easterly across the Congo basin to Uganda, Tanzania, and Malawi [9]. *C. congoensis* was previously named *Cassipourea africana* Benth. or *Weihea africana* (Benth.) Oliv. [10]. The fruits of *C. congoensis* are used in Nigeria as a substitute for tamarind (*Tamarindus indica*) L. (Fabaceae) in preparing local pap, a maize meal, and its nutritional content has been studied [11]. It is also regarded as a synonym of *Cassipourea malosana* (Baker) Alston (Rhizophoraceae) that is restricted to eastern Democratic Republic of Congo, Ethiopia, and south to South Africa. *C. malosana* is also reported to be closely related to, and often confused with, *Cassipourea flanaganii* Schinz (Alston) (Rhizophoraceae) and *C. flanaganii* is, used traditionally in South Africa as a skin-lightening agent [12]. The *Cassipourea* genus is reported to yield sulfur-containing compounds that include gerrardine [13], guinesine B, and guinesine C [14], sulfur-containing amides that include cassipoureamide A and B [15], euphane and lupane type triterpenoids [16, 17], flavonols, flavonol glycoside and biflavonoids [18–20] and bioactive monocyclic diterpenoids [16] . Extractives from the stem bark of *C. malosana*, including 2 novel flavan dimers, were tested against human ovarian cancer cells and showed very little activity [21].

Two previously unreported cycloartane triterpenoids, 26-hydroxy-3-keto-24-methylenecycloartan-30-oic acid **1** and 24-methylenecycloartan-3β,26,30-triol **2** along with the known mahuannin B **3**, 7-methoxymahuannin B **4**, 7-methoxygeranin A **5**, methyl-3-(4-hydroxy-3-methoxyphenyl)-2*E*-propenoate, glycerol-1-alkanoate, (*E*)-3-(4-hydroxy-3-methoxyphenyl) prop-2-enal **6,** (-)-syringaresinol **7**, and stigmast-5-en-3-O-β-D-glucoside, were isolated from the roots of *C. congoensis.* The compounds **1**–**7**, together with the crude extract, were screened for cytotoxicity against melanocytes (human primary epidermal melanocytes, ATCC) as well as for tyrosinase and melanin inhibitory activity. The crude extract and compounds **1** and **5** were subsequently found to inhibit the production of melanin at both 10 and 100 µM with low cytotoxicity validating the ethnomedicinal use of this plant. No previous studies have assessed the cytotoxicity, the effect on inhibition of tyrosinase, or melanin inhibitory activity of *C. congoensis*.

Results and Discussion

In the present study we report the isolation of 2 new cycloartane tritepernoids, 26 hydroxy-3-keto-24-methylenecycloartan-30-oic, acid **1** and 24-methylenecycloartan-3β,26,30-triol **2** together with the known proanthocyanidins, mahuannin B **3** [19], 7-methoxymahuannin B **4** [19], 7-methoxygeranin A **5**[20], methyl-3-(4-hydroxy-3-methoxyphenyl)-2*E*-propenoate, glycerol-1-alkanoate, (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal **6**, (-)-syringaresinol **7**, and stigmast-5-en-3-O-β-D- glucoside and screening for tyrosinase inhibitory activity and the subsequent inhibition of melanin formation. These compounds were isolated from the roots of *C. congoensis.* Both the roots and the bark of the plant are used as skin-lightening preparations.

