Phytochemistry Letters 10 (2014) 260–267

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Flavanoids from *Dalea elegans*: Chemical reassignment and determination of kinetics parameters related to their anti-tyrosinase activity

Mariana Andrea Peralta [1], María Daniela Santi [1], Alicia Mariel Agnese, José Luis Cabrera, María Gabriela Ortega [*]

Farmacognosia, Instituto Multidisciplinario de Biología Vegetal (IMBIV), CONICET and Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, X5000HUA Córdoba, Argentina

ARTICLE INFO

Article history:
Received 8 October 2014
Accepted 9 October 2014
Available online 23 October 2014

Keywords:
Dalea elegans
Fabaceae
Prenyalted flavanones
C-methyl flavonoids
Chemical reassignment
Antityrosinase activity

ABSTRACT

In the first chemical investigation developed on the species *Dalea elegans* twenty years ago, the occurrence of two prenylated derivatives of pinocembrin was reported: 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-6-prenylpinocembrin (6PP) given as a new structure in this family of compounds, and another derivative of already known structure, 6-prenylpinocembrin (6P). In the present paper, their structures were again analyzed by using spectroscopic techniques, especially 2D NMR. Based on the evidence obtained it is proposed the reassignment of both flavanones as: 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP) for the first and 8-prenylpinocembrin (8P) for the last. Additionally, triangularin, demethoxymatteucinol, comptonin and 7-hydroxy-5-methoxy-6,8-dimethylflavanone were isolated from aerial parts of *D. elegans* and informed by the first time for this species. All of these compounds were evaluated in vitro in relation to the antityrosinase effect by using a spectrophotometric method. Compound 8PP (IC_{50} 2.32 ± 0.01 μM) exhibited the most potency and was two times more active than Kojic acid (IC_{50} 4.93 ± 0.01 μM) used as a positive control. Triangularin also has shown an important inhibitory activity. Kinetic studies were performed for both compounds. Hence, new tyrosinase inhibitors with potential applications in pharmacy and cosmetic industry are presented.

1. Introduction

Tyrosinase, a copper-containing monooxygenase, is involved in melanin biosynthesis and catalyzes two different reactions in the melanin synthesis: formation of 3,4-dihydroxyphenylalanine (DOPA) by hydroxylation of L-tyrosine (monophenolase activity) and the subsequent dopaquinone formation (diphenolase activity). The spontaneous polymerization of dopaquinone conduces to form several brown or black pigments such as melanin, the present pigment in mammals and responsible for the color of skin, eyes and hair pigmentation. Melanin hyperpigmentation affects the skin producing age spots, melasma and malignant melanoma (Briganti et al., 2003; Seo et al., 2003). For these reasons, tyrosinase inhibitors are an alternative for these undesirable conditions.

Dalea L. (Fabaceae) is an exclusively American genus with more than two hundred and fifty species (Tropicos.org: http://www.tropicos.org/Name/40021469) distributed in arid regions from the southwest of United States to the central regions of Argentina and Chile (Burkart, 1952). Only *D. elegans* Gillies ex Hook & Arn. has its habitat in the province of Córdoba (central region of Argentina), among the four species mentioned for our country (Zuloaga and Morrone, 1999). In the 1990s, from the aerial parts and roots of this species, two prenylated flavanones derived from pinocembrin were isolated. Their structures were then reported as 2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-6-prenylpinocembrin (6PP), given as a new structure in this family of compounds (Cafaratti et al., 1994) and 6-prenylpinocembrin (6P) previously isolated from *Darris rariflora* (Braz Filho et al., 1975) (Fig. 1). These prenylated derivatives of pinocembrin have proven to be highly active in different biological activity assays (Ortega et al., 1996; Pérez et al., 2003; Elingold et al., 2008; Peralta et al., 2012). Another species of *Dalea* was studied in our group, *D. boliviana*, and showed the presence of new prenylated flavanones active as tyrosinase inhibitors (Peralta et al., 2011).

