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Chemical and bioactivity of flavanones obtained from roots of *Dalea pazensis* Rusby

María D. Santi [a,b], Mariana A. Peralta [a,b], Caterine S. Mendoza [c], José Luis Cabrera [a,b,*], María G. Ortega [a,b,*]

[a] Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Haya de la torre y Medina Allende, Edificio Ciencias II, X5000HUA Córdoba, Argentina
[b] Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET), Ciudad Universitaria, X5000HUA Córdoba, Argentina
[c] Departamento de Farmacia, Facultad de Ciencias Químico-Farmacéuticas y Bioquímicas, Universidad de San Francisco Xavier de Chuquisaca, Sucre, Bolivia

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ABSTRACT

Two new prenylated flavanones, pazentin A (3′,4′-dihydroxy-6,2′-diprenylpinocembrin, **1**) and pazentin B [4′-hydroxy-2′-methoxy-5′-(1‴, 1⁗-dimethylallyl)-6-prenylpinocembrin, **2**] together with two known ones (**3** and **4**) previously isolated from other *Dalea* species were obtained from the benzene extract of *Dalea pazensis* Rusby roots. The compounds were evaluated *in vitro* for their inhibition on mushroom tyrosinase enzyme and in relation to their effect on melanogenesis in B16 murine melanoma cells, by using a spectrophotometric method. The information obtained could be relevant to the knowledge of the structure-activity relationship for these flavonoids with the aim to explore the rational design for skin-whitening agents.

Dalea L. (Fabaceae) is an exclusively American genus with more than 172 species.[1] Its habitat extends from the central region of Argentina and Chile to the south-western United States.[2] Previously, we reported chemical and biological activity studies of two species of this genus from different geographical regions: *Dalea elegans* with habitat within the Andean region of Argentina and Bolivia, and *Dalea boliviana* whose presence extends from Argentina to southern Peru.[3] From both species we have isolated ten flavonoids, mostly belonging to the prenylated flavanones group,[4–6] with several biological activities, such as antioxidants,[7] toxic effects on isolated rat liver mitochondria and human tumor cells,[7] antimicrobials,[8–11] tyrosinase inhibitors.[5,6] The promising results of phytochemical and bioactivity studies of this genus encouraged us to begin the investigation of an endemic species of Bolivia: *Dalea pazensis* Rusby. This species is a shrub with yellow tap roots and violet flowers growing to about 1.30 m in height in the Andean region between 1000 and 3500 m. From the root benzene extract four compounds were isolated,[12,13] two new prenylated flavanones (**1** and **2**), and the two known ones (**3** and **4**) previously isolated from other *Dalea* species: *D. scandens* var *paucifolia* and *D. elegans*, respectively. Their structures were established by 1D and 2D NMR spectroscopy as well as HRMS analysis. Furthermore, all the compounds were evaluated *in vitro* for their activity on mushroom tyrosinase and B16 murine melanoma cell line melanogenesis. The results obtained led us to establish a structure-activity relationship for both activities of the isolated compounds.

Compound **1** was isolated as a yellow amorphous solid, and its molecular formula was established as $C_{25}H_{28}O_6$ from the analysis of its HRMS and NMR spectroscopic data. The UV absorption maxima at 293 and 330 (sh) nm were indicative of the presence of a flavanone skeleton.[14] ^{1}H and ^{13}C NMR data allowed the complete structure elucidation of **1** (Table 1). The ^{1}H NMR spectrum showed a signal at δ_H 12.48 (1H, s) that corresponds to a proton-bonded to the C-5 hydroxyl group. The presence of one aromatic proton singlet at δ_H 6.02 suggested a trisubstituted A-ring. The B-ring substitution was confirmed by two additional aromatic signals [δ_H 7.11 (1H, d, J = 8.4 Hz), 6.50 (1H, d, J = 8.4 Hz), which were assigned as H-5′ and H-6′, respectively. Furthermore, the spectrum showed signals for two prenyl moieties, which were unambiguously established to be attached at C-6 and C-2′, by the detection of HMBC correlations, as well as two broad singlets at δ_H 8.36 and 9.53 corresponding to 3′ and 4′-OH, respectively. Correlations between H-1″ (δ_H 3.24) belonging to the prenyl group, and H-5′ (δ_H 7.11) with C-3′ (δ_C 153.4) and C-4′ (δ_C 156.2), both linked to hydroxyl group, were observed in B ring. Additionally, H-6′ (δ_H 6.50) is correlated to C-1′ (δ_C 117.0) and C-2′ (δ_C 115.9). Moreover, in the A ring correlations between H-1″ (δ_H 3.24) belonging to the prenyl group

* Corresponding authors at: Farmacognosia, Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, Haya de la torre y Medina Allende, Edificio Ciencias II, X5000HUA Córdoba, Argentina.
E-mail addresses: jcabrera@fcq.unc.edu.ar (J.L. Cabrera), gortega@fcq.unc.edu.ar (M.G. Ortega).