Compound **1** was isolated as a white amorphous powder and the ESI(+)-HRMS indicated a molecular formula of $C_{31}H_{48}O_4$ and 8 degrees of unsaturation. The IR spectrum indicated the presence of a ketone group, a carboxylic acid and double bonds, shown by absorption bands at 3438, 1730, 1707, and 1621 cm^{-1}. The ^1H and ^{13}C NMR resonances for compound **1** were in close agreement with those of the reported 3-oxo-24-methylene cycloartan-26-ol [22, 23]. The ^{13}C NMR spectrum indicated the presence of a ketone (δ_C 216.9) and a carboxylic acid group carbon resonance (δ_C 181.1). The ^1H NMR spectrum showed a shielded pair of doublets (δ_H 0.87, d, J = 4.4 Hz; 0.50, d, J = 4.4 Hz) characteristic of the 2 H-19 cyclopropane ring protons. The corresponding C-19 resonance (δ_C 29.8) showed correlations in the HMBC spectrum with the H-8 (δ_H 1.83, m) and H-5 (δ_H 1.70) resonances. The H-5 resonance showed correlations with the C-3 keto group carbon resonance and the corresponding C-5 resonance with the 3H-28 (δ_H 1.09, s) and 3H-29 (δ_H 1.02, s) methyl group proton resonances. The H-8 resonance showed a correlation with the carboxylic acid group carbon resonance, and hence this group was placed at C-30. The H-8 resonance also showed a correlation with the C-13 resonance (δ_C 47.9), which, in turn, showed correlations with the 3H-18 (δ_H 1.05) and H-20 (δ_H 1.48) resonances. The H-20 resonance showed coupling in the COSY spectrum with the 3H-21 doublet resonance (δ_H 0.92, d, J = 6.3 Hz).

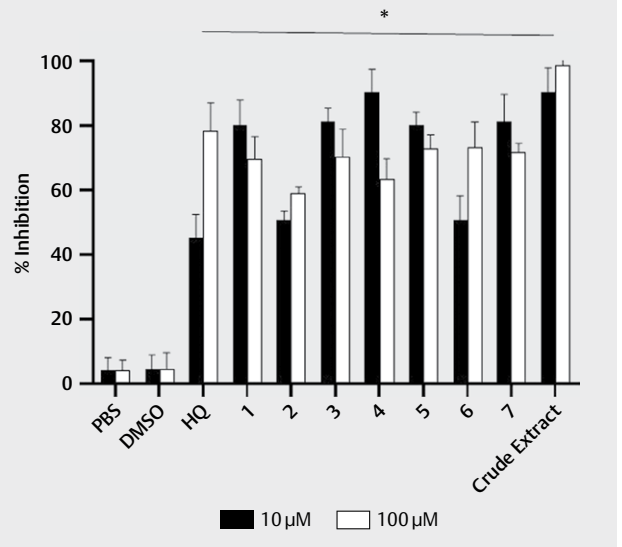
The ^{13}C NMR spectrum showed a hydroxymethylene carbon resonance (δ_C 66.1), which was assigned as C-26, with corresponding nonequivalent oxymethylene protons seen as a pair of double doublets (δ_H 3.56, dd, J = 7.0, 10.4 Hz; 3.51, dd, J = 10.4, 7.0 Hz). The H-26 resonances were seen to be coupled in the COSY spectrum with each other and the H-25 resonance (δ_H 2.36, sextet, J = 7.0 Hz) and this resonance was seen to be coupled to the 3H-27 doublet (δ_H 1.04, d, J = 7.0 Hz) and long range coupled to a pair of vinyl methylene protons (δ_H 4.88, s; 4.82, s) assigned as the 2 H-31 protons. The HMBC spectrum showed correlations between the corresponding C-31 resonance (δ_C 109.9) and the H-25 resonance and between the C-24 resonance (δ_C 152.0) and the 2 H-26, 3H-27 and 2 H-22 resonances. Based on the above spectral data, compound **1** was identified as 26-hydroxy-3-oxo-24-methylene cycloartan-30-oic acid. This compound has not been reported previously.

The IR spectrum of compound **2** showed the presence of a hydroxy group and double bond with absorption bands at 3432 and 1623 cm^{-1}, respectively. The ^1H and ^{13}C NMR resonances for compound **2** differed slightly from those of compound **1** as the ketone and carboxylic acid carbon resonances observed in the ^{13}C NMR spectrum of **1** were absent in **2** but 2 new oxygenated carbon resonances were present at δ_C 78.9 (CH) and δ_C 64.8 (CH_2). Correlations were observed in the HMBC spectrum between the 3H-28 (δ_H 0.81, s), the 3H-29 (δ_H 0.97, s), and H-5 (δ_H 1.33) resonances and the resonance at δ_C 78.9, indicating the presence of a hydroxy group at C-3. The hydroxy group at C-3 was assigned as β due to the chemical shift of δ_C 78.9, typical for a 3β-substituted hydroxy group as opposed to δ_C 76.8 when the 3-hydroxy group is in the α-position [22]. This assignment was confirmed by correlations seen in the