* Corresponding author at: IMBIV (CONICET) and Departamento de Farmacia, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre, Ciudad Universitaria, X5000HUA Córdoba, Argentina. Tel.: +54 0351 5353865.
E-mail address: gortega@fcq.unc.edu.ar (M.G. Ortega).
[1] These authors contributed equally to this publication.

http://dx.doi.org/10.1016/j.phytol.2014.10.012
1874-3900/

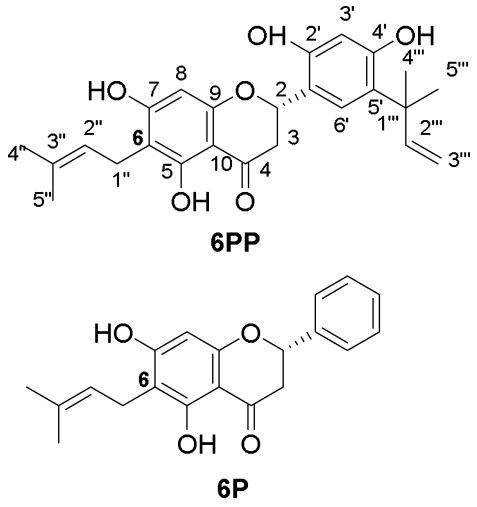

Fig. 1. Structures of compounds **6PP** and **6P**.

In this opportunity, the present investigation focuses in deepening the phytochemical study of *D. elegans*, and their evaluation as potential tyrosinase inhibitors.

Therefore, three C-methyl flavanones and one methyl chalcone besides the two prenylated flavanones above mentioned (6PP and 6P), were obtained. Their structures were established by 1D and 2D NMR spectroscopy as well as HRMS analysis. The analysis of 2D NMR spectroscopic data for 6PP and 6P gave us new chemical information specially for the structural analysis about A ring, in relation to some aspects referrals to the prenyl group substitution. Therefore, in the present paper the reassignment of both structures is proposed.

The antityrosinase activity of the six flavonoids obtained from *D. elegans* was evaluated, besides inquiring into the kinetic mechanism and the inhibition constant for the active flavonoids.

2. Results and discussion

6PP and 6P (Fig. 1) were obtained according to Cafaratti et al. (1994) and their spectroscopic data of UV-V, MS, and NMR ^{1}H for both compounds were coincident to those given by literature (Braz Filho et al., 1975; Cafaratti et al., 1994) and these compounds were compared with authentic samples of 6PP and 6P. In contrary, some uncertainty arose from the ^{13}C NMR data analysis in relation to the substitution on the A ring for both compounds. The doubt arises because the characteristic signal of the only methine of A ring at δC 95.5 ppm is not conclusive for a certain assignment, in view that it is in a resonance range that involves both, the free aromatic C-6 as well as the C-8 (Mabry et al., 1970). For this reason, for an unequivocal determination of the substitution pattern of A ring, 2D NMR experiments were developed. HMBC spectrum of 8PP (**1**) (Fig. 2) allows us to see the relationship between the signals for the proton of the hydroxyl at C-5 (OH-C5, δ 12.17 ppm) and the carbon at C-6 (δ 95.5 ppm). On the other hand, HSQC spectrum shows that the signal that corresponds to that carbon is indicative of the methine presence, so, on C-6 there is a proton instead a prenyl group. Other important correlations in the HMBC spectrum are shown in Fig. 3.

On the other hand, by the analysis of the carbon chemical shifts in B ring of the structure informed by Cafaratti et al. (1994) as 6PP, some mistakes were detected and corrected. These corrections correspond to the C-1′, C-5′, C-6′, C-2‴, C-3‴, C-4‴ and C-5‴ and are indicated in Table 1 for 8PP. Additionally, the absolute configuration at C-2 was determined to be *S* according to the positive Cotton effect at 334 nm and the negative Cotton effect at 294 nm in the CD spectrum (Slade et al., 2005). This new information allows reassign 6PP as (2*S*)-2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP, **1**) (Fig. 2). This structure has been reported in other species of *Dalea* genus (Nanayakkara et al., 2002; Belofsky et al., 2014).