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Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compounds **1**, **2, 3 and 4** [$(CD_3)_2CO$].

Position	**1**		**2**		**3**		**4**	
	δ_C, subst.	δ_H mult. (*J* in Hz)	δ_C, subst.	δ_H mult. (*J* in Hz)	δ_C, subst.	δ_H mult.(*J* in Hz)	δ_C, subst.	δ_H mult.(*J* in Hz)
2	78.7 CH	5.71 dd (2.8, 13.0)	74.4 CH	5.65 dd (3.0,13.0)	74.4 CH	5.64 dd (2.9, 12.9)	74.8 CH	5.68 dd (3.0,12.8)
3a	41.6 CH_2	2.70 dd (2.8, 17.3)	42.0 CH_2	2.64 dd (3.0,17.2)	42.0 CH_2	2.71 dd (3.0, 17.1)	40.3 CH_2	2.75 dd (3.0,17.1)
3b		3.20 dd (13.0, 17.3)		3.17 dd (13.0,17.1)		3.06 dd (12.8,17.1)		3.12 dd (12.8, 17.1)
4	196.9 qC*	–	197.0 qC	–	197.1 qC	–	196.7 qC	–
5	162.6 qC	–	161.5 qC	–	162.1 qC	–	162.3 qC	–
6	108.1 qC	–	108.0 qC	–	95.4 CH	6.02 s	95.5 CH	6.03 s
7	163.5 qC	–	163.8 qC	–	163.9 qC	–	163.7 qC	–
8	94.3 CH	6.02 s	94.4 CH	6.02 s	107.4 qC	–	107.5 qC	–
9	161.4 qC	–	161.6 qC	–	160.6 qC	–	160.7 qC	–
10	102.2 qC	–	102.2 qC	–	102.4 qC	–	102.4 qC	–
1′	117.0 qC	–	117.3 qC	–	117.6 qC	–	116.1 qC	–
2′	115.9 qC	–	156.0 qC	–	155.8 qC	–	153.4 qC	–
3′	153.4 qC	–	100.2 CH	6.54 s	100.1 CH	6.55 s	103.7 CH	6.48 s
4′	156.2 qC	–	156.6 qC	–	156.5 qC	–	156.3 qC	–
5′	124.7 CH	7.11d (8.4)	125.8 qC	–	125.7 qC	–	124.9 qC	–
6′	107.2 CH	6.50 d (8.4)	126.1 CH	7.38 s	125.6 CH	7.46 s	125.3 CH	7.40 s
1″	20.5 CH_2	3.24 d (7.0)	20.7 CH_2	3.24 d (7.2)	21.4 CH_2	3.22 d (7.5)	21.7 CH_2	3.26 d (7.3)
2″	122.7 CH	5.23 m (7.0)	122.8 CH	5.2 t (7.2)	122.9 CH	5.23 t (7.5)	122.9 CH	5.25 t (7.3)
3″	130.2 qC	–	130.3 qC	–	130.3 qC	–	130.5 qC	–
4″	24.8 CH_3	1.63 s	25.0 CH_3	1.63 s	25.0 CH_3	1.61 s	25.2 CH_3	1.62 s
5″	16.8 CH_3	1.74 s	16.9 CH_3	1.74 s	17.0 CH_3	1.61 s	17.1 CH_3	1.61 s
1‴	20.5 CH_2	3.44 d (7.0)	39.9 qC		39.9 qC		39.9 qC	
2‴	122.7 CH	5.23 m (7.0)	147.9 CH	6.27 dd (10.7,17.6)	148.0 CH	6.28 dd (10.7,17.6)	148.2 CH	6.2 (17.8,10.5)
3‴	130.9 qC		109.7 CH_2	5.00 d (17.6)	109.7 CH_2	5.04 d (17.6)	109.7 CH_2	5.00 d (17.8)
3‴				4.96 d (10.7)		4.96 d (10.7)		4.95 d (10.5)
4‴	24.8 CH_3	1.64 s	26.6 CH_3	1.28 s	26.6 CH_3	1.48 s	26.9 CH_3	1.47 s
5‴	16.8 CH_3	1.75 s	26.5 CH_3	1.46 s	26.6 CH_3	1.48 s	26.9 CH_3	1.47 s
5-OH		12.48 s		12.49 s		12.15 s		12.17 s
7-OH		7.38 s		8.24 s		8.17 s		
2′-OMe			55.0 CH_3	3.77 s	55.0 CH_3	3.79 s		
3′-OH		8.36 s						
4′-OH		9.53 s				9.51 s		

