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The effect of isolates from *Cassipourea flanaganii* (Schinz) Alston, a plant used as a skin lightening agent, on melanin production and tyrosinase inhibition

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ABSTRACT

Ethnopharmacological relevance: The Zulu and Xhosa people of South Africa use the stem bark of *Cassipourea flanaganii* as a skin-lightening cosmetic.

Aim of the study: To isolate and identify compounds responsible for the skin lightening properties from the stem bark of *Cassipourea flanaganii* and to evaluate their cytotoxicity towards skin cells, and potential to treat hyperpigmentation.

Materials and methods: Extracts from the stem bark of *Cassipourea flanaganii* were isolated using chromatographic methods and structures were determined using NMR, IR and MS analysis. The tyrosinase inhibitory activity and the ability to inhibit the production of melanin were determined using human primary epidermal melanocyte cells. Cytoxicity was established using the same melanocytes and a neutral red assay.

Results: One previously undescribed compound, *ent*-atis-16-en-19-al (**1**) along with the known *ent*-atis-16-en-19-oic acid (**2**), *ent*-atis-16-en-19-ol (**3**), *ent*-kaur-16-en-19-oic acid (**4**), *ent*-kaur-16-en-19-al (**5**), *ent*-manoyl oxide (**6**), guinesine A (**7**), guinesine B (**8**), guinesine C (**9**), lichenxanthone (**10**), 2,4-dihydroxy-3,6-dimethyl benzoic acid methyl ester (**11**), lynoside (**12**), lupeol (**13**), β-amyrin (**14**), docosyl ferulate (**15**), stigmasterol, sitosterol and sitosterol-*O*-glucoside were isolated in this investigation. An impure fraction containing compound **3** was acetylated to obtain 19-acetoxy-*ent*-atis-16-ene (**3a**). Compounds **10** and **11** are usually isolated from lichen, hence they are possible contaminants of lichen harvested with the bark. Compounds **1**, **3a**, **5**-**14** were not significantly cytotoxic to the primary epidermal melanocyte cells ($P > 0.05$) when compared to the negative and positive controls (DMSO, 0.1% and hydrogen peroxide, 30 wt% in water). Inhibition of tyrosinase was significantly greater with respect to the negative control ($P < 0.001$) for compounds **3a**, **5**-**8** and **9**-**10** at 10 μM and for compounds **5**-**8** and **9**-**10** at 100 μM. Compared to hydroquinone (the positive control) at 10μM, the level of inhibition was comparable or to that of compounds **3a**, **5**, **6**, and **8**-**10** at 10μM, with **9** and **10** showing a greater level of inhibition. Inhibition of melanin was both concentration and time dependent for all compounds tested with higher melanin content at 24 hours compared to 48hrs and at 10mM compared to100 mM at both time points; melanin content was significantly lower for hydroquinone at both time points and concentrations.

Conclusions: Compounds **1**, **5-14**, isolated from *Cassipourea flanaganii* and the derivative **3a** showed low cytotoxicity. All compounds had a clear time and concentration dependent effect on melanin content which did not appear to be dependent on their inhibition of tyrosinase.

Keywords

Cassipourea flanaganii, *ent*-atisane diterpenoids, *ent*-atis-16-en-19-al and melanin inhibition

1. Introduction

Cassipourea flanaganii (Schinz) Alston (Rhizophoraceae) is one of several plant species in southern Africa employed traditionally as skin lighteners; others include *Sideroxylon inerme* L. (Momtaz et al., 2008), *Garcinia livingstonei* T.Anderson (Mulholland et al., 2013), *Rapanea melanophloeos* (L.) Mez. and *Calodendrum capense* (L.f.) Thunb. (Cocks and Dold, 2004). *C. flanaganii* is a rare, small to medium-sized forest tree growing to about 12 m tall with slender stems and dark grey, wrinkled bark. The species is endemic to South Africa and occurs mainly in the north-eastern region of the Eastern Cape Province, with a few records from southern KwaZulu-Natal. Although currently recognised as a valid species, its distinction from the more northerly *Cassipourea malosana* (Baker) Alston (syn. *C. gerrardii* (Schinz) Alston) has been questioned (Boon, 2010). The stem bark of *C. malosana* (as *C. gerrardii*) is similarly exploited in South Africa for its skin lightening qualities, on which account its phytochemistry has been investigated previously and a flavanol glycoside, (+)-afzelechin 3-*O*-α-L-rhamnopyranoside and an A-type proanthocyanidin, epiafzelechin-(4β→8,2β→0→7)-*ent*-afzelechin isolated (Drewes et al., 1992a, 1992b). More recently, 11 compounds were isolated from the bark of a Kenyan sourced *Cassipourea malosana*: 2α,3α-epoxyflavan-5,7,4'-triol-(4β → 8)-afzelechin, 2β,3β-epoxyflavan-5,7,4'-triol-(4α → 8)-epiafzelechin, methyl 4-ethoxy-2-hydroxy-6-propylbenzoate, ethyl 3-formyl-2,4-dihydroxy- 6-methylbenzoate, (–)-angolensin-4-methyl ether, lichexanthone, docosyl ferulate, tetracosyl ferulate, methyl 2,4-dihydroxy-3,6-di-methylbenzoate, 7-oxo-stigmasterol and 7-oxo-β-sitosterol (Nishiyama et al., 2019). Investigation of *Cassipourea guianensis* Aubl. yielded sulphur-containing amides (Ichimaru et al., 2000), *Cassipourea gummiflua* Tul. yielded sulphur containing bases (Cooks et al., 1967; Drewes et al., 1994; Williams et al., 2008), *C. madagascariensis* (Thouars) DC. Yielded diterpenoids, a lupane triterpenoid and combretol (Chaturvedala et al., 2006) and *C. lanceolata* Tul. yielded euphane triterpenoids (Hou et al., 2010).