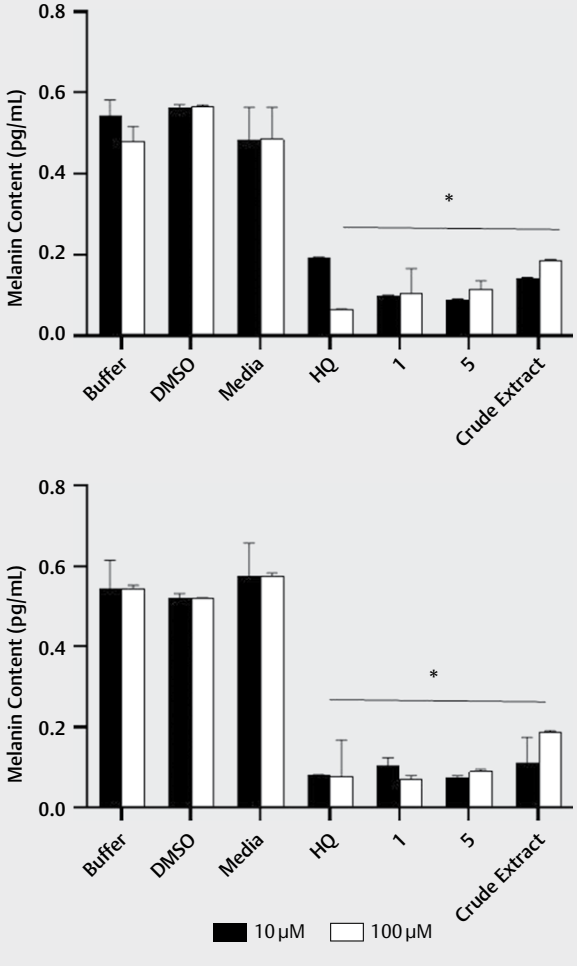
NOESY spectrum between the H-3/H-5 and H-3/3H-28 resonances. Instead of the C-30 methyl group being oxidized to a carboxylic acid as in **1**, it was oxidized to an oxymethylene (δ_C 68.2) group in **2**. The C-30 resonance showed a correlation in the HMBC spectrum with the H-8 resonance (δ_H 1.56, m). The side chain remained the same as for compound **1**. This compound was found to be the previously unreported 24-methylenecycloartan-3β,26,30-triol.

C. congoensis is closely related to and is often confused with *C. flanaganii*, a plant used in South Africa as a skin-lightening agent [12]. The widespread use of this plant and its related species for skin-lightening purposes make the investigation into both its efficacy and safety of importance. Tyrosinase is a key enzyme in the production of melanin as it catalyzes the rate-limiting step in the production of this pigment. The inhibition of tyrosinase by the test compounds would indicate that tyrosinase inhibition may be one of the mechanisms by which these compounds operate and it would give a good indication of which compounds are responsible for the skin-lightening effect of the plant extract. All compounds tested as well as the crude extract showed significant inhibition of tyrosinase ($p < 0.01$) when compared to the negative controls (PBS and DMSO). However, the effect of the crude extract was greater than that of any of the individual compounds tested. Furthermore, unlike the isolated compounds, the inhibitory effect of the crude extracts on tyrosinase exceeded that of the positive control, *p*-hydroquinone, which is also used as a skin-lightening agent, (▶ **Fig. 1**). Further characterization showed that the crude extract and a selection of its isolated compounds (compounds **1** and **5**, selected based on their very differing levels of potency on tyrosinase activity) are significantly ($p < 0.001$) inhibiting the production of melanin in the human primary epidermal melanocytes, compared to the negative controls, over both 24 and 48 h at the concentrations used (▶ **Fig. 2**). Their effect, however, did not appear to be time-dependent. There was no significant increase in melanin production in either the crude extract or isolated compounds when compared to *p*-hydroquinone ($p \leq 0.1$), thus demonstrating the inhibitory potential of both the crude extract and the compounds. Interestingly, the extent of inhibition of melanin synthesis did not mirror that of tyrosinase activity for the crude extract and compounds **1** and **5**. For the latter compound, the inhibition of melanin was far greater than expected based on its effect on tyrosinase activity which was relatively low compared to that of the crude extract and compound **1**. This result may suggest that the crude extract's, and some of its isolated compounds', mechanism of action may also involve their inhibition of other enzymes involved in melanin synthesis such as tyrosinase related proteins 1 and 2 (TYP-1 and TRP-2) [24]. To shed further light on how these compounds behave in relation to melanin synthesis their effect on the activities of tyrosinase, TRP-1 and TRP-2 in melanocytes needs to be investigated.

