JOURNAL OF NATURAL PRODUCTS

Article

Cite This: J. Nat. Prod. XXXX, XXX, XXX−XXX

pubs.acs.org/jnp

Isolation of Flavonoids and Flavonoid Glycosides from *Myrsine africana* and Their Inhibitory Activities against Mushroom Tyrosinase

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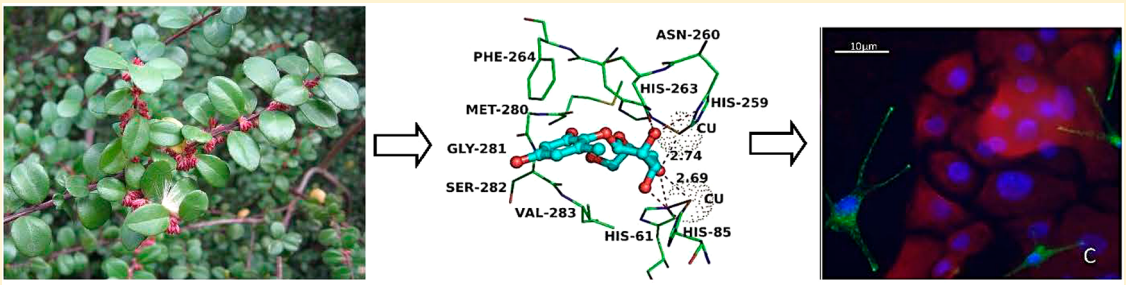
Supporting Information



Myrsine africana (MA) | Molecular docking of Myrsinoside A in the tyrosinase enzyme | MA inhibiting melanin transfer in human cells

ABSTRACT: Bioassay-guided fractionation of the methanol extract of the shoots of *Myrsine africana* led to the isolation of the new compound myricetin 3-*O*-(2″,4″-di-*O*-acetyl)-*α*-L-rhamnopyranoside (**9**) and 11 known compounds. The known compounds quercetin 3-*O*-(3″,4″-di-*O*-acetyl)-*α*-L-rhamnopyranoside (**8**), rutin (**10**), quercetin 3-*O*-*α*-L-rhamnopyranoside (**11**), and myricetin 3-*O*-*α*-L-rhamnopyranoside (**12**) are reported for the first time from the methanol extract of the shoots of *M. africana*. Compounds **10** and **12** showed significant inhibition of tyrosinase with 50% inhibition (IC_{50} values) of the enzyme at 0.13 ± 0.003 and 0.12 ± 0.002 mM, respectively, which was supported by the docking fitness scores obtained through molecular docking analysis. In addition, compounds **1**−**12** displayed significant antioxidant activity with IC_{50} values ranging 1.90 to 3.90 *μ*M.

Melanin is a pigment produced in the melanocytes and is responsible for the variation in the colors of eyes, skin, and hair.[1] Melanin protects against photodamage by absorbing ultraviolet (UV) rays and scavenging reactive oxygen species (ROS). The exposure to sunlight induces an increase in melanin production, leading to a protective phenomenon termed skin tanning. However, an overproduction of melanin can lead to a condition known as skin hyperpigmentation.[2] UV radiation increases the presence of free radicals, which are involved in the melanin biosynthesis pathway and consequently stimulates melanogenesis.[3] In a previous study, Fujiwara et al. concluded that UVB-induced pigmentation in guinea pigs could be reduced by the daily consumption of vitamin C, a well-known antioxidant;[4] therefore, the scavenging of free radicals by antioxidants is an important factor to inhibit melanogenesis. There are several significant tyrosinase inhibitors obtained from natural sources.[5−7] These are mainly used to treat skin hyperpigmentation or depigmentation conditions.[8] Several plant species are being explored in South Africa due to their traditional use against hyperpigmentation and skin problems.[2]

Myrsine africana L. (Myrsinaceae) is a small shrub that is widely distributed in South Africa and Europe. The leaf decoction of *M. africana* is traditionally used by the Southern Sotho, Tswana, and Kwena tribes of South Africa for skin disorders such as acne, pigmentation, wound healing, and cellulitis and for its properties as a blood purifier.[9,10] *M. africana* has several other traditional uses, such as the treatment of diarrhea, rheumatism, toothache, and pulmonary tuberculosis.[10] It is also used as a flavorant, appetizer, carminative, and spice.[11] Local people in the Samburu district in Kenya use the hot decoction of ground seeds of *M. africana* for treating wounds.[12] The inhabitants of Pakistan use a leaf decoction of *M. africana* as a blood purifier and for skin allergies.[13]

The blood purifying properties of *M. africana* play an important role in ensuring healthy and beautiful skin.[14] Natural blood purifiers fight skin microorganisms, reduce acne and

Received: July 13, 2017

DOI: 10.1021/acs.jnatprod.7b00564
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pimples, and prevent aging.[15] Poor blood circulation results in pigmentation changes, which ultimately leads to age spots.[16]

Therefore, for the present study *M. africana* was selected to evaluate its effect on skin hyperpigmentation. These studies also revealed the presence of flavonoids, benzoquinones, and triterpenoids in the shoots of the plant that led to the investigation of their therapeutic use against hyperpigmentation. The study aimed at determining the antityrosinase activity of the methanol extract of the shoots of *M. africana*. The bioassay-guided fractionation was performed to identify compounds responsible for tyrosinase inhibition and antioxidant activity. This led to the isolation of a new and 11 known compounds. The biological activity of the methanol extract of the shoots of *M. africana* and its constituents on melanin production and its effect on mushroom tyrosinase activity, the tyrosinase enzyme (through molecular docking), the 1,2-diphenyl-1-picrylhydrazyl (DPPH) free radical, and melanin production were explored. The mechanism of action was determined by evaluating melanin transfer using immunofluorescence on a coculture of human melanocytes with keratinocytes.

RESULTS AND DISCUSSION

Tyrosinase Enzyme Inhibition Assay and Bioassay-Guided Fractionation. The inhibitory effect of the methanol extract of the shoots of *M. africana* on the rate-limiting enzyme of melanogenesis, tyrosinase, was determined. The extract showed good inhibition of tyrosinase with an IC_{50} value of 0.12 ± 0.001 mg/mL when L-tyrosine was used as the substrate (Table 1). In a similar study by Momtaz et al.[17] an IC_{50} value of 0.02 ± 0.42 mg/mL for a methanol extract of the aerial parts and bark of *M. africana* was reported.