The stem bark of *C. flanaganii* (also known as the Cape Onionwood) is traded extensively under the name *umemezi*, mainly in the Eastern Cape province (Dold and Cocks, 2002), although reportedly as far afield as the Western Cape and KwaZulu-Natal (Cunningham, 1988; Khan, 1996). Material in trade is mainly sourced from the Pirie State Forest near King William's Town, or the Gatyana Forest in the Willowdale district (Cocks and Dold, 2004). These authors recounted how the bark of this species is sold unprocessed, and subsequently ground by the user to a paste with water, before being applied daily to the face as a skin lightener.

Cocks and Dold (2004) reported that the name *umemezi* derives from the Xhosa verb *ukumemeza*, meaning to 'attract attention' or to 'call aloud', a reference to its effective complexion lightening quality. A random survey undertaken in six Cape Town townships in the mid-1990's revealed that over half of the respondents used *umemezi* (Khan, 1996). The reason for such use of skin lightening agents in South Africa was linked by Khan to feelings of social inferiority by Africans with dark complexions, which she interpreted as a psychological outcome of apartheid. The cosmetic use of *C. flanaganii* was first documented by Earle (1976), and concerns about the ecological and socio-economic impacts of its use have since been well-documented by Cocks and Dold (2004), who have shown that although harvesting of the species provides an important means of income for poverty-stricken gatherers, such extraction has become unsustainable with the species now threatened with extinction. This plant is currently on the International Union of Conservation of Nature's (IUCN) lists Red List of threatened species falling under category (A4acd; C1+2a(i)) (Williams et al. 2008), and fewer than 2500 mature individuals remaining in the wild. Harvesting of the bark is usually destructive and results in the death through ring barking of individuals, the stems of which may be as little as 5 cm in diameter when targeted (Cocks and Dold 2004). Should constituents from the Cape Onionwood be demonstrated to have industrial/commercial value, efforts to conserve this species through large-scale cultivation could be motivated, as a component of a formal Biodiversity Management Plan for the species (DEAT, 2009).

In contrast to ecological and social aspects, little is known of the safety of use of *C. flanaganii*, or the basis for its efficacy. The issue of safety is a key one due to concerns on the widespread use of skin lightening agents that contain hydroquinone, a compound associated with several undesirable cutaneous or systemic side effects. These include contact dermatitis and exogenous ochronosis (Findlay and de Beer, 1980; Hardwick et al., 1989; Olumide et al., 2008). This study

aimed to isolate and identify compounds responsible for reported skin lightening properties in the stem bark of *Cassipourea flanaganii*, to evaluate their cytotoxicity towards skin cells, and potential to treat hyperpigmentation.

2. Materials and methods

2.1 General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. 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 $CDCl_3$, 4.87 and 49.15 ppm for CD_3OD, for ^{1}H and ^{13}C NMR spectra respectively. HRMS spectra were recorded using an Agilent 1260 Infinity II coupled to an Agilent 6550 Quadrupole Time-of-Flight mass spectrometer using electrospray ionisation. Column chromatography was done using SiO_2 packed to 4 or 1cm diameter gravity columns. Analytical and preparative TLC was undertaken on aluminium precoated SiO_2 plates (Merck 9385) and visualised using anisaldehyde spray reagent.

2.2 Plant material

The stem bark of *C. flanaganii*, purchased at the King Williams Town medicinal plant market, reportedly had been harvested at Mdantsane in the Eastern Cape Province, South Africa, in June 2009. Plant material was identified and confirmed by Professor Neil Crouch at the KwaZulu-Natal Herbarium (NH) in Durban. A voucher specimen has been retained for verification purposes (N. Dlova 1, NH).