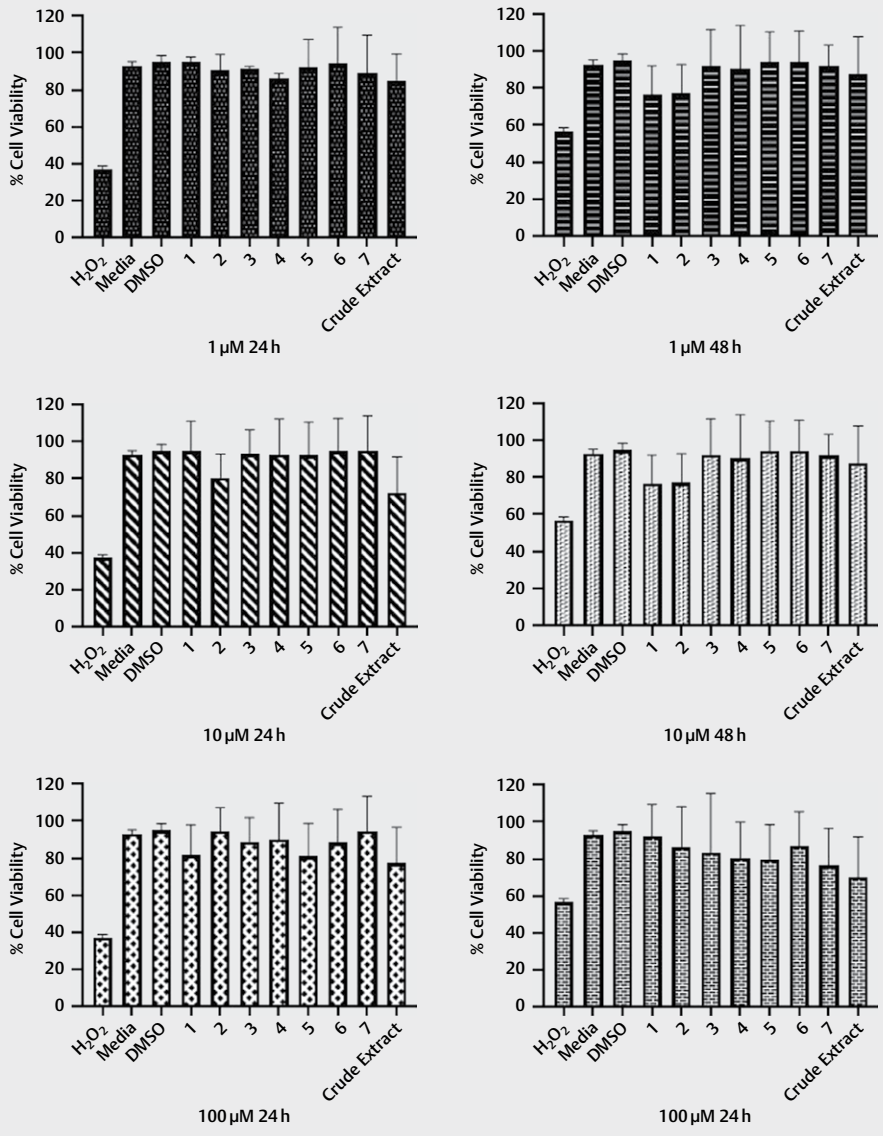
Cytotoxicity towards melanocytes was determined using a neutral red assay (▶ **Fig. 3a–c**) over 3 independent experiments (n = 3).