* qC: quaternary carbon.

with C-7 (δ_C 163.5) and C-6 (δ_C 108.1) were observed. In addition, H-8 (δ_H 6.02) is correlated with C-7 (δ_C 163.5) and C-9 (δ_C 161.4) (Fig. 1). Other correlations are shown in the Fig. 1.

The absolute configuration at C-2 was determined to be *S* according to the positive Cotton effect at 330 nm and the negative Cotton effect at 293 nm in the CD spectrum and the levorotatory optical rotation.[15] So, the structure of **1** was established as (−)-(2*S*)-3′,4′-dihydroxy-6,2′-diprenylpinocembrin (pazentin A).

Compound **2** was obtained as a pale yellow, amorphous solid. Its molecular formula was determined as $C_{26}H_{30}O_6$ consistent with the analysis of its HRMS and NMR spectroscopic information. The UV, IR, and ^{1}H and ^{13}C NMR spectra (Table 1) were consistent with a flavanone skeleton. The UV absorption maxima was at 291 and 330 (sh) nm. A characteristic flavanone ABX spin system is observed in the ^{1}H NMR spectrum of **2** (Table 1): [δ_H 5.65, *J* = 13.0, 3.0 Hz, H-2), 3.17 (*J* = 17.1, 13.0 Hz, H-3ax), and 2.64 (*J* = 17.2, 3.0 Hz, H-3 eq)]. The signal at δ_H 12.49 was assigned at the hydrogen-bonded at hydroxy group attached at C-5. The presence of singlet at δ_H 6.02 (1H, s, H-8) was indicative of a trisubstituted aromatic A-ring. The aromatic ^{1}H NMR signals of the B-ring δ_H 6.54 (1H, s) and 7.38 (1H, s) were assigned to H-3′ and H-6′ respectively.

In addition, the spectrum showed signals for prenyl and α, α-dimethylallyl moieties, which were located at C-6 and C-5′, respectively, based on HMBC correlations.

HMBC correlations between H-3′ (δ_H 6.54) with C-4′ (δ_C 156.6) linked to hydroxyl group, and C-2′ (δ_C 156.0) linked to methoxy group were observed in B ring. Also, in this ring it was observed the correlation between H-6′ (δ_H 7.38) with C-1‴ (δ_C 39.9) belonging to the dimethylallyl group and C-2 (δ_C 74.4). In A ring, the same correlations of the compound **1** were observed so, the compound **2** presents the same substitution pattern in this ring. Other considered correlations were shown in the Fig. 1. The levorotatory optical rotation of **2** in addition to the positive Cotton effect at 330 nm and negative Cotton effect at 291 nm in its CD spectrum indicated a 2*S* absolute configuration.[15] Compound **2** was therefore identified as (−)-(2*S*)-4′-hydroxy-2′-methoxy-5′-(1‴, 1‴-dimethylallyl)-6-prenylpinocembrin (pazentin B).