2.3 Extraction and isolation

The dried stem bark of *C. flanaganii* was milled using a cross beater mill to yield 176 g. The powdered material was soaked in CH_2Cl_2 and MeOH successively to yield 2.3 g and 4.7 g extracts respectively. Compounds **1** – **10**, **13**-**15**, stigmasterol, sitosterol and sitosterol *O*-glucoside were isolated from the CH_2Cl_2 extract of *C. flanaganii* (Fig. 1). Silica gel (Merck 9385) gravity column chromatography was undertaken for the CH_2Cl_2 and MeOH extracts separately using the indicated step gradient elution. For the CH_2Cl_2 extract a mobile phase of hexane and increasing quantities of CH_2Cl_2 was used whereas for the MeOH extract a mobile phase of CH_2Cl_2 and increasing quantities of MeOH was used. Each time 75 mL fractions were collected and monitored using TLC technique. The structures of the compounds were

established on the basis of their 1D and 2D NMR spectroscopic data and comparison with the published data. Compound **1** and **4** were eluted using 20% CH_2Cl_2 in hexane as fractions 20 – 25 and were purified using preparative thin layer chromatography (pTLC Merck 9385) over SiO_2 with 30% CH_2Cl_2 in hexane as the mobile phase to yield **1** (1.7 mg) and **4 (**3.0 mg) (Langat et al., 2018). Compound **2** (2.0 mg) (Ungur et al., 2013) and **5** (32.2 mg) (Langat et al., 2018) were eluted as fraction 59 – 60 in 80 CH_2Cl_2 in hexane and were purified using 100% CH_2Cl_2. Fractions 57-58 contained the impure compound **3** (Grande et al., 1991; Zdero et al., 1990). This fraction was acetylated in acetic anhydride and pyridine and purified to yield compound **3a** (Zdero et al., 1990). The acetylated derivative **3a** (1.5 mg) was purified using 50% CH_2Cl_2 in hexane. Compound **6** (3.2 mg) (Langat et al., 2018) was eluted in fractions 26-27 using 20% CH_2Cl_2 in hexane and purified using the same solvent system by preparative TLC over SiO_2 to yield 3.2 mg. Compound **8** was eluted in fraction 152 using 1% MeOH in CH_2Cl_2 and further purified using the same solvent system to give compound **8** (10.8 mg) (Kato et al., 1989). Compound **9** (38.2 mg) (Kato et al., 1989) was eluted as a pure fraction, 160, over 1% MeOH in CH_2Cl_2. Compound **10** (1.5 mg) (Goel et al., 2011) was eluted in fractions 37 – 39 using 30% CH_2Cl_2 in hexane and was purified using pTLC with the same solvent system. Compounds **13** (1.6 mg) and **14** (7.2 mg) co-occurred in fractions 30 – 34 that were separately eluted using 100 % CH_2Cl_2. Compound **15** (16.6 mg), was eluted as a pure fraction using 100% CH_2Cl_2. Compound **7**, **11** and **12** were isolated from the MeOH extract of *C. flanaganii*. Compound **7** (11.9 mg) (Kato et al., 1989) was eluted from fractions 98-104 using 1% MeOH in CH_2Cl_2 as a pure compound. Compound **11** (6.7 mg) (Goel et al., 2011) was eluted from 180-186 using 2% MeOH in CH_2Cl_2. Compound **11** was purified using a gravity column packed with Sephadex LH20 eluted using. Compound **12** (12.2 mg) eluted from fraction 198 - 200 using 5% MeOH in CH_2Cl_2, and it was purified using a gravity column packed with Sephadex LH20 eluted using 1:1mixture of mixture of MeOH: CH_2Cl_2.

*2.3.1 Ent-atis-16-en-19-al (**1**):*

White amorphous powder; $[\alpha]_D^{25}$ – 61.00 (c 0.0168, $CHCl_3$);IR (neat) ν_{max} 2928, 2855 and 1722 cm^{-1}; HR-ESIMS m/z 309.2197 $[M+Na]^+$ (calcd for $C_{20}H_{30}ONa$, 309.2189); ^1H and ^{13}C NMR data for Compound **1** see Table 1.

2.4 In vitro screening of compounds

2.4.1 Cell culture

Human primary epidermal melanocytes (American Type Culture Collection, ATCC) were cultured in dermal cell basal media (ATCC) supplemented with adult melanocyte growth kit and 2% penicillin-streptomycin-amphorectin B Solution (ATCC) and were grown as per the supplier's instructions. On reaching 90% confluency, cells were passaged once prior to plating for the cell culture experiments. The positive controls, hydroquinone (purity >99 %) and hydrogen peroxide (30 wt% in water) were purchased from Sigma-Aldrich (Poole, Dorset).

2.4.2 Toxicity assay

Compounds **1**, **3a**, **5-14** and the positive control were resuspended from a stock solution (DMSO, >99.7%, Sigma Aldrich) in 16 nM K_3PO_4 buffer (both from Sigma Aldrich, Dorset, UK) at concentrations of 1 μM, 10 μM and 100 μM. Human primary epidermal melanocytes were plated in 96 well plates at a concentration of 1 x 10^5 cells/well. Following a 24 hour incubation, cells were treated with respective concentrations of each compound (concentrations as given 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 24hr and 48hr 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 was removed from cells and neutral red solution (20 μL) was added to the cells. Following a 2-hour incubation at 37 °C, the neutral red solution was removed, and cells rinsed with DPBS. A solution containing 1% acetic acid in 50% EtOH/deionized H_2O (200μL) was added to solubilise the dye. Following a 20-minute incubation, supernatants were read using a Tecan Infinate Pro 200 plate reader at 540 nm and the cell viability determined.