▶ **Fig. 1** The effect of compounds **1**–**7** and HQ (*p*-hydroquinone) at 10 and 100 µM and crude extract at 1 and 10 mg/mL of *C. congoensis* on tyrosinase activity. * $p < 0.001$ compared to the negative controls. Each data point represents n = 3 and is representative of 3 independent experiments.



▶ **Fig. 2** The effect of compounds **1** and **5** at 10 and 100 µM and crude extract at 1 and 10 mg/mL of *C. congoensis* for 24 h (top) and 48 h (bottom) on melanin content in primary melanocytes. * $p < 0.001$ compared to the negative controls. Each data point represents n = 3 and is representative of 3 independent experiments.



▶ **Fig. 3** Percentage viability of human primary epidermal melanocytes exposed to crude extracts of 1 and 10 mg/mL and compounds 1 µM a, 10 µM b, and 100 µM c from *C. congoenesis* for 24 and 48 h. No significant cytotoxicity was observed when compared to the negative controls ($p < 0.05$). Each data point represents n = 3 and is representative of 3 independent experiments.

No significant cytotoxicity was detected for the test compounds or crude extract relative to the negative controls at a confidence limit of 95 % (media, and vehicle control, $p < 0.05$). The crude extract did show a greater cytotoxicity than the individual compounds isolated.

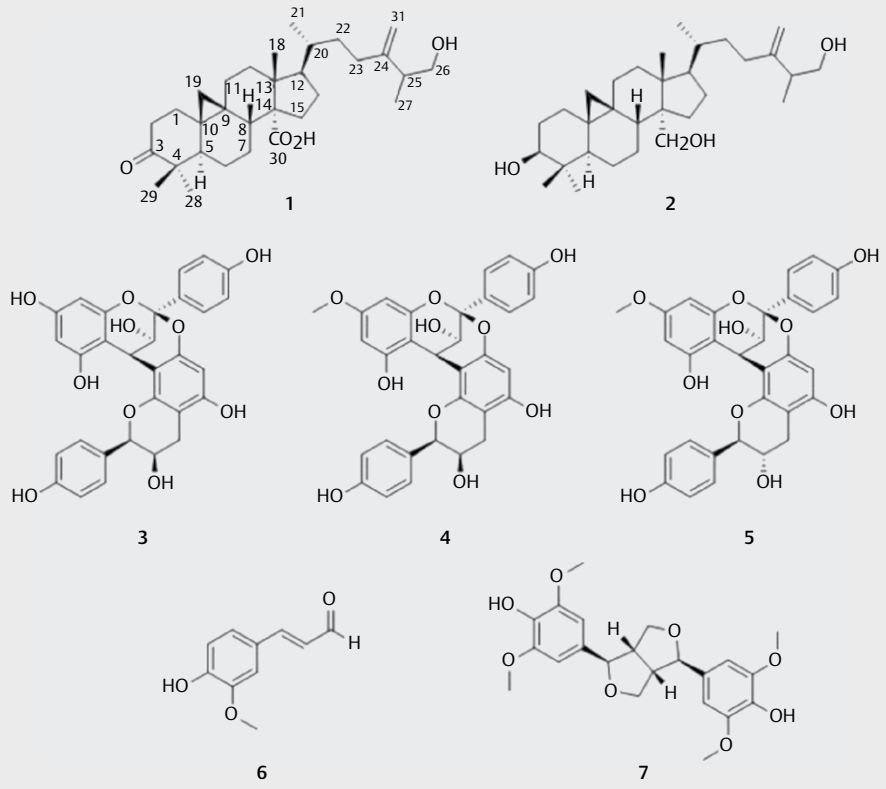
The results of this study show that the crude extract (as used for skin-lightening purposes) as well as the isolates studied had no significant cytotoxicity when compared to the negative controls while still showing significant inhibition of tyrosinase and, in the case of the crude extracts and compounds **1** and **5**, melanin production by primary melanocytes. Overall, this result suggests this plant, and its compounds, may be a good alternative to preparations containing *p*-hydroquinone.