In a similar way to the study for compound **1**, the spectral data of HMBC (Fig. 3) and HSQC for 8P (**2**) were analyzed. The results obtained have shown that the substitution of the prenyl group on

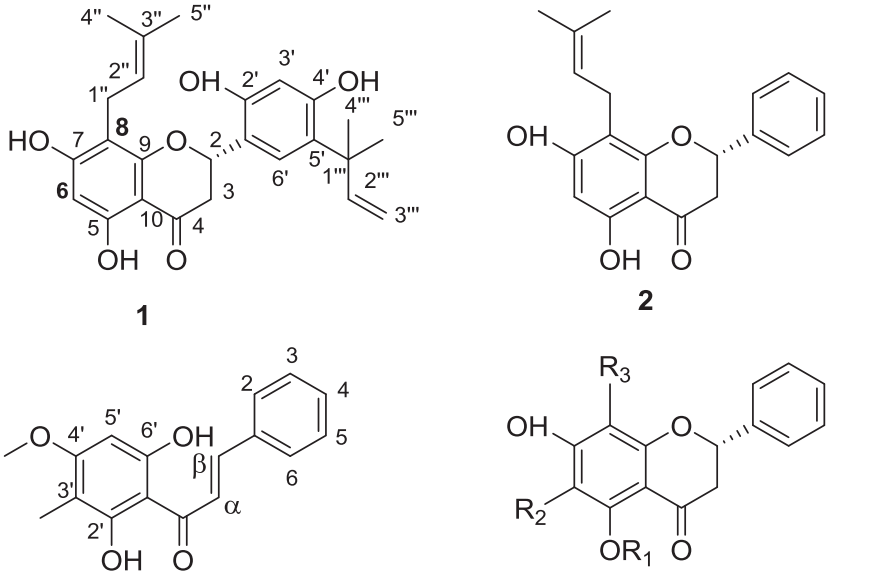

Fig. 2. Structures of compounds **1**–**6**.

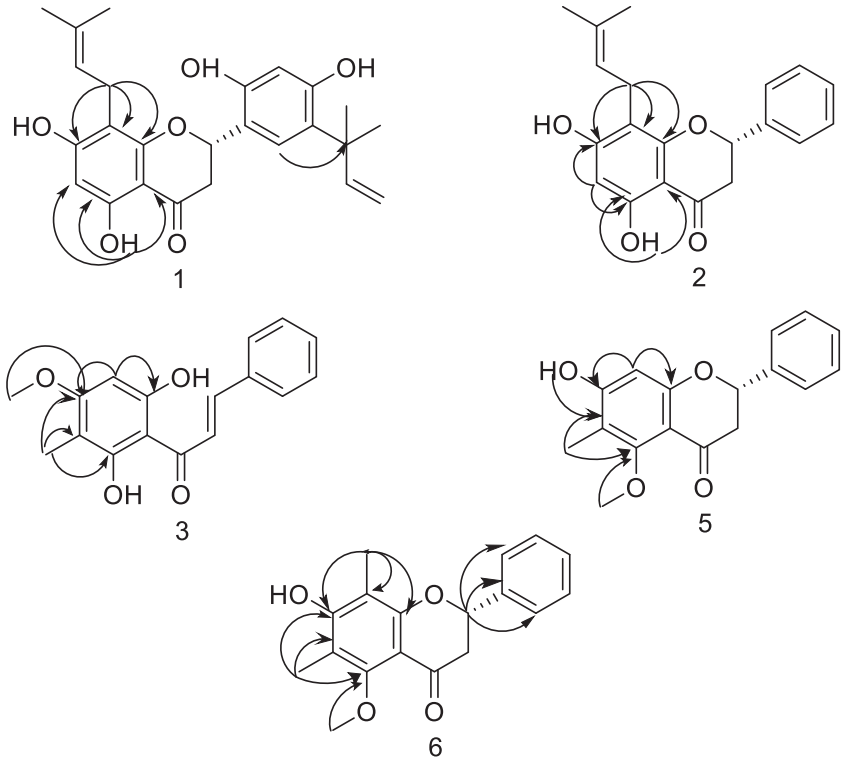

Fig. 3. Key HMBC correlations of compounds **1**–**3** and **5**–**6**.