2.4.3 Tyrosinase assay

The tyrosinase assay used is one previously described by Chou et al. (2013). L-Dopa (210 μL) (>98%, Sigma Aldrich) was dissolved in 16 ηM K_3PO_4 buffer and incubated at 25°C for 10 minutes with 7.5 μL of each compound (10μM and 100μM), DMSO 0.1%, 16ηM K_3PO_4 buffer (the negative control and blank, respectively) or hydroquinone (>99%, Sigma Aldrich) (10μM or 100 μM) (the positive control as hydroquinone is a known inhibitor of tyrosinase) (Palumbo et al., 1991), 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. Compounds **1**, **3a**, **5, 6, 9 and 10** were tested in this assay.

Percentage inhibition of tyrosinase activity was determined using the following equation

((1-B/A) X 100 where A= change in optical density at *475nm (OD_{475})/minute without sample (negative controls) and B = change in OD_{475}/minute with sample.*

*2.4.4 Determination of Melanin Content in Primary Melanocytes treated with compounds **1**, **3a**, **5-14**.*

Primary melanocytes were seeded in 24 well plates at a density of $1x10^5$ cells per well and following a 24 hr incubation were treated with compounds **1**, **3a**, **5-14** and the positive control, hydrogen peroxide (30 wt% in water), DMSO (0.1%) and K_3PO_4 buffer as negative controls and the media alone to serve as a blank.

Following 24 hr and 48 hr incubations, cells were rinsed with Dulbecco's phosphate buffered saline (PBS) (Sigma Aldrich) and removed from the base of the well using a cell scraper. Cells were pelleted in an Eppendorf tube and solubilised in a solution of 1 N NaOH (Sigma Aldrich) and10% DMSO (>99.7%, Sigma Aldrich) and incubated at 80°C for 2 hours. Following this, cells were centrifuged at 12000 G for 10 minutes 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.

2.4.5 Statistical Analysis

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

3. Results and discussion

Compound **1** was isolated as a white amorphous powder and the HRMS gave a molecular ion peak at m/z 309.2197 $[M+Na]^+$ which indicated a molecular formula of $C_{20}H_{30}O$, (calcd.309.2189) and 6 degrees of unsaturation. The FTIR spectrum indicated the presence of a carbonyl group shown by an absorption band at 1722 cm^{-1}. The ^1H NMR spectrum showed an formyl proton resonance at δ_H 9.07 (s), two doublets for a double bond at δ_H 4.74 (d, J = 2.0

Hz) and 4.58 (d, J = 2.0 Hz), an allylic proton resonance at δ_H 2.24 (m), and two singlet methyl proton resonances at δ_H 1.01 (s) and 0.83 (s). The ^{13}C and DEPT spectrum supported 20 carbon resonances including a carbon resonance at δ_C 206.2 (CH) for an aldehyde group, δ_C 152.7 (C) and δ_C 105.0 (CH_2) for a terminal double bond. Overall, the ^{13}C and DEPT spectra supported two methyl groups (CH_3), ten methylene groups (CH_2), four methine groups (CH) and 4 fully substituted carbons. The aldehyde carbonyl and one double bond accounted for 2 double bond equivalents hence 4 remained to be accounted for, therefore compound **1** was determined to be a tetracyclic diterpenoid consistent with the atisane and kaurane classes of diterpenoids. The presence of nine aliphatic methylene groups supported an atisane class of diterpenoids. A correlation in the HMBC spectrum between the aldehyde carbon resonance at δ_C 206.1 and a methyl group proton resonance at δ_H 1.01 (s) suggesting that the aldehyde group was attached to the C-4 position of ring A of the atisane skeleton. The methyl group proton resonance at δ_H 1.01 (s) was assignable to the 3H-19 group. In addition, the correlation in the HMBC spectrum between a methine carbon resonance at δ_C 57.1 and the two methyl group proton resonances at δ_H 1.01 (s) and δ_H 0.83 (s) allowed for the assignment of 3H-20. The ^1H NMR methyl group proton resonance at δ_H 0.83 (s) was assigned to 3H-20. A correlation in the NOESY spectrum between the 3H-20 and the aldehyde proton resonance suggested that they are on the same face of the molecule. A terminal double bond was assigned as 2H-17, a characteristic group of the atisane diterpenoids. A specific rotation of – 61.00° suggested that compound 1 belonged to the *ent* series. The above spectroscopic and spectrometric data were consistent with *ent*-atis-16-en-19-al (**1**). A search in the literature showed that compound **1** has not been previously reported. The related *ent*-atis-16-en-19-oic acid (**2**) and *ent*-atis-16-en-19-ol (**3**) were also isolated in this study. A comparison of the published data for **2** and **3** showed consistency to those of **1**. In addition, *ent*-kaur-16-en-19-oic acid (**4**), *ent*-kaur-16-en-19-al (**5**) and *ent*-manoyl oxide (**6**) were isolated from this plant representing the second time that diterpenoids are reported from *Cassipourea* (Hou et al 2010). The isolation of guinesine A (**7**), guinesine B (**8**) and guinesine C (**9**) is consistent with the previous reports from the *Cassipourea* genus (Chaturvedala et al 2006; Cooks et al., 1967; Drewes et al 1994; Williams et al., 2008).