Spectra for compounds **1** and **2** are available as Supporting Information.

Materials and Methods

General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. ECD spectra were obtained on an Applied Photophysics Chirascan CD spectrometer using a 1-mm cell and acetonitrile as the solvent. FTIR spectra were recorded using a Perkin-Elmer (2000) spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ on a 500 MHz Bruker AVANCE NMR instrument at room temperature. Chemical shifts (δ) are expressed in ppm and were referenced against the solvent resonances at 7.26 and 77.23 ppm for ^1H and ^{13}C NMR spectra, respectively. ESI(+)-HRMS mass spectra were recorded on a Bruker MicroToF mass spectrometer using an Agilent 1100 HPLC to introduce samples (University of Oxford). Column chromatogra-



▶ **Fig. 4** Structures of compounds **1**–**7**.

phy was done using SiO_2 packed to 4- or 1-cm diameter gravity columns. TLC was done on aluminum-backed precoated SiO_2 plates (Merck 9385) visualized using anisaldehyde spray reagent.

Plant material

The roots of *C. congoensis* were collected by Mr. Nana Victor in November 2010 at Mbalmayo, southern Cameroon. A voucher specimen (1773SRF/CAM) was deposited at the National Herbarium, Yaoundé, Cameroon.

Extraction and isolation of compounds from C. congoensis

The powdered root of *C. congoensis* (1.3 kg) was extracted successively with refluxing CH_2Cl_2 and MeOH for 48 h each. The solvents were evaporated under reduced pressure using a rotary evaporator (Büchi type R-205) to give brownish extracts of 16.2 g and 47.2 g of extract respectively. The CH_2Cl_2 extract was separated using column chromatography over silica gel (Merck 9385) eluting with n-hexane with increasing amounts of CH_2Cl_2. Once 100 % CH_2Cl_2 had been reached, a step gradient elution using methanol was used to yield several fractions (75 mL each). Combined fractions 32–39 were purified using a n-hexane-CH_2Cl_2(1:1) system to give 26-hydroxy-3-oxo-24-methylene cycloartan-30-oic acid **1** (10.5 mg). Purification of combined fractions 66–69 using a n-hexane-CH_2Cl_2 system (1:1) gave 24-methylenecycloartan-3β,26,30-triol **2** (12.5 mg) whereas purification of combined fractions 105–110 using 100 % CH_2Cl_2 gave mahuannin B **3** (10.3 mg), 7-methoxymahuannin **4** (17.5 mg), and glycerol 1-alkanoate. The MeOH extract (47.2 g) was separated using column chromatography over silica gel using CH_2Cl_2-EtOAc mixtures of increasing polarity to yield several fractions (75 mL each) that were combined following analytical TLC analysis. Fractions 47–59, 126–131, and 139–154 were purified using CH_2Cl_2-EtOAc (3:1), CH_2Cl_2-EtOAc (1:5), and EtOAc 100 % to give 7-methoxygeranin A **5** (12.8 mg), (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal, **6** (15.2 mg), and (-)-syringaresinol **7** (18.2 mg), respectively. Stigmast-5-en-3-O-β-D-glucoside (25.6 mg) was obtained from combined fractions 139–154 alongside **1**. Structures are shown in ▶ **Fig. 4**.

26-Hydroxy-3-oxo-24-methylenecycloartan-30-oic acid (**1**):

White amorphous powder; (10.5 mg, >98 % pure; from NMR) $[\alpha]^{25}_{D+}$19 (c 0.0270, $CHCl_3$); IR (neat) ν_{max} 3438, 2957, 2853, 1707, 1621 cm^{-1}; ESI(+)-HRMS m/z 507.3447 $[M+Na]^+$(calcd for $C_{31}H_{48}O_4Na$, 507.3450); see ▶ **Table 1** for ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data.