The two known compounds **3** and **4** were identified by means of spectroscopic methods including HRMS, 1D and 2D NMR analysis and also by comparing experimental data with those previously described in the literature as 4′-hydroxy-2′-methoxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (**3**)[16,17] and 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (**4**).[6,18]

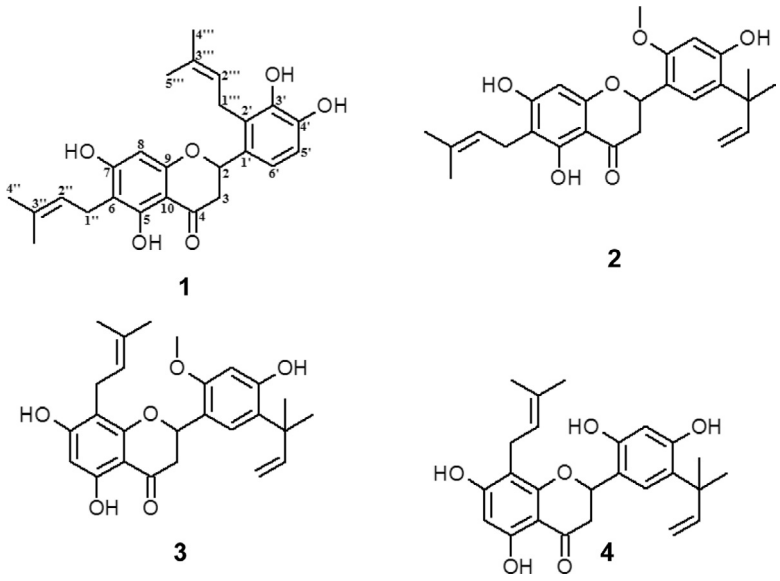

Fig. 1. Structures of compounds **1, 2, 3** and **4.**

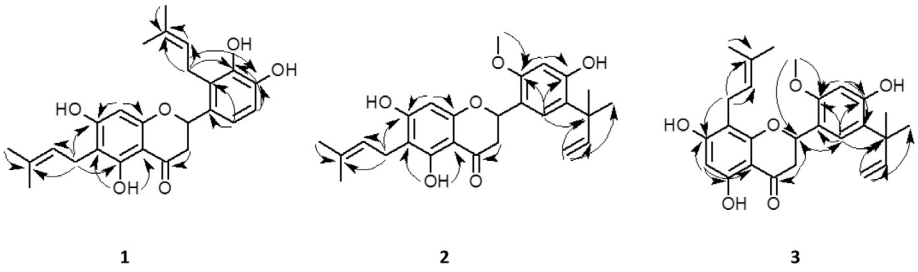

Fig. 2. Key HMBC correlations of compounds **1, 2, 3.**

Table 2
Inhibitory activity on mushroom tyrosinase for compounds **1–4**.

Compound	Inhibition at 10 μM (%)	Inhibition at 100 μM (%)	IC_{50} (μM)
1	2.84 ± 0.4	9.4 ± 0.3	n.d
2	0	0	n.d
3	21.5 ± 1.8	63.8 ± 4.4	49.80 ± 0.09
4	90.5 ± 5.0	95.0 ± 0.6	2.32 ± 0.01*

Positive control Kojic acid IC_{50} = 4.93 ± 0.01 μM. Media ± SD of at least 3 determinations.
n.d not determined.
* Peralta et al., 2014.[6]

The enzymatic oxidation of l-tyrosine to melanin synthesis is of considerable importance since melanin has many functions and alterations in melanin synthesis occur in many disease states. Tyrosinase inhibitors perform a downregulation of melanin formation, therefore, they have become increasingly important in medicinal and cosmetic products.[19]

Different concentrations of compounds **1–3** were tested in order to evaluate the inhibitory activity against mushroom tyrosinase enzyme using l-tyrosine as substrate (Table 2).[20] A concentration-response effect was studied on the flavonoids that showed tyrosinase inhibition by more than 50% at 100 μM and were compared with the activity of compound **4**, previously reported by our group and kojic acid, the reference inhibitor. All the experimental conditions were reproduced (Fig. 2).

Compound **3** showed a significant inhibition on tyrosinase enzyme at 100 μM (64 ± 4%), while **1** at the same concentration, demonstrated very low inhibition (9.4 ± 0.3%), and **2** did not inhibit the activity of tyrosinase (Table 2). The IC_{50} values for **3** and the reference inhibitor Kojic acid were estimated using nonlinear fitting of concentration–response data.[21] The IC_{50} value for **3** was 49.80 ± 0.09 μM and for Kojic acid was 4.93 ± 0.01 μM. (Table 2 and Fig. 3).