The long-term use of skin lightening agents, in particular those containing hydrogen peroxide, is a concern due to the damage caused to the skin (Shroff et al., 2017). The extensive use of *C. flanaganii* as a skin lightening agent warrants investigation as a potentially safer alternative to hydroquinone. Compounds **1**, **3a**, **5-14** were tested at two concentrations (10 and 100 mM) and at two-time intervals (24 and 48 hr). Compounds **1**, **3a**, **5-14** were assessed for their

cytotoxicity towards the cells of interest, the primary epidermal melanocytes. Over three concentrations (1, 10 and 100 μM) and two time intervals (24 and 48 hours) no significant cyctotoxicity was observed ($P < 0.001$); cell viability was in excess of 80% for all the compounds which is similar to the effect of compounds *Cassipourea congoensis* as well as the crude extract reported by the authors (Takou et al., 2019). In contrast, the cell viability was slightly below 60% for the hydrogen peroxide positive control.

Tyrosinase is a key enzyme in the production of melanin as it catalyses the rate limiting step in the production of this pigment and as such compounds **1**, **3a**, **5-8** and **9-10** were tested for their ability to inhibit tyrosinase. The isolates were tested at 10 and 100 μM. Compounds **3a** at 10 μM and compounds **5-8** and **9-10** showed a significant inhibition of tyrosinase relative to the negative control ($P < 0.001$) while compound **1** did not. At 100 μM, neither compound **1** nor compound **3a** significantly inhibited tyrosinase ($P > 0.05$).

When considering the effect of the isolates on melanin inhibition, the results were found to be strongly dependent on time and concentration. After 24 hours, all compounds at both concentrations, with the exception of compound **13** at 100 μM, significantly increased the production of melanin relative to the negative control ($P < 0.001$). At 48 hours, all compounds showed a significant increase in melanin production at 10 μM compared to the negative controls ($P < 0.001$) but compounds **1**, **3a**, **5** and **6** showed a significant decrease in melanin concentration at 100 μM, relative to the negative control ($P < 0.001$). As such it would appear that the isolates do inhibit melanin production, but only at a higher concentration and over a longer time period, 48 hours. These results do no conclusively indicate that the mechanism of action is the inhibition of tyrosinase, as compounds **1** and **3a** did not significantly inhibit tyrosinase at the 100 μM, while compounds **5** and **6** did. All 4 inhibited the formation of melanin. The results would, however, show that for the plant to be effective as a skin lightening agent, the extract would need to be applied for at least 48 hours to show any effect. The isolates were found to be non-toxic to these skin cells at this concentration and time interval.

4. Conclusion

The results would indicate that the isolates from *C. flanaganii* show low cytotoxicity towards melanin producing skin cells, and that at the higher concentration (100μM) and time interval (48hr) melanin content in these cells is significantly decreased by 4 of the compounds isolated **1**, **3a**, **5** and **6**. The decrease in melanin is both concentration and time dependent, indicating

that a higher concentration over a longer time period is more effective. The low toxicity observed in conjunction with the decrease in melanin content, specifically at 100µM, suggest that these compounds could be used in place of hydroquinone to treat hyperpigmentation. Accordingly, *C. flanaganii* warrants further investigation as a safe and effective skin lightening agent. However, the current conservation status of the species would preclude its use as a commercial source of the actives, unless cultivated sustainably at scale.

Author contributions

Ncoza Dlova and Dulcie A. Mulholland designed the study. Ncoza Dlova provided the plant material which was identified and preserved by Neil R. Crouch. Moses K Langat and Ncoza Dlova extracted the plant materials, isolated and purified compounds. Moses K Langat and Dulcie A. Mulholland characterized the structures of the compounds. Sianne L. Schwikkard and Elizabeth I. Opara designed the tyrosinase inhibitory assay, inhibition of the production of melanin assay and cytotoxicity experiments. Lauren E. Mulcahy-Ryan and Jacob D. Hiles conducted the biological assays and analysed the data. Moses K Langat, Sianne Schwikkard and Neil Crouch wrote the paper. All authors edited the paper and have given their approval for submission of the paper.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online xxxxxx