24-Methylenecycloartan-3β,26,30-triol (**2**)

White amorphous powder; (12.5 mg, >90 % pure; from NMR) $[\alpha]^{25}_{D+}$19.5 (c 0.0350, $CHCl_3$); IR (neat) ν_{max} 3432, 2957, 2853, 1623 cm^{-1}; ESI(+)-HRMS m/z 495.3793 $[M+Na]^+$(calcd for $C_{31}H_{52}O_3Na$, 495.3814); see ▶ **Table 1** for ^1H NMR (500 MHz, $CDCl_3$ and ^{13}C NMR (125 MHz, $CDCl_3$) data.

▶ **Table 1** ^{1}H and ^{13}C NMR data for compounds **1** and **2** ($CDCl_3$, 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR spectra).

	Compound 1		Compound 2	
No	**δ_C**	**δ_H (*J* in Hz)**	**δ_C**	**δ_H (*J* in Hz)**
1α	33.5	1.87 m	32.3	1.60 m
β		1.61 m		1.26 m
2*α*	37.5	2.72 dt (6.2, 15.0)	30.4	1.77 m
β		2.31 dt (4.4, 15.0)		1.58 m
3	216.9	–	78.9	3.28 dd (4.3, 11.1)
4	50.1	–	40.7	–
5	47.8	1.70 m	47.5	1.33 m
6*α*	21.0	1.49 m	21.6	1.63 m
β		1.01 m		0.80 m
7*α*	28.4	2.23 m	27.5	1.93 m
β		1.35 m		1.46 m
8	45.4	1.83 m	48.9	1.56 m
9	20.5	–	19.8	–
10	27.7	–	26.5	–
11*α*	29.6	2.15 m	27.2	1.91 m
β		1.40 m		
12*α*	33.8	1.86 m	32.4	1.59 m
β		1.71 m		1.57 m
13	47.9	–	46.2	–
14	63.0	–	54.2	–
15*α*	31.5	2.18 m	28.1	1.91 m
β		1.28 m		1.44 m
16*α*	27.0	1.53 m	27.7	1.95 m
β		1.13 m		1.12 m
17	52.5	1.52 m	52.5	1.60 m
18	18.0	1.11 s	19.5	1.05 s
19A	29.8	0.87 d (4.0)	30.7	0.59 d (4.0)
B		0.50 d (4.0)		0.38 d (4.0)
20	35.8	1.48 m	36.4	1.44 m
21	18.6	0.92 (6.3)	18.6	0.90 d (6.3)
22*α*	34.8	1.60 m	35.1	
β		1.18 m		1.16 m
23*α*	31.2	2.10 m	31.4	2.11 m
β		1.85 m		1.88 m
24	152.0	–	152.2	–
25	42.7	2.36 sextet (6.5)	42.8	2.36 m
26A	66.1	3.56 dd (7.0, 10.4)	66.1	3.55 dd (7.0, 10.4)
B		3.51 dd (10.4, 7.0)		3.51 dd (10.4, 7.0)
27	16.6	1.04 d (7.0)	16.6	1.05 d (7.0)
28	22.4	1.02 s	14.2	0.81 s
29	21.1	1.09 s	25.6	0.97 s
30	181.1	–	64.8	3.83 d (11.8)
				3.68 d (11.8)
31	109.9	4.88 s	109.8	4.87 s
		4.82 s		4.82 s

Pharmacological assays

Cell culture

Human primary epidermal melanocytes (ATCC) were cultured in Dermal Cell Basal Media (ATCC) supplemented with Adult Melanocyte Growth Kit Components and 2 % penicillin-streptomycin-amphorectin B Solution (ATCC) and were grown as per manufacturer's instructions. On reaching 90 % confluency, cells were passaged once prior to plating for cell culture experimentation. Positive controls, *p*-hydroquinone (purity > 99 %) and hydrogen peroxide (30 wt % in water) used were purchased from Sigma-Aldrich.