The results obtained highlight some structural requirements[22,23] related to the anti-tyrosinase activity previously observed in prenylflavonoids, as the presence of the 4-substituted phloroglucinol moiety when it is part of the A ring.[5] Compound **3** shows this structural condition and exhibited moderate activity as tyrosinase inhibitor. The activity observed for compound **3** was lower than compound **4** (Table 2) which exhibits a strong activity as tyrosinase inhibitor, due to the presence of resorcinol moiety in B ring[22,23], and the 4-substituted phloroglucinol moiety in A-ring in this compound.[5,6]

Compounds **1** and **2**, having a 6-substituted phloroglucinol moiety in the A ring of their structures, showed very low or inexistent activity, respectively. This fact could be related to the absence of the 4-substituted phloroglucinol moiety in the A ring and the 4-substituted resorcinol moiety in B-ring, both structural conditions already demonstrated to be important in order to observe anti-tyrosinase activity.[22,23] However, we consider that more studies about these structural factors are necessary to reveal inhibitory activity on tyrosinase of these compounds.

B16 murine melanoma tyrosinase has a high homology with human melanoma tyrosinase, so that cell line offers a valid model for the evaluation of inhibitory or stimulatory activity of tyrosinase, approaching the behavior that would occur in humans.

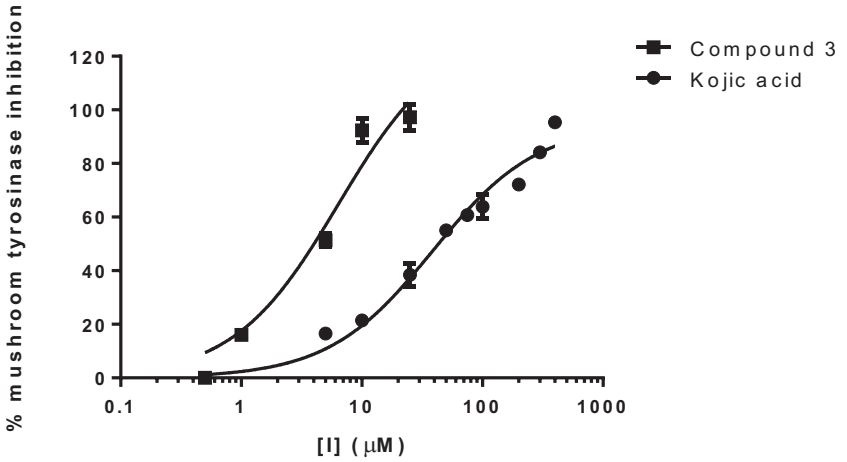

Fig. 3. Concentration-dependent inhibition of mushroom tyrosinase activity by compound **3** and positive control Kojic acid (N = 3).

The cellular viability in the presence of these natural compounds was first determined, and subsequently the effect on tyrosinase and melanin biosynthesis on B16 murine melanoma cells was tested.[24,25] After 24 h culture of melanoma cells with the compounds **1**–**4** at different concentrations, the cell viability was determined using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay,[26] and the maxima no cytotoxic concentration (MNCC), was estimated.[21] The descending order of their MNCC was **2** (50.0 ± 8.0 μM), **4** (10.0 ± 1.0 μM), **3** (5.0 ± 0.5 μM), and **1** (1.0 ± 0.3 μM). Kojic acid proved to be cytotoxic at (5000.0 ± 10.0 μM). All the compounds were cytotoxic in a concentration-dependent manner.

Later, the inhibition of melanin production in B16 cells was examined.[27] The concentration of the flavonoids and the reference inhibitors that induced the 50% inhibition of melanin production can be sort as continue: **1** (0.75 ± 0.2 μM) > **4** (1.0 ± 0.4 μM) > **2** (5.0 ± 1.0 μM) = **3** (5.0 ± 1.8 μM) > Kojic Acid (2000.0 ± 5.0 μM).

In the above conditions, compound **1** was shown to be two thousand and seven hundred fold more active than the reference inhibitor, while compound **4** proved to be approximately two thousand fold more active. Compounds **2** and **3** were four hundred fold more active than kojic acid. These results showed the relevant activity on the inhibition of the melanin biosynthesis in B16 melanoma cells by these compounds, which present in their structures two prenyl groups and hydroxyl groups on both A and B rings. These findings are according with those informed by Arung et al., (2007),[28] who suggested that both prenyl and OH groups, as well as the type of substitution pattern in flavones, are crucial for the inhibition of melanin production in B16 melanoma cells, and their potency was related to the number of isoprenoid moiety substitutions.