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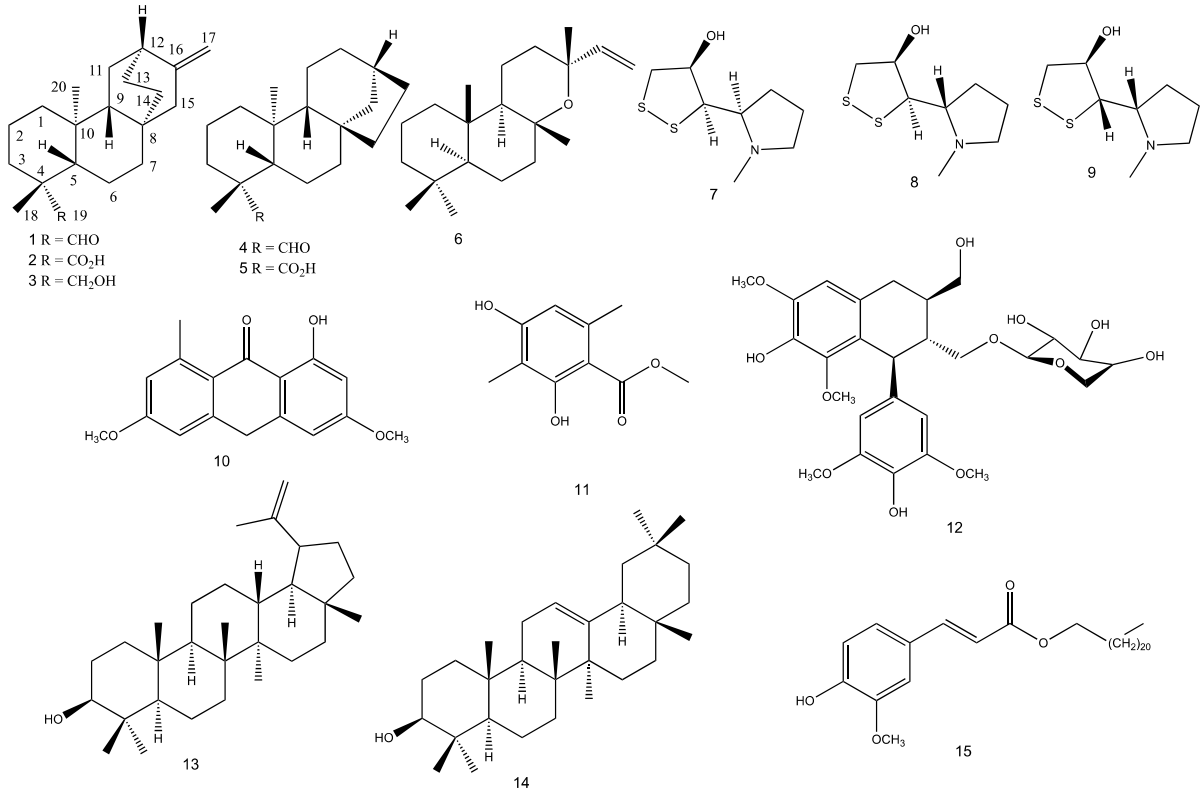


Figure 1: Compounds isolated from the stem bark of *C. flanaganii*

Table 1: ^{1}H and ^{13}C NMR spectroscopic data for compound 1 ($CDCl_3$)

Carbon number	^{13}C NMR (125 MHz)	^{1}H NMR(500 MHz)
1α	39.1	1.58 m
β		0.86 m
2α	18.2	1.43 m
β		1.63 m
3α	34.7	2.15 m
β		1.03 m
4	48.6	-
5	57.1	1.18 m
6α	18.6	1.73 m
β		1.82 m
7α	39.8	1.19 m
β		1.57 m
8	33.6	-
9	51.7	1.17 m
10	38.3	-
11α	27.4	1.56 m
β		1.64 m
12	36.6	-
13α	29.1	2.24 m
β		1.64 m
14α	28.8	1.96 m
β		1.39 m
15α	48.4	1.93 m
β		2.08 m
16	152.7	-
17A	105.0	4.74 d J = 2.0 Hz
B		4.58 d J = 2.0 Hz
18	24.4	1.01 s
19	206.2	9.07 s
20	13.2	0.83 s