The methanol extract of the shoots of *M. africana* was subjected to bioassay-guided fractionation using various chromatographic techniques. It resulted in the isolation of one new and 11 known compounds. The structural assessment of these compounds was performed using HR-MS and ^{1}H and ^{13}C NMR data. The assignment of signals was facilitated by COSY, HSQC, and HMBC experiments. The known compounds were (Chart 1) myrsinoside A (**1**), myrsinoside B (**2**),[18] quercetin (**3**), myricetin (**4**),[19] mearnsetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**5**),[18] mearnsitrin (**6**),[11] myricetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**7**), quercetin 3-*O*-(3″,4″-di-*O*-acetyl)-α-L-rhamnoside (**8**),[20] rutin (**10**),[21] quercetin 3-*O*-α-L-rhamnopyranoside (**11**),[22] and myricetin 3-*O*-α-L-rhamnopyranoside (**12**).[23] The compounds were identified based on the acid hydrolysis, physical data analysis, and comparison with reported data. Compounds **1**−**7** have previously been identified from the shoots and/or leaves of *M. africana*; however, compounds **8** and **10**−**12** were identified for the first time from the methanol extract of the shoots of *M. africana*. Compounds **8** and **10**−**12** have previously been reported from other plants.[20−23]

Compound **9** was isolated as a yellowish, amorphous powder. The molecular formula was established as $C_{25}H_{24}O_{14}$ from the protonated molecular ion peak $[M + H]^+$ at *m/z* 549.1238 (calcd for $C_{25}H_{25}O_{14}$ 549.1239) in the HRESIMS and from 25 carbon resonances in the ^{13}C NMR spectrum. The IR spectrum (KBr) exhibited absorption bands at 3417 and 1719 cm^{-1} along with other absorption bands, indicative of the presence of hydroxy and carbonyl groups, respectively, in the molecule. The UV spectrum showed absorption bands at 257 and 354 nm which gave bathochromic shifts with the addition of shift reagents, indicating the attachment of the sugar residue at C-3 of the myricetin moiety.[24] The acidic hydrolysis of **9** yielded an aglycone and rhamnose[25] as the only sugar moiety (identified by TLC after comparison with authentic samples). An MS fragment at *m/z* 319.04 indicated the loss of the di-*O*-acetylrhamnose moiety, which indicated myricetin as the aglycone. The ^{1}H NMR spectrum (500 MHz in DMSO-d_6) provided further evidence for myricetin as the aglycone of compound **9** via the singlet resonance at δ_H 6.86 attributed to H-2′ and H-6′ and the characteristic two-proton AX system of the A-ring of 5,7-dihydroxyflavonols. The signals at δ_H 5.34 (1H, d, *J* = 2.0 Hz) and 0.88 (3H, d, *J* = 6.8 Hz) were assigned to the anomeric and 6″-methyl protons of a rhamnopyranosyl unit, respectively. The glycosidic linkage of the rhamnopyranoside was determined based on the coupling constant of the anomeric proton. The α-configuration of the L-rhamnose was deduced from the coupling constant (*J* = 2.0 Hz) of the anomeric proton signal at δ_H 5.34 (Table 1). The small *J* values indicated α-glycosidic linkages in all cases. The spectrum also showed signals for a myricetin 3-*O*-L-rhamnoside possessing two acetyl groups resonating at δ_H 2.07 and 2.04 as three-

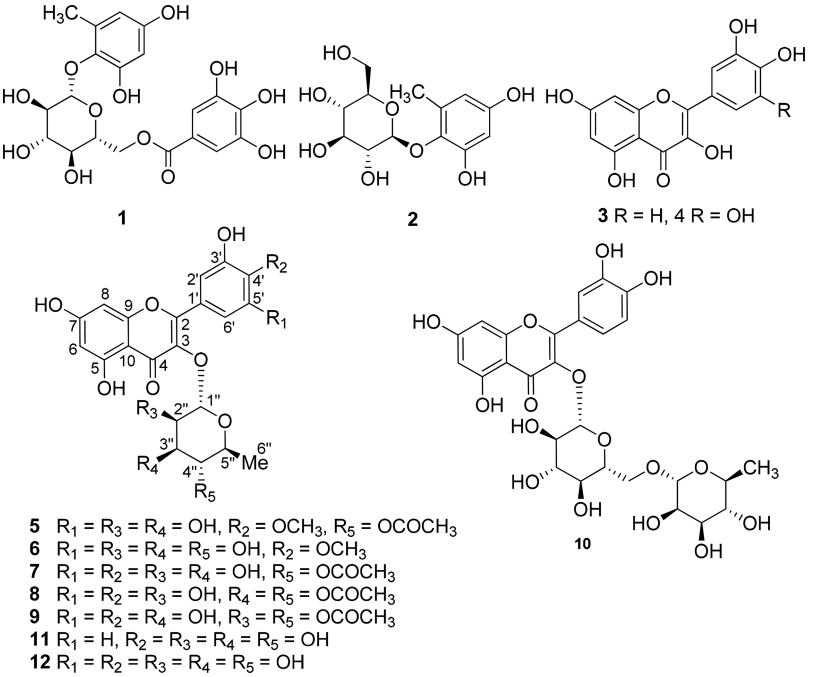
Table 1. Antityrosinase and Antioxidant Activity of the Partitions Obtained through Liquid−Liquid Partitioning, Major Fractions, and Isolated Compounds from the Methanol Extract of the Shoots of *Myrsine africana*