Effect of *C. congoensis* crude extract and compounds on the viability of human primary epidermal melanocytes

Compounds were resuspended from a stock solution (DMSO, > 99.7 %, Sigma-Aldrich) in 16 nM K_3PO_4 buffer (both from Sigma-Aldrich) at concentrations of 1, 10, and 100 µM. The crude extract was tested at concentrations of 1 mg/mL and 10 mg/mL. Human primary epidermal melanocytes were plated in 96-well plates at a concentration of 1×10^5 cells/well. Following a 24-h incubation, cells were treated with respective concentrations of compound and crude extracts (as above), as well as DMSO (the final concentration of DMSO ± cells was 0.1 %), hydrogen peroxide (30 wt % in water), which was used as the positive control and media alone and further incubated for 24 h and 48 h to determine the extent of any toxicity over time. Neutral red (Sigma-Aldrich) was dissolved in water at a concentration of 3.3 µg/mL. Cell culture media with compound or crude extract was removed from cells and 20 µL neutral red solution was added to cells. Following a 2-h incubation at 37 °C, the neutral red solution was removed and cells rinsed with DPBS. Two hundred microliters of a solution containing 1 % acetic acid in 50 % EtOH/deionized H_2O was added to solubilize the dye. Following a 20-min incubation, supernatants were read using a Tecan Infinate Pro 200 plate reader at 540 nm and the cell viability determined.

Effect of *C. congoensis* crude extract and compounds on tyrosinase activity

The tyrosinase assay used is one previously described by Chou et al. [25]. Two hundred ten microliters of L-Dopa (> 98 %, Sigma-Aldrich) was dissolved in 16 ηM K_3PO_4 buffer and incubated at 25 °C for 10 min with 7.5 µL of crude extract (1 and 10 mg/mL) each compound (10 and 100 µM), DMSO 0.1 %, 16 ηM K_3PO_4 buffer (the negative control and blank, respectively) or *p*-hydroquinone (> 99 %, Sigma-Aldrich) (10 or 100 µM) (the positive control as *p*-hydroquinone is a known inhibitor of tyrosinase) [26], plus 7.5 µL of tyrosinase (mushroom, lyophilized powder, ≥ 1000 unit/mg solid, Sigma-Aldrich) (or 20 mM K_3PO_4 buffer as a blank). The absorbance was determined at 475 nm using a Tecan Infinate Pro 200 plate reader.

Percentage inhibition of tyrosinase activity was determined using the following equation

$$((1-B/A) \times 100)$$

where A = change in optical density at 475 nm (OD_{475})/min without sample (negative controls) and B = change in OD_{475}/min with sample.

Melanin determination in primary melanocytes treated with crude extracts and active compounds

Primary melanocytes were seeded in 24-well plates at a density of 1×10^5 cells per well and following a 24-h incubation were treated with crude extract (1 and 10 mg/mL, compounds **1**, **5**, and the positive control, *p*-hydroquinone (10 and 100 µM each), DMSO, and K_3PO_4 buffer as negative controls and the media alone to serve as a blank.

Following 24-h and 48-h incubations, cells were rinsed with Dulbecco's PBS (Sigma-Aldrich) and removed from the base of the well using a cell scraper. Cells were pelleted in an Eppendorf tube and solubilized in a solution of 1 N NaOH (Sigma-Aldrich) and 10 % DMSO (> 99.7 %, Sigma-Aldrich) and incubated at 80 °C for 2 h. Following this, cells were centrifuged at 12 000 g for 10 min at room temperature, and the absorbance of 200 µL of the supernatants was determined at 470 nM using a Tecan Infinate Pro 200 plate reader. Melanin content of the supernatants was determined against a standard curve of synthetic melanin (synthetic, Sigma-Aldrich) (0–20 ng/mL) and was expressed as ρg/cell.

Statistical analysis

Cell viability, tyrosinase activity, and melanin content data are presented as mean ± standard error of the mean (SEM) for 3 independent experiments (n = 3). Data were analyzed for significance ($p \leq 0.05$) using one-way analysis of variance; *post hoc* Tukey test was then used to compare the means.

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Conflict of Interest

The authors declare there are no conflict of interest.

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