the A ring is at C-8 instead of C-6 as it was reported for 6P (Cafaratti et al., 1994). Besides, according to the 2D NMR data analysis of the compound **2**, some differences on the assignments of the aromatic B ring carbons, the oxygenated carbons C-5, C-7 and C-9 as well as the prenyl moiety (C-1″, C-3″ and C-4″), with respect to those previously reported by Mitscher et al. (1983) were observed. The changes in assignments of these carbon signals for compound **2** are presented in Table 1 and key HMBC correlations are shown in Fig. 3.

The absolute configuration of 8P at C-2 was determined to be *S* according to the positive Cotton effect at 334 nm and the negative Cotton effect at 294 nm in the CD spectrum (Slade et al., 2005). Thus, the reassignment as (2*S*)-8-prenylpinocembrin (8P, **2**) (Fig. 2) is proposed.

In order to search other bioactive metabolites from this plant, fractionation of benzene extract of aerial parts of *D. elegans* led to the isolation of another four known compounds. They 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 2′,6′-dihydroxy-4′-methoxy-3′-methylchalcone (triangularin, **3**) (Mustafa et al., 2005), 6,8-dimethylpinocembrin (demethoxymatteucinol, **4**) (Basnet et al., 1993); 7-hydroxy-5-methoxy-6-methylflavanone (comptonin, **5**) (Kenneth et al., 1987); 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**6**) (Amor et al., 2005). Based on 2D NMR data analysis of the last compound, we observed some differences on the assignments of the oxygenated carbons C-5, C-7 and C-9 as well as the aromatic carbons of B ring, with respect to those previously reported. In fact, the HMBC experiment showed correlation between the signal of the methoxyl group protons (δ 3.81 ppm) and the quaternary carbon at δ 157.7 ppm. Also, methyl protons at C-6 (δ 2.14 ppm) correlate with two oxygenated carbons signals at δ 157.7 and δ 158.8 ppm allowing us to assign these signals as C-5 and C-7 respectively. Besides, other differences in assignments of signals that correspond to aromatic carbons of the B ring were observed. The strong correlations observed between the methine proton at C-2 and aromatic non-substituted carbons at δ 125.9 ppm allowed us to assign these signals as C-2′ and C-6′ methines. The other aromatic carbons of the B ring were assigned according to well known chemical shifts for non-substituted B ring flavanones (Harbone and Mabry, 1982). The mentioned changes in assignments of carbon signals for compound **6** are presented in Table 3 and key HMBC correlations are shown in Fig. 3. On the other hand, there are not previous reports about 2D NMR data for compounds compound **3** and **5**, so the most important HMBC correlations for these compounds are given for the first time (Fig. 3). As far as we know this is the first report of these C-methylated flavanones in *Dalea* genus.