	antityrosinase activity	DPPH activity
compound[a]	IC_{50}[b] ± SD	IC_{50} ± SD
Myrsineafricana crude extract	0.12 ± 0.001 mg/mL	8.65 ± 0.230 μg/mL
petroleum ether partition	0.29 ± 0.001 mg/mL	nt[c]
$CHCl_3$ partition	0.28 ± 0.001 mg/mL	nt
EtAc partition	0.12 ± 0.001 mg/mL	nt
n-BuOH partition	>0.30 mg/mL	nt
MF-1	>0.30 mg/mL	nt
MF-2	>0.30 mg/mL	nt
MF-3	0.08 ± 0.001 mg/mL	nt
MF-4	0.10 ± 0.001 mg/mL	nt
MF-5	>0.30 mg/mL	nt
MF-6	>0.30 mg/mL	nt
MF-7	>0.30 mg/mL	nt
1		3.00 ± 0.007 μM**
2	0.41 ± 0.002 mM	3.90 ± 0.462 μM**
3	0.24 ± 0.003 mM	3.20 ± 0.040 μM**
4	0.21 ± 0.003 mM	4.20 ± 0.003 μM**
5	0.19 ± 0.004 mM	2.20 ± 0.019 μM**
6	0.31 ± 0.003 mM	1.90 ± 0.021 μM**
7	0.28 ± 0.002 mM	3.50 ± 0.040 μM**
8	0.18 ± 0.002 mM	3.40 ± 0.751 μM**
9	0.52 ± 0.002 mM	3.20 ± 0.009 μM**
10	0.13 ± 0.003 mM	2.30 ± 0.002 μM**
11	0.15 ± 0.003 mM	2.20 ± 0.022 μM**
12	0.12 ± 0.003 mM	2.00 ± 0.006 μM**
kojic acid[d]	0.01 ± 0.001 mM	
ascorbic acid (vitamin C)[e]	0.144 ± 0.004 mM	11.20 ± 1.36 μM

[a]Myrsinoside A (**1**), myrsinoside B (**2**), quercetin (**3**), myricetin (**4**), mearnsetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**5**), mearnsitrin (**6**), myricetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**7**), quercetin 3-(3″,4″-di-*O*-acetyl-α-L-rhamnoside) (**8**), myricetin 3-*O*-(2″,4″-di-*O*-acetyl)-α-L-rhamnopyranoside (**9**), rutin (**10**), quercetin 3-*O*-α-L-rhamnopyranoside (**11**), and myricetin 3-*O*-α-L-rhamnopyranoside (**12**). [b]50% inhibitory concentration. [c]nt, not tested. [d]Positive control for antityrosinase activity. [e]Positive control for antioxidant activity.

DOI: 10.1021/acs.jnatprod.7b00564
J. Nat. Prod. XXXX, XXX, XXX−XXX

Chart 1. Chemical Structures of Isolated Compounds from the Methanol Extract of the Shoots of *Myrsine africana*



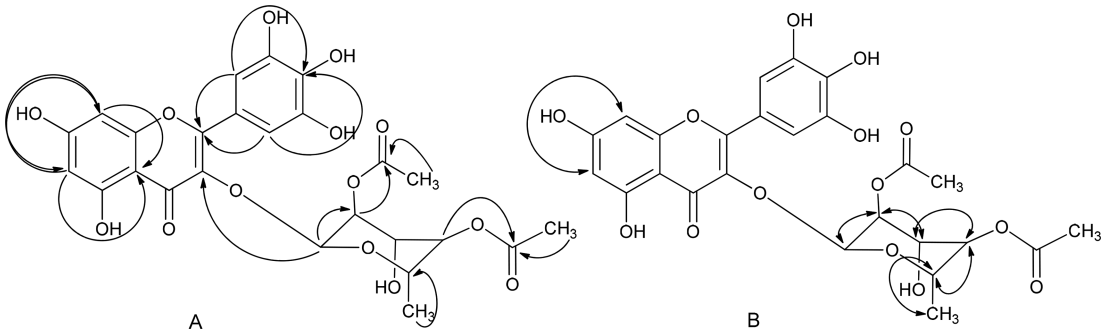


Figure 1. Key HMBC [H → C] (A) and ^{1}H–^{1}H COSY (B) correlations of compound **9** isolated from the methanol extract of the shoots of *Myrsine africana*.

proton singlets. The presence of the two acetyl groups was further supported by the ^{13}C NMR resonances at δ_C 170.1, 170.0, 21.1, and 20.9 assigned to the carbonyl and methyl carbons. COSY correlations were observed between H-6 (δ 6.18) and H-8 (δ 6.36) and between H-5″ (δ 3.34) and H_3-6″ (δ 0.88). COSY correlations were also observed between H-1″ (δ 5.34) and H-2″ (δ 5.01), H-2″ (δ 5.01) and H-3″ (δ 3.95), H-3″ (δ 3.95) and H-4″ (δ 4.63), and H-4″ (δ 3.95) and H-5″ (δ 3.34). In the HMBC spectrum, H-1″ (δ_H 5.34) correlated to C-3 (δ_C 134.8). Similarly, H-2″ (δ_H 5.01) showed connectivity to C-2″-$\underline{C}OCH_3$ (δ_C 170.1) and H-4″ (δ_H 4.63) with C-4″-$\underline{C}OCH_3$ (δ_C 170.0). HMBC correlations were observed from the methyl group protons (δ_H 0.88) to C-5″ (δ_C 69.9), (δ_H 2.07) to 2″-$\underline{C}OCH_3$ (δ_C 170.1), and (δ_H 2.04) to 4″-$\underline{C}OCH_3$ (δ_C 170.0) (Figure 1). On the basis of NMR shift values the acetyl substituents were located at C-2″ and C-4″, which was confirmed by the deshielded H-2″ (δ_H 5.01) and H-4″ (δ_H 4.63) resonances. Hence, the structure of compound **9** was defined as the new myricetin 3-*O*-(2″,4″-di-*O*-acetyl)-α-L-rhamnopyranoside.