Additionally, in order to investigate the possible mechanism that involves the diminished melanin content, we evaluated the inhibitory effect of different concentrations of **1**–**4**, on tyrosinase intracellular activity in murine B16 melanoma cells after 24 h of incubation.[29] Table 3 shows the intracellular tyrosinase inhibition of flavanones **1**–**4** and the reference inhibitor, at their MNCC concentration. Compound **4** exhibited inhibition activity on intracellular tyrosinase.

As can be shown, flavanone **4** and kojic acid produced a tyrosinase inhibition of 34.2 ± 0.8% and 45.4 ± 0.1%, respectively at their MNCC. In order to compare the activity between **4** and kojic acid, in Table 4 we indicate several concentrations at the same % of tyrosinase inhibition for these compounds. At concentrations 0.05 μM and 5 μM compound **4** exerted 20.6[a]% and 31.8[b]% of inhibition of intracellular tyrosinase, respectively while kojic acid produced the same inhibition (21.6[a]% and 28.6[b]; [a,b]$p > 0.05$, the values are not significantly different statistically) at 5 μM and 1000 μM, respectively.

Table 3
Inhibition of intracellular tyrosinase of murine B16 melanoma cells for compounds **1**–**4**.

Compound	MNCC (μM)	% intracellular tyrosinase inhibition
1	1.0 ± 0.3	0
2	50.0 ± 8.0	0
3	5.0 ± 0.5	0*
4	10.0 ± 1.0	34.2 ± 0.2
Kojic acid	5000.0 ± 10.0	45.4 ± 0.1

* Compound **3** showed a stimulation of 18.9 ± 0.6%. Media ± SD of at least 9 determinations.

Table 4
Inhibition of intracellular tyrosinase in murine B16 melanoma cells for compound **4** and kojic acid.

Compound	Concentration (μM)	% intracellular tyrosinase inhibition
4	0.05	20.6 ± 2.0[a]
Kojic acid	500	21.6 ± 1.2[a]
4	5	31.8 ± 4.0[b]
Kojic acid	1000	28.6 ± 2.8[b]

Media ± SD of at least 9 determinations.
[a,b]$p > 0.05$, the values are not significantly different statistically.

This fact indicates that **4** is more active than kojic acid and the tyrosinase inhibition could be related with the decrease observed in melanin production in B16 melanoma cells in presence of **4**.

On the other hand, it was observed that compounds **1** and **2** were inactive on intracellular tyrosinase and compound **3**, presented a slight stimulation (Table 3). Interestingly, compounds **1**, **2** and **3** diminished extracellular melanin, but in an independent tyrosinase manner. Other stages related to the melanin biosynthesis, such as protein or/and transcriptional expression of the enzymes that regulate its biosynthesis (Tyrp1 and Tyrp2) or signaling pathways could be implicated in the action of compounds **1**–**3**. Further studies should be carried out in order to establish the mechanisms through which these compounds would exert their action.

In conclusion, this is the first chemical and biological activity study of *D. pazensis*. Four compounds were isolated: two new prenylated flavanones: pazentin A (**1**) and pazentin B (**2**) together with the known flavanones (**3** and **4**). The mushroom tyrosinase inhibition activities of compounds **1**–**3** were evaluated. Compound **3** showed a moderate inhibition on tyrosinase activity while **1** and **2** were inactive. Recently, we informed that **4** showed a strong

inhibition on tyrosinase activity two times more active than Kojic acid.[6]

Furthermore, we have provided data concerning to the activity of these prenylated flavanones on melanogenesis on murine B16 melanoma cells. With regard to decreased extracellular melanin, it was observed that all natural compounds were significantly more active than the reference inhibitor kojic acid. In terms of inhibition activity on intracellular tyrosinase, we observed that the most active was compound **4** compared to the reference inhibitor; compounds **1** and **2** were inactive and compound **3** showed a slight stimulation. Although these compounds displayed cytotoxicity at concentrations greater than their MNCC, could be necessary other studies in order to demonstrate safety in their clinical use as whitening agents in cosmetic products.

The results herein provide additional information related to the structure-activity relationship for flavonoids showing inhibition of mushroom tyrosinase and melanin biosynthesis in an *in vitro* system, in order to explore the rational design for skin-whitening agents.