Tyrosinase acts as a rate-limiting enzyme in melanin biosynthesis. Therefore, tyrosinase inhibitors are important substances to treat abnormal pigmentation disorders, such as melasma, age spots and sites of actinic damage, arising from accumulation of an excessive level of epidermal pigmentation. Compounds **1**–**6** were tested for inhibitory activity against the tyrosinase enzyme using L-tyrosine as substrate. Different concentrations of flavonoids were evaluated as seen in Table 4 and the flavonoids that showed tyrosinase inhibition by more than 50% at 100 μM, a dose-response effect were studied. 8PP (**1**) and triangularin (**3**) at 100 μM showed an important inhibition on tyrosinase enzyme (95.0 ± 0.6% and 98.6 ± 0.6%, respectively), while the activity of 8P (**2**) and demethoxymatteucinol (**4**) were moderated (73.7 ± 0.6% and 50.0 ± 0.6%, respectively). Comptonin (**5**) and 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**6**) demonstrated very low inhibition (22.7 ± 0.6% and 6.97 ± 0.6%, respectively). The IC_{50} values for **1**–**4** and the reference inhibitor Kojic acid were estimated using nonlinear fitting of concentration–response data. Among the compounds tested, compound **1** showed strong tyrosinase inhibition activity (IC_{50} 2.32 ± 0.01 μM), which is approximately twofold more active than Kojic acid, the positive control (IC_{50} 4.93 ± 0.01 μM), followed by **3** (33.3 ± 0.1 μM). On the other hand, **2** and **4** showed weak inhibitions (80.6 ± 0.3 and 97.6 ± 0.3 μM, respectively) (Table 4 and Fig. 4). Since

Table 1
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compounds **1**, **2** and **4** ($CDCl_3$).

Position	8PP (**1**)		8P (**2**)		**4**	
	δ_C, mult.	δ_H mult. (*J* in Hz)	δ_C, mult.	δ_H mult. (*J* in Hz)	δ_C, mult.	δ_H mult. (*J* in Hz)
2	74.8 CH	5.68 dd (3.0,12.8)	79.0 CH	5.43 dd (3.1, 12.9)	78.7 CH	5.40 dd (3.3, 12.1)
3a	40.3 CH_2	2.75 dd (3.0,17.1)	43.3 CH_2	2.85 dd (3.1, 17.1)	43.5 CH_2	2.85 dd (12.1, 17.1)
3b		3.12 dd (12.8, 17.1)		3.05 dd (12.9, 17.1)		3.04 dd (3.1, 17.1)
4	196.7 qC	–	196.2 qC	–	196.3 qC	–
5	162.3 qC	–	162.3[h] qC	–	159.3 qC	–
6	**95.5** CH	**6.03** s	**96.9** CH	**6.03** s	102.9 qC	–
7	163.7 qC	–	163.7[i] qC	–	160.8 qC	–
8	**107.5** qC	–	**106.1** qC	–	102.9 qC	–
9	160.7 qC	–	159.6[j] qC	–	157.7 qC	–
10	102.4 qC	–	103.3 qC	–	103.0 qC	–
1′	116.1[a] qC	–	138.7[k] qC	–	138.9 qC	–
2′	153.4 qC	–	125.9[l] CH	7.59 m	125.9 CH	7.50 m
3′	103.7 CH	6.48 s	128.8 CH	7.46 m	128.8 CH	7.44 m
4′	156.3 qC	–	128.7[m] CH	7.40 m	128.6 CH	7.40 m
5′	124.9[b] qC	–	128.8 CH	7.46 m	128.8 CH	7.44 m
6′	125.3[c] CH	7.40 s	125.9[l] CH	7.59 m	125.9 CH	7.50 m
1″	21.7 CH_2	3.26 d (7.3)	21.8[n] CH_2	3.33 d (7.0)		
2″	122.9 CH	5.25 t (7.3)	121.5 CH	5.20 d (7.0)		
3″	130.5 qC	–	135.1[o] qC	–		
4″	25.2 CH_3	1.62 s	25.8[p] CH_3	1.73 s		
5″	17.1 CH_3	1.61 s	17.8 CH_3	1.73 s		
1‴	39.9 qC	–				
2‴	148.2[d] CH	6.29 dd (10.5,17.8)				
3‴	109.7[e] CH_2	5.00 *cis* d (17.8)				
3‴		4.95 *trans* d (10.5)				
4‴	26.9[f] CH_3	1.47 s				
5‴	26.9[g] CH_3	1.47 s				
6-Me					7.6 CH_3	2.08 s
8-Me					6.8 CH_3	2.07 s
5-OH	–	12.17 s	–	12.12 s		12.27 s