All the isolated compounds were tested for antityrosinase activity (Table 1). Compounds **10**, **11**, and **12** showed the highest tyrosinase inhibition with IC_{50} values of 0.13 ± 0.003, 0.15 ± 0.003, and 0.12 ± 0.002 mM, respectively. Compounds **3**–**7** showed tyrosinase inhibition with IC_{50} values ranging from 0.21 to 0.50 mM. In the present study, the IC_{50} value of quercetin was found to be 0.24 ± 0.003 mM. According to earlier reports, the IC_{50} value of quercetin in the oxidation of L-DOPA by mushroom tyrosinase was found to be 0.13 mM.[26] Rutin has been previously reported to inhibit tyrosinase, and it showed an IC_{50} value of 0.52 ± 0.002 mM against tyrosinase in the present study. On the contrary, another study reported an IC_{50} value of 0.07 ± 0.3 mM for rutin against tyrosinase.[27] Quercetin 3-*O*-α-L-rhamnopyranoside and myricetin 3-*O*-α-L-rhamnopyranoside were previously investigated for their antityrosinase potential and showed IC_{50} values of 0.10 and 0.32 mM, respectively.[28] However, in the present study, quercetin 3-*O*-α-L-rhamnopyranoside and myricetin 3-*O*-α-L-rhamnopyranoside showed IC_{50} values of 0.13 ± 0.003 and 0.15 ± 0.003 mM, respectively. It is interesting to note that the antityrosinase activities of myrisinosides A and B, myricetin, mearnsetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside, mearnsitrin, myricetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside, and

DOI: 10.1021/acs.jnatprod.7b00564
J. Nat. Prod. XXXX, XXX, XXX−XXX

quercetin 3-*O*-(3″,4″-di-*O*-acetyl)-α-L-rhamnopyranoside have not previously been reported.

Measurement of DPPH Radical Scavenging Activity. There are a number of intrinsic and extrinsic stresses that constantly affect the skin and, therefore, contribute to an increase in the reactive oxygen species. ROS induced through UV radiation are known to play a role in skin pigmentation by inducing melanogenesis or increasing melanocyte proliferation. Antioxidants, which are able to scavenge ROS, could therefore possibly inhibit messenger molecules, which stimulate melanogenesis. The copper ion present in the active site of the tyrosinase enzyme could also provide a binding site for antioxidants and in turn regulate skin pigmentation.[29−31] In the molecular docking study, ascorbic acid and kojic acid showed similar docking fitness scores of 48.25 and 46.15, respectively, with the active site of the tyrosinase enzyme. The well-known antioxidant ascorbic acid is furthermore known to inhibit tyrosinase monophenolase activity.[32] Therefore, the *M. africana* extract and isolated compounds were tested for their antioxidant activity against the DPPH free radical. The extract showed strong DPPH radical scavenging capacity with an IC_{50} value of 8.65 ± 0.23 μg/mL (Table 1). There are no published reports on the DPPH activity of the constituents of *M. africana*. Compounds **1**−**12** showed IC_{50} values statistically lower ($p < 0.01$) than that of ascorbic acid (IC_{50} value of 11.2 μM) with mearnsitrin having the highest activity (Table 1). The DPPH radical scavenging activities of myrisinoside A, myrisinoside B, mearnsetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside, and myricetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside have not previously been reported, whereas quercetin, myricetin, myricetin 3-*O*-α-L-rhamnopyranoside, mearnsitrin, rutin, and quercetin-3-*O*-α-L-rhamnopyranoside are well known as antioxidants and have previously been reported.[31−35]

Molecular Docking Analysis. The molecular docking study was performed to identify the possible orientations and binding interactions of the molecules in the active site of the tyrosinase enzyme (Table 3; Figure 2). Corresponding to the tyrosinase inhibitory activity, kojic acid showed the highest docking fitness score of 48.25, followed by ascorbic acid (vitamin C) at 46.14, as compared to the isolated compounds. Despite the presence of only two H-bond interactions (Figure 3), there is a minimal distance between the polar interaction of kojic acid and the Cu^{2+} ions and the primary hydroxy group of ascorbic acid and the Cu^{2+} ions, which could potentially explain the high docking score. In contrast to this, despite having three to four H-bond interactions with the active site residues, compounds **1**, **2**, **8**, and **9** showed lower docking fitness scores when compared to kojic acid. This could also potentially be due to the distances of the polar interactions with the Cu^{2+} ions. The distances of the polar interactions in **1**, **2**, **8**, and **9** were marginally larger than the corresponding distances of kojic acid. Similar interactions were seen for compound **10**; however, five H-bond interactions with the site residues were observed, of which four bonds were on the same two residues. Compound **10**, however, showed good antityrosinase results that substantiated its docking fitness scores. Compounds **3**−**7** showed moderate antityrosinase activity that corresponded to their docking scores. These molecules showed moderate distances for polar interactions with the Cu^{2+} ions and also showed more H-bond interactions when compared to compounds **1**, **2**, **8**, and **9**. Corresponding to tyrosinase inhibitory activity, compounds **11** and **12** showed better docking fitness scores, which was supported by reasonably short polar interaction distances and large H-bond interactions. The docking study clearly indicated that distances for polar interactions with Cu^{2+} ions play a crucial role in controlling the tyrosinase inhibitory activity.

Table 2. ^{1}H and ^{13}C NMR Data (DMSO; 500 and 125 MHz) of Compound 9

position	δ_H (J in Hz)	δ_C, type	HMBC	^{1}H−^{1}H COSY
2		158.4, C		
3		134.8, C		
4		177.6, C		
5		161.3, C		
6	6.18, d (2.1)	98.7, CH	8, 10	8
7		164.2, C		
8	6.36, d (2.1)	93.6, CH	6, 10	6
9		156.4, C		
10		108.6, C		
1′		119.6, C		
2′	6.86, s	107.8, CH	2, 4′	
3′		145.8, C		
4′		136.5, C		
5′		145.8, C		
6′	6.86, s	107.8, CH	2, 4′	
1″	5.34, d (2.0)	101.6, CH	3, 2″	2″
2″	5.01, dd (3.9, 2.0)	73.2, CH	2″-COCH$_3$	1″, 3″
3″	3.95, dd(9.8, 4.4)	68.1, CH		2″, 4″
4″	4.63, t (9.8)	74.0, CH	4″-COCH$_3$	3″, 5″
5″	3.34, dd (9.8, 6.8)	69.9, CH		5″, 6″
6″	0.88, d (6.8,)	17.3, CH$_3$	5″	5″
2″-COCH$_3$		170.1, C		
4″-COCH$_3$		170.0, C		
2″-COCH$_3$	2.07, s	20.9, CH$_3$	2″-COCH$_3$	
4″-COCH$_3$	2.04, s	21.1, CH$_3$	4″-COCH$_3$	

Measurement of Melanin Content. The effect of the methanol extract of the shoots of *M. africana* on melanin production was determined by observing the effect on melanin production in B16F10 cells and melanin transfer between human melanocytes and keratinocytes. The extract was found to be nontoxic to melanocyte cells at the highest concentration (100 μg/mL) tested. At a concentration of 50 μg/mL, the extract inhibited 50% of melanin production as compared to untreated cells as determined from a bovine serum albumin standard curve.