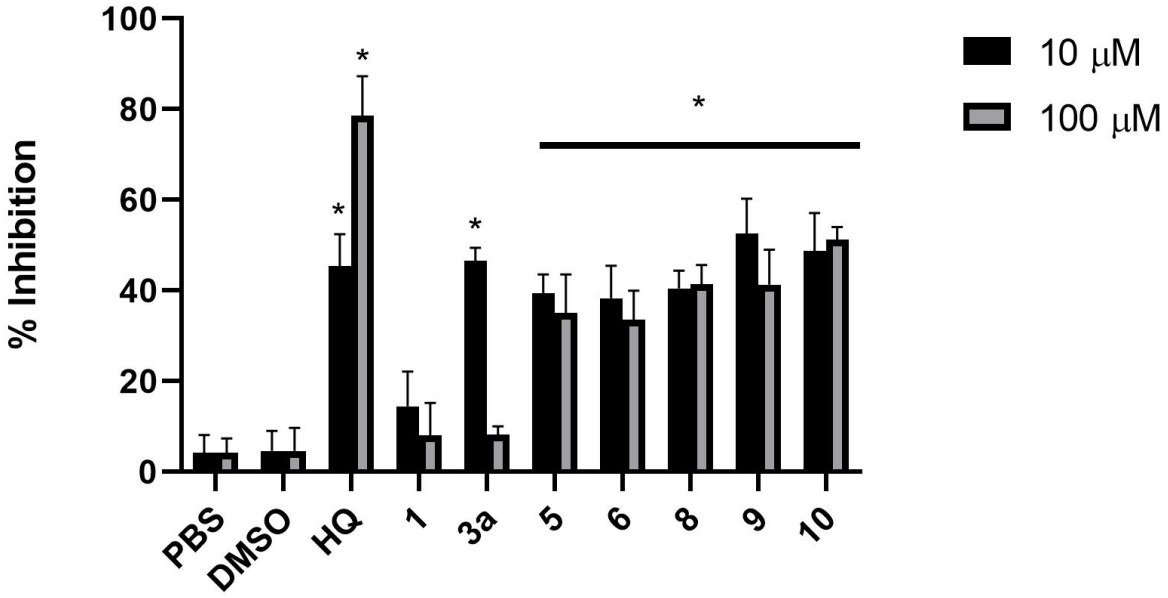
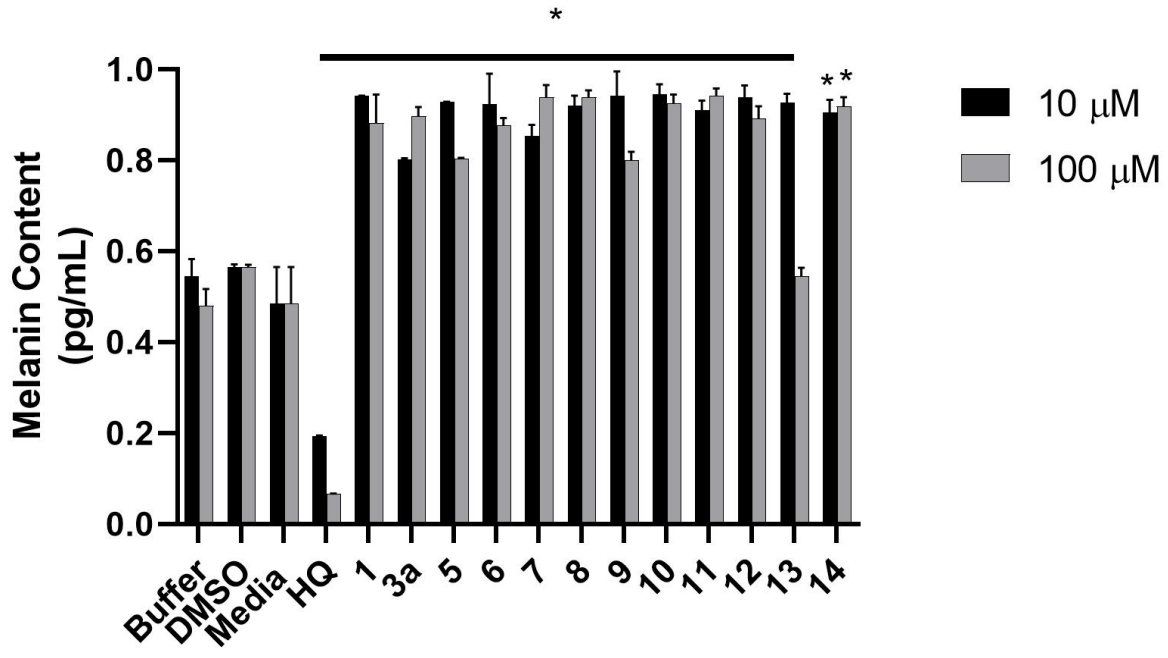


Figure 2: The effect of compounds **1, 3a, 5-6** and **8-10** of *C. flanaganii* and HQ (*p*-hydroquinone) at 10 μM and 100 μM on tyrosinase activity. * $P < 0.001$ compared to the negative controls. Each data point represents n = 3, and is representative of three independent experiments.



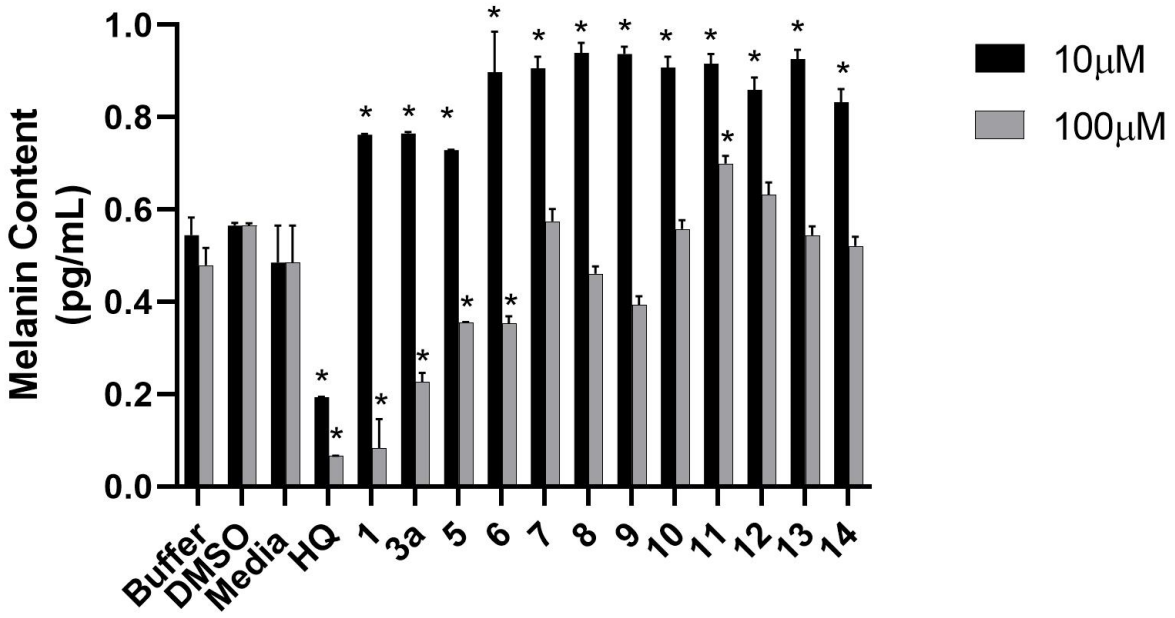
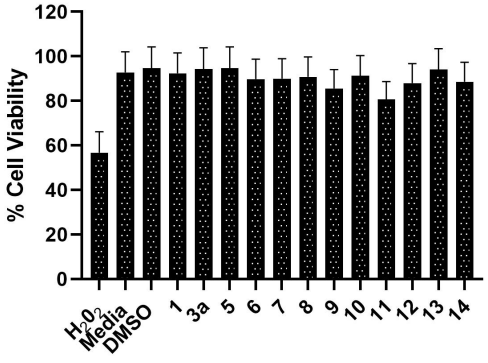
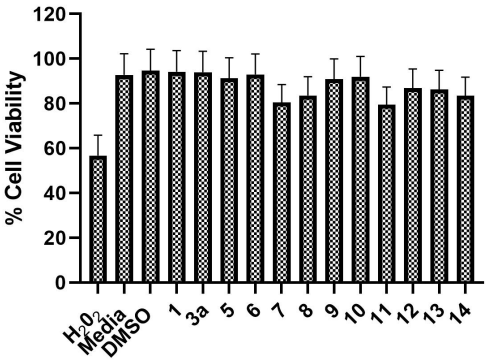