[a] δ 116.1 ppm instead of δ 124.8 ppm as reported for 6PP (Cafaratti et al., 1994).
[b] δ 124.9 ppm instead of δ 103.2 ppm as reported for 6PP (Cafaratti et al., 1994).
[c] δ 125.3 ppm instead of δ 116.7 ppm as reported for 6PP (Cafaratti et al., 1994).
[d] δ 148.2 ppm instead of δ 27.1 ppm as reported for 6PP (Cafaratti et al., 1994).
[e] δ 109.7 ppm instead of δ 27.1 ppm as reported for 6PP (Cafaratti et al., 1994).
[f] δ 26.9 ppm instead of δ 149.9 ppm as reported for 6PP (Cafaratti et al., 1994).
[g] δ 26.9 ppm instead of δ 113.4 ppm as reported for 6PP (Cafaratti et al., 1994).
[h] δ 162.3 ppm instead of δ 160 ppm as reported by Mitscher et al. (1983).
[i] δ 163.7 ppm instead of δ 162 ppm as reported by Mitscher et al. (1983).
[j] δ 159.6 ppm instead of δ 164 ppm as reported by Mitscher et al. (1983).
[k] δ 138.7 ppm instead of δ 135 ppm as reported by Mitscher et al. (1983).
[l] δ 125.9 ppm instead of δ 128 ppm as reported by Mitscher et al. (1983).
[m] δ 128.7 ppm instead of δ 126 ppm as reported by Mitscher et al. (1983).
[n] δ 21.8 ppm instead of δ 26 ppm as reported by Mitscher et al. (1983).
[o] δ 135.1 ppm instead of δ 138 ppm as reported by Mitscher et al. (1983).
[p] δ 25.8 ppm instead of δ 22 ppm as reported by Mitscher et al. (1983).

Table 2
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for chalcone triangularin (**3**) ($CDCl_3$).

Position	**3**	
	δ_C, mult.	δ_H mult. (*J* in Hz)
1	135.3 qC	–
2	128.5 CH	7.60 m
3	129.0 CH	7.40 m
4	130.3 CH	7.40 m
5	129.0 CH	7.40 m
6	128.5 CH	7.60 m
1′	109.6 qC	–
2′	161.4 qC	–
3′	110.2 qC	–
4′	161.5 qC	–
5′	99.8 CH	6.24 s
6′	164.2 qC	–
α	143.3 CH	7.84 d (15.8)
β	126.5 CH	7.97 d (15.8)
2′-OH	–	13.32 s
4′-OMe	62.3 CH_3	3.69 s
3′-Me	8.04 CH_3	2.12 s

the compounds **1** and **3** showed important tyrosinase inhibition activity, we decided to investigate the inhibitory mechanism exerted by them on mushroom tyrosinase. The plots of the remaining enzyme activity versus concentrations of enzyme in the presence of different concentration of **1** and **3** gave a series of straight lines, which all passed through the origin (Fig. 5A and B). The increasing concentration of **1** and **3** resulted in the decrease of the line slope, indicating that the inhibition on the enzyme was reversible for both. Then, we obtain information about the inhibition type exerted by compound **1** and **3** in the oxidation of L-tyrosine analyzing the kinetic behavior of mushroom tyrosinase at different concentrations of both. The results analyzed by Lineweaver–Burk plotting $1/v$ versus $1/[S]$ are shown in Figs. 6 and 7. For compound **1**, the plot showed a family of straight lines with different slopes but they intersected one another in the *Y*-axis. V_{max} value remained the same and the values of K_m augmented with increasing concentrations of the inhibitor which indicates that **1** is a competitive inhibitor (Fig. 6). The behavior observed suggested that this prenyl flavanone inhibited only the activity of the free enzyme. The equilibrium constant for inhibitor binding with free enzyme, K_I, was obtained from the plot of K_m versus the concentrations of **1**, as shown in Fig. 6. The results obtained are given in Table 5.