Measurement of Melanin Transfer. During the mechanistic studies, the effect of the methanol extract of the shoots of *M. africana* on melanin transfer was analyzed using immunofluorescence. The cocultures of normal human melanocytes (NHMs) and normal human keratinocytes (NHKs) treated with the *M. africana* extract showed an increase in contact between NHMs and NHKs as compared to the DMSO-treated cells as well as an increase in dendrite length (Figure 3). However, no melanosome transfer was detected at a concentration of 20 μg/mL ($^1/_4IC_{50}$) as compared to cells treated with α-melanocyte stimulating hormone (α-MSH). Furthermore, there were no melanosomes present around the nucleus of the keratinocytes. This confirmed our findings that the *M. africana* extract was able to inhibit melanin production.

During the tyrosinase and molecular docking studies it was evident that kojic acid and ascorbic acid had the highest docking scores, which correlated with their tyrosinase inhibition. However, the docking fitness scores and tyrosinase

DOI: 10.1021/acs.jnatprod.7b00564
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 3. Antityrosinase Activity and Docking Scores of Compounds Isolated from the Methanol Extract of the Shoots of *Myrsine africana*

compound[a]	IC_{50} (mM)	dock score (gold fitness score)	distance for polar interactions with Cu^{2+} ions (Å)		active site residues involved in H-bond
kojic acid	0.01 ± 0.001	48.25	2.19	2.37	His61, His259
1		26.49	2.74	2.69	His61, His85, His259, Asn260
2	0.41 ± 0.002	31.01	2.19	3.32	His259, Asn260, His263, Met280
3	0.24 ± 0.003	40.40	2.41	2.42	His61, His85, His263, Met280, Gly281
4	0.21 ± 0.003	39.58	2.03	2.79	His61, His259, His263 (2)[b], Asn260 (2)[b], Met280
5	0.19 ± 0.004	33.81	2.36	2.54	His61, His85, His263, Met280, Val283
6	0.31 ± 0.003	35.69	2.16	3.04	His61, His263, Met280, Gly281
7	0.28 ± 0.002	35.92	2.20	2.45	His61, His263, Met280, Gly281, Val283
8	0.18 ± 0.002	30.56	2.22	3.15	His61, His85, His263
9	0.52 ± 0.002	29.38	1.99	3.37	His259, His263, Met280, Val283
10	0.13 ± 0.003	30.24	3.43	2.96	His85 (2)[b], Met280, Gly281 (2)[b]
11	0.15 ± 0.003	37.58	2.25	2.41	His61, His263, Met280, Val283
12	0.12 ± 0.003	37.54	2.21	2.72	His61, His263 (2)[b], Met280, Gly281
ascorbic acid (vitamin C)		46.15	2.12	2.41	

[a]Myrsinoside A (**1**), myrsinoside B (**2**), quercetin (**3**), myricetin (**4**), mearnsetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**5**), mearnsitrin (**6**), myricetin 3-*O*-(4″-*O*-acetyl)-α-L-rhamnopyranoside (**7**) quercetin 3-(3″,4″-di-*O*-acetyl)-α-L-rhamnoside (**8**), myricetin-3-*O*-(2″,4″-di-*O*-acetyl)-α-L-rhamnopyranoside(**9**), rutin (**10**), quercetin 3-O-α-L-rhamnopyranoside (**11**), and myricetin 3-*O*-α-L-rhamnopyranoside (**12**). [b]Number of H-bonds with respective residues.

inhibition of the isolated compounds were not as high as those of the two positive controls. The compounds, however, had significant antioxidant activity, even higher than that of ascorbic acid, and, therefore, the mechanism of these compounds could be due to the inhibition of melanin transfer and not due to tyrosinase inhibition. Thus, the activity of compounds to inhibit melanin transfer should be considered for future studies.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured in MeOH using a PerkinElmer polarimeter with a sodium lamp operating at 598 nm. UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer, and the λ values are reported in nm. IR spectra were recorded on a Nexus 670 FT-IR instrument using KBr pellets. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data were recorded using an Agilent NMR spectrometer (Central Analytical Facilities, Stellenbosch, South Africa), and the chemical shifts were reported as δ values with tetramethylsilane as an internal standard at 30 °C (measured in methanol-d_4 and DMSO-d_6). Inverse detected heteronuclear correlations were measured using HMQC and HMBC pulse sequences with a pulsed-field gradient. HR-ESIMS data were obtained using a Waters UPLC-MS system with PDA detector and Waters Synapt G2 QTOF mass spectrometer.

Plant Material. The shoots of *M. africana* were collected from the Manie van der Schijff botanical garden of the University of Pretoria (S 25°45′21″, E 28°13′51″) in January 2013 and identified by Ms. Magda Nel at the H.G.W.J. Schweickerdt Herbarium (PRU), where a voucher specimen (MA-S-2013-1) was deposited.