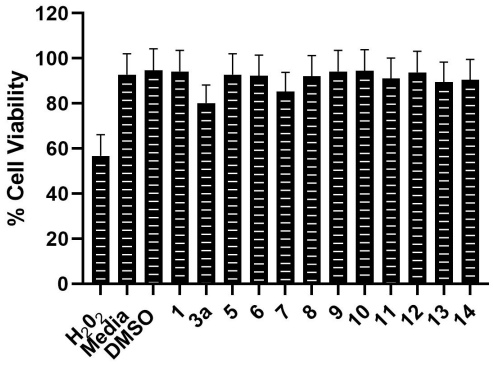
Figure 3: The effect of compounds **1, 3a** and **5** to **14** of *C. flanaganii* at 10 μM and 100 μM for 24 hr (top) and 48 hr (bottom) on melanin content in primary melanocytes. * $P < 0.001$ compared to the negative controls. Each data point represents n = 3, and is representative of three independent experiments.



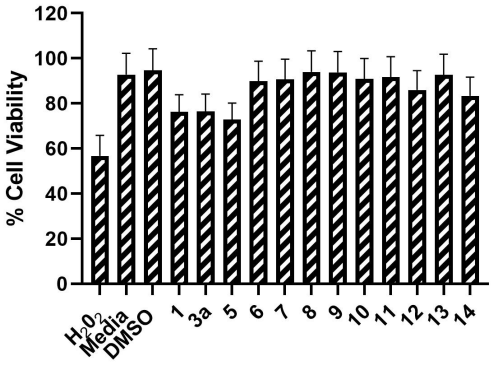
1μmol at 24hr



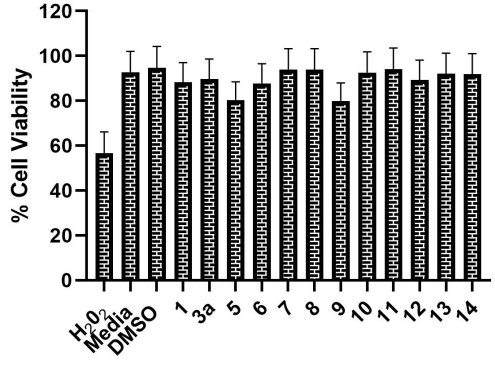
1μmol at 48hr



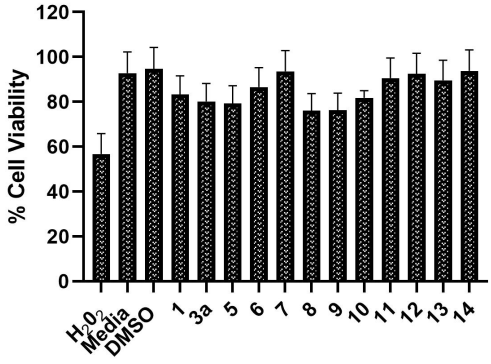
10 µmol at 24hr



10 µmol at 48hr



100 µmol at 24hr



100 µmol at 48hr

Figure 4: Percentage viability of human primary epidermal melanocytes exposed to compounds from *C. flanaganii* at 1 µM, 10 µM and 100 µM for 24 and 48hr. No significant cytotoxicity was observed when compared to the negative controls ($P < 0.001$). Each data point represents n = 3 and is representative of three independent experiments.