Table 3
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for compounds **5** and **6** ($CDCl_3$).

Position	**5**		**6**	
	δ_C mult.	δ_H mult. (*J* in Hz)	δ_C mult.	δ_H mult. (*J* in Hz)
2	79.0 CH	5.40 dd (3.0, 13.0)	78.6 CH	5.41 dd (3.1, 13.0)
3a	45.7 CH_2	3.01 dd (16.9, 13.2)	45.7 CH_2	2.97 dd (13.0, 16.6)
3b		2.79 dd (16.8, 3.1)		2.83 dd (3.1, 16.6)
4	189.3 qC	–	189.6 qC	–
5	160.4 qC	–	157.7 qC	–
6	112.6 qC	–	111.2 qC	–
7	161.2 qC	–	158.8 qC	–
8	99.5 CH	6.30 s	106.9 qC	–
9	162.2 qC	–	159.6 qC	–
10	109.3 qC	–	109.2 qC	–
1′	138.8 qC	–	139.3 qC	–
2′	126.1 CH	7.42 m	125.9 CH	7.40 m
3′	128.8 CH	7.42 m	128.7 CH	7.40 m
4′	128.7 CH	7.42 m	128.4 CH	7.40 m
5′	128.8 CH	7.42 m	128.7 CH	7.40 m
6′	126.1 CH	7.42 m	125.9 CH	7.40 m
5-OMe	61.3 CH_3	3.84 s	61.3 CH_3	3.81 s
6-Me	7.7 CH_3	2.11 d (2.1)	7.8 CH_3	2.14 d (2.1)
7-OH	–	6.03 s	–	5.33 s
8-Me			8.1 CH_3	2.13 d (2.2)

Table 4
Anti-tyrosinase activity of compounds **1**–**6**.

Compound	Inhibition at 10 μM (%)	Inhibition at 100 μM (%)	IC_{50} (μM)
1	90.5 ± 5.0	95.0 ± 0.6	2.32 ± 0.01
3	24.7 ± 0.6	98.6 ± 0.6	33.3 ± 0.1
2	8.4 ± 1.6	73.7 ± 0.6	80.6 ± 0.3
4	6.3 ± 1.3	50.0 ± 0.6	97.6 ± 0.3
5	6.7 ± 0.9	22.7 ± 0.6	
6	5.5 ± 0.3	7.0 ± 0.6	

Positive control Kojic acid IC_{50} = 4.93 ± 0.01 μM.

In Fig. 7 is illustrated the inhibition of mushroom tyrosinase by **3**. In this plot is observed a family of parallel straight lines with the same slopes. As the concentration of the inhibitor is increasing, the values of K_m and V_{max} are decreased, indicating that triangularin (**3**) is an uncompetitive inhibitor of tyrosinase. This kind of inhibition is observed when the inhibitor joins with the enzyme–substrate complex but not with the free enzyme.

K_{IS}, the equilibrium constant for inhibitor binding with the enzyme–substrate complex, was obtained from a plot of $1/V_m^{app}$ versus different concentration of **3** as seen in Fig. 7. The kinetics parameters are informed in Table 5.

As a result of the evaluation of the compounds under study for their antityrosinase activity, it was observed that **1** showed an important inhibitory activity on the tyrosinase enzyme followed by compound **3**, while **2** and **4** were notably fewer actives.

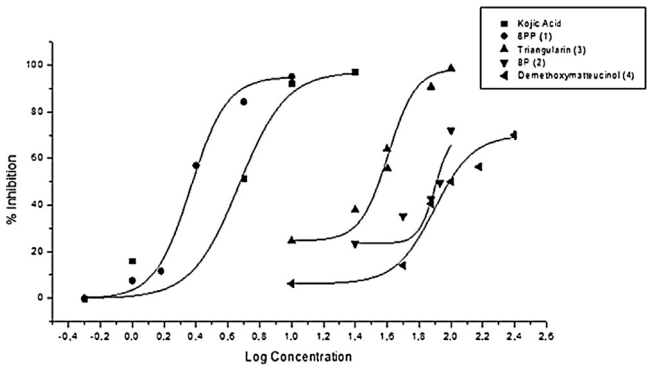

Fig. 4. Dose-dependent inhibition of mushroom tyrosinase activity by compounds **1**–**4** and positive control Kojic acid (*N* = 3).