Extraction and Isolation. The air-dried shoots (2.3 kg) were ground to a fine powder and soaked in MeOH (5 L) on a rotatory shaker for 3 days. The filtrate was collected using a Buchner funnel with Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Büchi R-200) at 40 °C. The extract (88 g, 3.83% yield of dried plant material) was redissolved in MeOH (100 mL) and suspended in 800 mL of distilled H_2O. The aqueous layer extract (88 g) was suspended in different solvents and successively extracted three times in petroleum ether (pet. ether) (3 × 1.2 L), $CHCl_3$ (3 × 1.2 L), EtOAc (3 × 1.2 L), and *n*-BuOH (3 × 1.2 L). The organic layers were evaporated to give 2.30 g (2.61% yield of extract) of pet. ether, 4.70 g (5.34% yield of extract) of $CHCl_3$, 18.70 g (21.25% yield of extract) of EtOAc, and 40.00 g (45.45% yield of extract) of *n*-BuOH extracts and were tested for tyrosinase inhibition (Table 1). The EtOAc fraction (18.70 g) showed the highest tyrosinase inhibition and was subjected to silica gel column chromatography using *n*-hexane, MeOH, and distilled H_2O in ratios of increasing polarity. Similar fractions were pooled based on TLC profiles to obtain seven major fractions (MF-1 to MF-7). MF-3 and MF-4 showed the highest tyrosinase inhibition, with IC_{50} values of 0.08 ± 0.001 and 0.10 ± 0.001 mg/mL, respectively (Table 1) and, therefore, were subjected to further scrutiny. MF-2 (1.25 g, 1.42% yield) was subjected to silica gel CC with an eluent of CH_2Cl_2/MeOH of increasing polarity (0−100%). Frs. 2−4 (500 mg, 0.57% yield of extract) were chromatographed using silica gel with CH_2Cl_2/MeOH (7:1, 1 L), which afforded compound **1** (257 mg, 0.29% yield of extract). Frs. 5−7 (205 mg, 0.23% yield of extract) were subject to repeated silica gel column chromatography (CC) with $CHCl_3$/MeOH (6:1, 800 mL), to afford compound **2** (62 mg, 0.07% yield of extract).

MF-3 (3.82 g, 4.34% yield of extract) was subjected to silica gel CC using CH_2Cl_2/MeOH of increasing polarity (0 to 100%) as an eluent. Similar fractions were pooled according to TLC profiles to give six fractions (1−6). Fr. 2 (625 mg, 0.71% yield of extract) was chromatographed on a Sephadex LH-20 column using CH_2Cl_2/MeOH. Frs. 4−7 were pooled (210 mg, 0.24% yield of extract) and chromatographed twice using Sephadex LH-20 with $CHCl_3$/MeOH (25:1, 500 mL) to yield compound **3** (21 mg, 0.02% yield of extract). Subfr. 9 (95 mg, 0.11% yield of extract) of fr. 2 was further purified using Sephadex LH-20 with $CHCl_3$/MeOH (16:1, 500 mL) to yield compound **4** (25 mg, 0.03% yield of extract). Fr. 3 (118 mg, 0.13% yield of extract) was chromatographed on Sephadex LH-20 using $CHCl_3$/MeOH as an eluent. Subfrs. 2−4 from fr. 3 were pooled (80 mg, 0.09% yield of extract) and chromatographed using Sephadex LH-20 with $CHCl_3$/MeOH (6:1, 500 mL) to yield compound **5** (10 mg, 0.01% yield of extract). Fr. 5 (0.28 mg, 0.23% yield of extract) was further chromatographed on Sephadex LH-20 using $CHCl_3$/MeOH (3:1, 800 mL) to yield compound **6** (9 mg, 0.01% yield of extract). Fr. 6 (728 mg, 0.83% yield of extract) was chromatographed on Sephadex LH-20 using $CHCl_3$/MeOH, affording subfr. 2 (225 mg, 0.26% yield of extract), which was further purified using Sephadex LH-20 with $CHCl_3$/MeOH (2:1, 800 mL) to yield compounds **7** (26 mg, 0.03% yield of extract) and **8** (17 mg, 0.02% yield of extract). Subfr. 3 (110 mg, 0.13% yield of extract) from fr. 6 yielded compound **9** via repeated Sephadex LH-20 CC eluted with $CHCl_3$/MeOH (2.1:1, 600 mL) (13 mg, 0.02% yield of extract).

MF-4 (2.58 g, 2.93% yield of extract) was subjected to a Sephadex LH-20 column using MeOH/H_2O of decreasing polarity (100 to 0%

DOI: 10.1021/acs.jnatprod.7b00564
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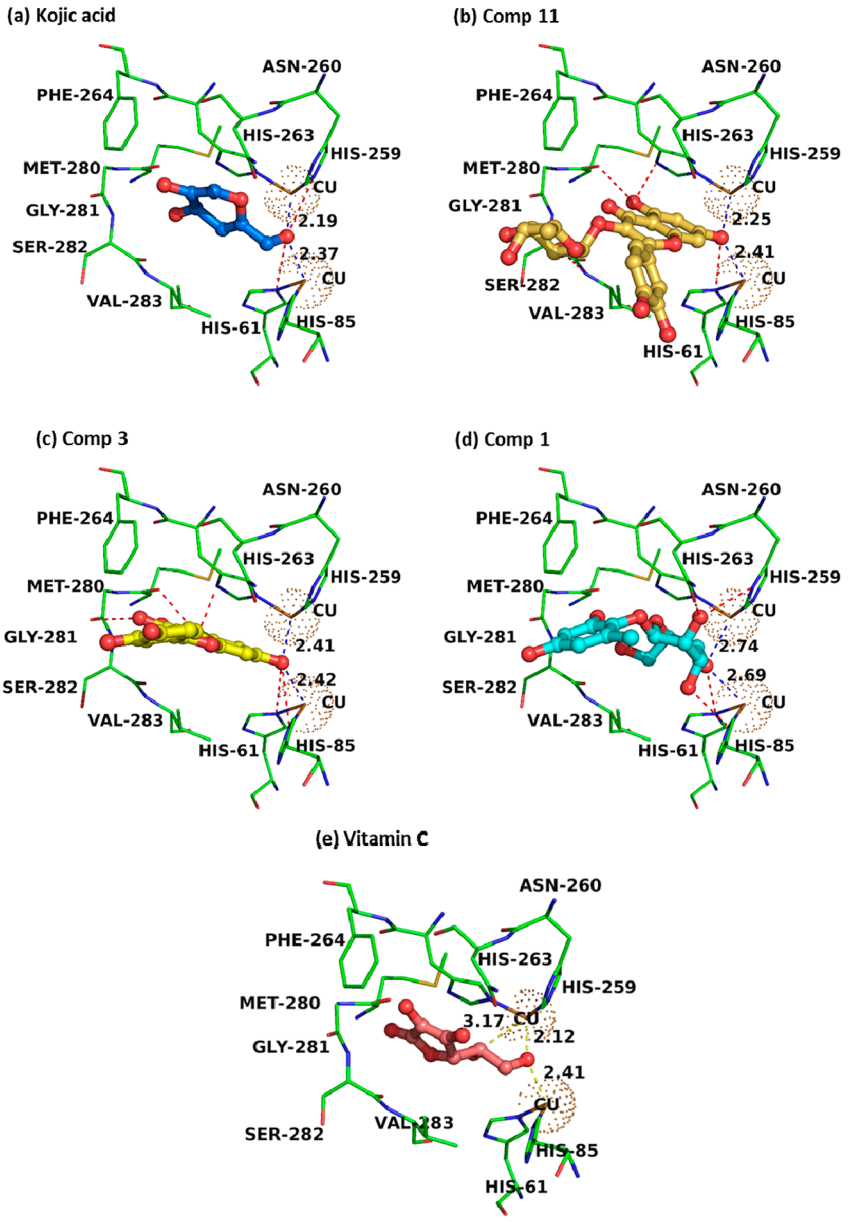