(−)-(2S)-3′,4′-Dihydroxy-6,2′-diprenylpinocembrin (***1***) was obtained as a yellow amorphous solid; $[\alpha]_D^{25}$ −17 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 293 (3.69), 330 (sh) (3.13) nm; CD (c 0.004, MeOH) $[\theta]_{293}$ −0.46, $[\theta]_{330}$ +0.05; IR (KBr) ν_{max} 3644, (OH), 2921, 1644 (C=O), 1601, 1455 (C=C) 2852 (OCH_3), cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6, 100 MHz), see Table 1. HRMS *m/z* 447.1775 $[M+Na]^+$ (calcd. for $C_{25}H_{27}O_6$, 447.1778).

(−)-(2S)-4′-Hydroxy-2′-methoxy-5′-(1‴,1‴-dimethylallyl)-6-prenylpinocembrin (***2***) was obtained as a pale yellow amorphous solid; $[\alpha]_D^{25}$ -23.2 (c 0.095,MeOH); UV (MeOH) λ_{max} (log ε) 291 (4.08), 330 (sh) (3.38) nm; CD (c 0.002, MeOH) $[\theta]_{293}$ −0.18, $[\theta]_{330}$ +0.03; IR (KBr) *v*max 3454 (OH), 2925, 1635 (C=O), 1604, 1453 (C=C), 2854 (OCH_3) cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6,100 MHz), see Table 1. HRMS *m/z* 461.1944 $[M+Na]^+$ (calcd. for $C_{25}H_{27}O_6$, 461.1935).

(−)-(2S)-4′-Hydroxy-2′-methoxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (***3***) was obtained as a white needles; m.p 80–82 °C; $[\alpha]_D^{25}$ -16.5 (c 0.075,MeOH); UV (MeOH) λ_{max} (log ε) 290 (4.72), 330 (sh) (4.04) nm; CD (*c* 0.001, MeOH) $[\theta]_{290}$ −0.41, $[\theta]_{330}$ +0.56; 3733, (OH), 2929, 1635 (C=O), 1504, 1445 (C=C), 2858 (OCH_3), cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) and ^{13}C NMR (acetone-d_6,100 MHz), were coincident with those previously reported;[14,15] HRMS *m/z* 461.1922 $[M+Na]^+$ (calcd. for $C_{25}H_{27}O_6$, 461.1935).

Conflict of interest

The authors declare that they have no conflict of interest.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.02.058.