Figure 2. H-bond interactions with tyrosinase active site residues and polar interactions with Cu^{2+} ions in various complexes with (a) kojic acid, (b) compound **11**, (c) compound **3**, (d) compound **1**, and (e) vitamin C.

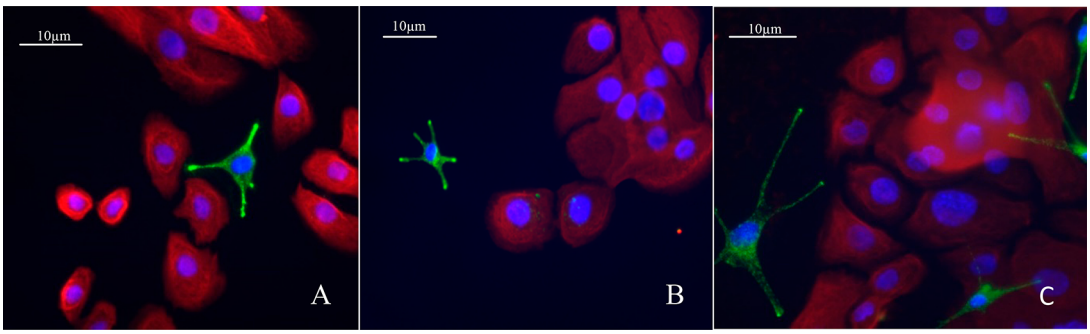

Figure 3. Untreated cells (A), cells treated with the positive control α-MSH (B), and cells treated with *Myrsine africana* extract (C). Analyses with double immunolabeling. Melanosomes (small green dots) observed around the nucleus (stained blue) confirm melanin transfer. No melanin transfer was observed between the normal human melanocytes (stained green) and normal human keratinocytes (stained red) treated with *M. africana*.

H_2O) as an eluent. Similar fractions were pooled according to TLC profiles to give three main fractions (1−3). Fr. 1 (0.315 g, 0.36% yield of extract) was chromatographed on a Sephadex LH-20 column using MeOH/H_2O and rechromatographed using SephadexLH-20 with

DOI: 10.1021/acs.jnatprod.7b00564
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MeOH/H_2O (1:9, 800 mL) to yield compound **10** (11 mg, 0.01% yield). Fr. 2 (521 mg) was chromatographed using a Sephadex LH-20 column with MeOH/H_2O. The subfractions were combined (120 mg, 0.14% yield of extract) and rechromatographed twice using Sephadex LH-20 with MeOH/H_2O (1:6, 500 mL) to yield compounds **11** (31 mg, 0.04% yield of extract) and **12** (18 mg, 0.02% yield of extract).

*Myricetin 3-O-(2″,4″-di-O-acetyl-α-L-rhamnopyranoside (**9**):* yellowish, amorphous powder; $[\alpha]_D$ −187.9 (*c* 0.020, MeOH), R_f 0.60, silica gel 60 F_{254}, CH_2Cl_2/MeOH (82:18); UV (MeOH) λ_{max} 257, 354 nm; IR (KBr) ν_{max} 3417, 1719, 1611, 1439 cm^{-1}; ^1H and ^{13}C NMR data see Table 2; HR-ESIMS *m/z* 549.1238 ($[M + H]^+$) (calcd for $C_{25}H_{25}O_{14}$, 549.1239); HR-ESIMS *m/z* 547.1090 $[M - H]^-$.

Tyrosinase Enzyme Inhibition Assay. The tyrosinase assay was conducted as described by Curto et al.[33] with slight modifications. The enzymatic rate of mushroom tyrosinase (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) was evaluated spectrophotometrically through the color change and absorbance increase when L-tyrosine (substrate) was converted into L-DOPA. A stock solution containing 600 μg/mL of extract, 0.38 mM DMSO, and 970 μL of K_3PO_4 buffer (pH 6.5) was prepared. In a 96-well plate, 70 μL of each sample stock solution was combined with 30 μL of tyrosinase (333 units/mL in K_3PO_4 buffer) in triplicate, and after 5 min of incubation at room temperature, 110 μL of substrate (2 mM L-tyrosine) was added to each well. Final concentrations of the extract, positive control (kojic acid), major fractions, and pure compounds ranged from 1.5 to 200 μg/mL. The final concentrations of DMSO present in the samples ranged from 0.007 to 0.895 μg/mL. The absorbance of the sample wells (Abs_{sample}) and the DMSO vehicle control wells ($Abs_{control}$) were determined kinetically over a period of 30 min at 492 nm using the BIO-TEK Power-Wave XS multiwell plate reader (A.D.P., Weltevreden Park, RSA). The percentage tyrosinase inhibition was calculated as follows: Inhibition percent (%) = 100 − ($Abs_{sample}/Abs_{control}$ × 100). Kojic acid (purity >98%) was used as the positive control (Sigma-Aldrich Co.). All samples were tested in triplicate, and the IC_{50} values were calculated using GraphPad Prism 4 software.

Measurement of DPPH Radical Scavenging Activity. The method, described by Berrington and Lall,[34] was followed to determine the antioxidant (DPPH radical scavenging) activity of the shoot extract of *M. africana* and the isolated compounds. The samples and positive control, ascorbic acid, were prepared at a stock concentration of 2 mg/mL in EtOH. EtOH was used as the control, as no DPPH inhibition was expected. In a 96-well plate, serial dilutions of the samples were prepared, in triplicate, to obtain final concentrations ranging from 0.78 to 100 μg/mL for the extract, positive control, and isolated compounds. To all the wells was added 90 μL of a DPPH solution in EtOH (0.04 M), except for the blank plates, where distilled H_2O was added instead. The plates were incubated for 30 min in a dark room for the DPPH to develop. The absorbance was measured at a wavelength of 515 nm using the BIO-TEK PowerWave XS multiwell plate reader. The results given as IC_{50} values indicated the concentration required to bind 50% of the DPPH free radicals. The percentage inhibition was calculated as follows: DPPH radical scavenging activity (%) = [($Abs_{control}$ − Abs_{sample})/($Abs_{control}$)] × 100, where $Abs_{control}$ is the absorbance of DPPH radical + EtOH control; Abs_{sample} is the absorbance of (DPPH radical + sample/positive control) − (blank values of corresponding sample). All samples were tested in triplicate, and the IC_{50} values were calculated using GraphPad Prism 4 software. Ascorbic acid (purity >99%) was used as the positive control (Sigma-Aldrich Co.).

Molecular Docking Analysis. Molecular docking was performed using the GOLD program. It uses a genetic algorithm considering ligand flexibility and partial protein flexibility.[35] The default docking parameters were employed for the docking study. The crystal structure of *Agaricus bisporus* tyrosinase was used and obtained from the protein data bank (pdb id: 2Y9X). Protein Preparation Wizard version 1.8.2 of the GOLD program was used to correct the interaction between residues and topology. All ligands were corrected for bond order and minimized to get optimized conformation. The docking protocol was set by extracting and redocking the tropolone in the tyrosinase crystal structure with rmsd < 1.0 Å. It was followed by docking of all molecules in the active site defined as 6 Å regions around the cocrystal ligand in tyrosinase protein.

Cell Culture and Chemicals. B16F10 murine melanoma cells (Highveld Biological, Johannesburg, RSA) were grown in Eagle's minimum essential medium (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated fetal bovine serum, 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 1% fungicide (250 μg/mL fungizone) (Life Technologies, Johannesburg, RSA) at 37 °C with 5% CO_2. 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide salt (XTT), actinomycin D, phenylthiorea, and α-MSH were obtained from Sigma-Aldrich Co. Cells were subcultured after an 80% confluent monolayer had formed using trypsin−EDTA (0.25% trypsin containing 0.01% EDTA).

Cell Viability Assay. Cytotoxicity was measured by the XTT method using the Cell Proliferation Kit II on B16-F10 mouse melanoma cells. The method described by Berrington and Lall[34] was used to perform the assay. Briefly, 100 μL of cells was seeded in 96-well plates (1 × 10^5 cells/mL) and incubated for 24 h at 37 °C in 5% CO_2 for cell adherence. Cells were treated with *M. africana* extract for 72 h. Actinomycin D (purity >95%) was used as the positive control at concentrations ranging from 0.00039 to 0.05 μg/mL. After treatment, XTT (50 μL) was added to a final concentration of 0.30 mg/mL for 2 h. Blank plates were included that were prepared in the same manner, but did not contain cells. Absorbance was measured at 490 and 690 nm (reference wavelength) using a BIO-TEK Power-Wave XS multiwell plate reader.

Measurement of Melanin Content. The melanin inhibition assay was conducted as described by Hall and Orlow[36] with slight modification. The *M. africana* extract was tested at a concentration of 50 μg/mL ($^1/_2IC_{50}$). Mouse melanoma B16-F10 cells were plated at 1 × 10^6 cells per 500 μL of culture medium, treated with 50 μg/mL extract, and incubated for 72 h at 5% CO_2 at 37 °C. After incubation, cells were centrifuged at 1100 rpm for 10 min, and the supernatant was removed and examined for protein concentration using the Bradford protein assay as described below. The remaining pellet was washed twice with phosphate-buffered saline and dissolved in 230 μL of 2 M NaOH with 20% DMSO at 60 °C. A 200 μL amount of the dissolved mixture, which contained melanin, was measured for absorbance at 490 nm. The melanin content was determined as the absorbance of melanin per total protein concentration. Phenylthiourea (stock concentration of 0.100 mM) was used as the positive control (Sigma-Aldrich Co.).

Measurement of Melanin Transfer. Normal human melanocytes (grown from skin biopsies at the San Gallicano Dermatological Institute, Rome, Italy) were seeded (2500 cells/well) on coverslips previously coated with 2% gelatin into 24-well plates and were incubated at 37 °C for 72 h. Normal human keratinocytes were added to NHMs and incubated in Medium 154 with a higher Ca^{2+} concentration (0.15 mM) and keratinocyte growth supplement for 24 h. The *M. africana* extract (20 μg/mL) was added to the coculture and incubated at 37 °C for 24 h. Control cells were treated with DMSO control (0.1%), the volume equal to that present in the cells incubated with *M. africana* extract. α-MSH (purity >97%) (100 mM) was added as the positive control for 24 h (Sigma-Aldrich Co.). Melanin transfer was analyzed by immunofluorescence.[37]

Statistical Analysis. Data recorded are presented as mean ± SD (n = 3). Results were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the GraphPad Prism 4 statistical software. Differences with $p < 0.01$ (**), where samples were statistically lower than the positive controls, were considered to be significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00564.

^1H and ^{13}C NMR and COSY spectroscopic data for compound **9** (PDF)

DOI: 10.1021/acs.jnatprod.7b00564
J. Nat. Prod. XXXX, XXX, XXX−XXX

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the University of Pretoria and the National Research Foundation for financial support and the Central Analytical Facilities (CAF), Stellenbosch, South Africa, for acquiring the IR, ^{1}H and ^{13}CNMR, and MS data.

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DOI: 10.1021/acs.jnatprod.7b00564
J. Nat. Prod. XXXX, XXX, XXX−XXX