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12. General experimental Procedures: Optical rotations were measured on a Jasco P-1010 polarimeter (Tokyo, Japan). UV spectra were recorded on a Cary Win UV–VIS spectrophotometer, Varian Inc, Agilent Technologies (Santa Clara, USA). CD spectra were obtained with a Jasco J-810 spectropolarimeter (Tokyo, Japan).1H and 13C NMR spectra were acquired in Acetone-d6 on a Brüker Avance II 400 (400 MHz for 1H and 100 MHz for 13C) spectrometer (Rheinstetten, Germany) using TMS as internal standard. HRMS were obtained in a QTOF mass spectrometer (micrOTOF-Q11 Series, Brüker) equipped with an electrospray ionization (ESI) interface (Bruker Daltonics, USA). FTIR spectra were recorded as KBr disks, using a Nicolet 5SXC spectrophotometer (Madison, Wi, USA). Column chromatography was performed on silica gel Merck. TLC was carried out using 20 cm x 20 cm plates with 0.5 mm layer of silica gel GF254 (Merck). Spots were visualized by UV illumination (254 nm).
13. Plant material: Roots of *D. pazensis* (Fabaceae) were collected in March 2013, in Yotala, locality close to Sucre city (19°08′53″ S 65°15′48″ W at 2543 m above sea level). Plant material was identified by specialized personnel belonging at the Herbario del Sur de Bolivia (HSB) (Portal E. & López C.D.) and a voucher specimen was deposit as 961A. Extraction and isolation: Roots of *Dalea pazensis* (35 g) were dried at room temperature, powdered and extracted with benzene using a soxhlet extractor. The solvent was subsequently removed to yield crude extract (0.51 g). This extract was subjected to column chromatography (CC) using silica gel as stationary phase and eluted with n-hexane/ethyl acetate (100:0 to 0:100) giving five fractions that were combined on basis of their TLC profiles on n-hexane/ethyl acetate (70:30). Fraction 2 (Rf = 0.40, 23.1 mg) was purified over TLC with silica gel using chloroform/ethanol (95:5) as mobile phase to yield the compound **4** (Rf = 0.6, 4 mg). Fraction 4 (Rf = 0.38, 131 mg) was chromatographed using preparative TLC with benzene/ethyl acetate (95:5) giving the compounds: **1** (Rf = 0.58, 4 mg), **2** (Rf = 0.55,6 mg) and **3** (Rf = 0.68,16 mg).
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20. Mushroom tyrosinase inhibition assay: The assay was performed as previously described by Peralta et al., 2014.[6] Briefly, the assay medium consisting of 0.25 mL of mushroom tyrosinase solution (200 U/mL), and 0.75 mL of the control solution [Na_3PO_4 buffer (0.1 M, pH 6.8)] or the sample solution [prepared with each flavonoid **1**–**4** dissolved in DMSO (final concentration 0.1% v/v) and subsequently diluted to the appropriate concentrations with the above buffer] were mixed and preincubated at 25 °C for 10 min. Then, 0.50 mL of L-tyrosine solution (1.7 mM, Sigma) was added. The absorbance was measured at 475 nm after 20 min of incubation. Kojic acid was used as positive control agent. Each treatment was replicated three times. The percent inhibition of tyrosinase activity was calculated as follows: % inhibition = $[(Abs_{control} \times Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control solution and Abs_{sample} is the absorbance of the sample solution.
21. Calculations and statistics: All assays were independently performed in triplicate, and results were expressed as media ± SD of three separate experiments. The IC_{50} values were estimated using the *GraphPad Prism 6* software on a compatible computer.
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24. Cell culture: B16 murine melanoma cell line was maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% streptomycin/penicillin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.
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26. MTT assay on B16 melanoma cells: B16 melanoma cells (1×10^5) were cultured in 96 well-plates in the same condition described above and incubated for 24 h. After that, cells were incubated with 100 μl the control solution (fresh media with DMSO 0.5% v/v) or 100 μl the test solution [prepared with each stock solution of flavonoid **1**–**4** (100 mM) dissolved in DMSO (final concentration 0.5% v/v) at several concentrations (0.01–500 μM, obtaining by diluting the stock solution in DMEM) for 24 h. The treatment was replicated for triplicate. After incubation, cells were rinsed with PBS and 100 μl of MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide in PBS (5 mg/mL)] was added to each well. The plates were incubated in a humidified atmosphere of 5% of CO_2 at 37°C for 30 min. After that, MTT reagent was removed, the cells were washed with PBS and then, 100 μl of isopropyl alcohol was added into the plate and was stirred for 15 minutes. The absorbance was measured at 595 nm in a microplate reader (BioTek ELx800). The concentration MNCC (concentration that the cells are maintained at 90% of viability) was determined.
27. Measurement of melanin content: The assay was performed as previously described by Yamauchi et al., 2014.[25] B16 cells were seeded in 24-well plate at a density of 1×10^5 cells per well and incubated overnight. The cells were treated with each compound of different concentrations of for 24 h. The culture medium, containing the extracellular melanin, was removed and the melanin content was measured at 510 nm using a microplate reader (BioTek ELx800). The culture medium of untreated cells was considered as 100% of production of melanin.
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29. Intracellular tyrosinase inhibition assay: Tyrosinase enzyme activity was estimated by measuring the formation of a stable dark pink adduct between 3-methyl-2-benzothiazolinone (MBTH) and dopaquinone, as described previously by W inder et al., 1991[30] with slight modification. Briefly, cells (1×10^5) were treated with different concentrations of each compound for 24 h, the cells were then solubilized in phosphate buffer (0.1 M; pH 6.8) containing 0.1% Triton ×100. Lysate was clarified by centrifugation at 10,000g for 10 min at 4 °C; 60 μL of supernatant was mixed with 40 μL of L-DOPA (10 mM) and 100 μL of MBTH (5 mM) and incubated for 20 min at 37 °C. The absorbance was followed spectrophotometrically at 490 nm, every minute for 10 min, since the substrate added to the reaction mixture. The % inhibitions of intracellular tyrosinase were calculated as the mushroom tyrosinase assay. The results were analyzed by unidirectional analysis of variance (ANOVA) followed by the *Tukey* test for multiple comparisons using *GraphPad InstStat* software.
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