Compounds **5** and **6** demonstrated very low inhibition of tyrosinase, even at 100 μM (Table 4). Analyzing the structural requirement for inhibitory activity against the tyrosinase enzyme for the active compounds, previously has been reported that the presence of 4-substituted resorcinol moiety in B-ring of flavones and flavanones is essential for antityrosinase activity (Lee et al., 2004). In recent studies we showed the importance of the 4-substituted phloroglucinol moiety with two free hydroxyl groups when it is part of the A ring (Peralta et al., 2011). Compound **1** presents both structural features and stands out because its strong activity on tyrosinase enzyme, surpassing even to the Kojic acid.

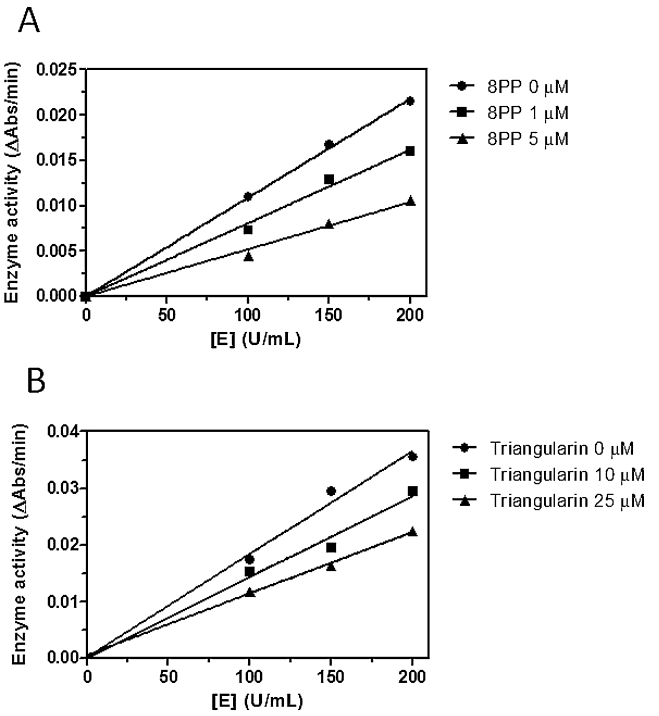

Fig. 5. Effect of 8PP (**1**) and triangularin (**3**) on the activity of mushroom tyrosinase for the catalysis of L-tyrosine (250 μM) at 25 °C, pH 6.8. Different enzyme concentrations (100, 200 and 500 U/mL) and various concentrations of inhibitors were utilized. (A) Compound **1**. (B) Compound **3**. Values are mean ± SD of three separate experiments.

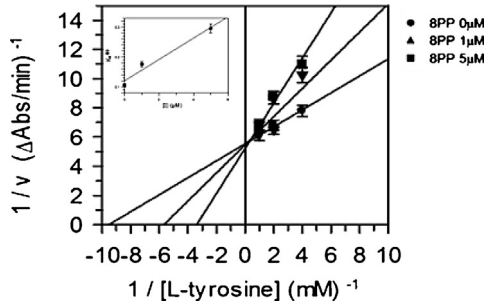

Fig. 6. Lineweaver–Burk plot for inhibition of 8PP (**1**) on mushroom tyrosinase for the catalysis of L-tyrosine at 25 °C, pH 6.8. Substrate concentrations were 250–1000 μM and the enzyme concentration was 200 U/mL. The inset represents the plot of apparent Michaelis constant (K_m^{app}) versus the concentration of **1** for determining the inhibition constant K_I. Values are mean ± SD of three separate experiments.

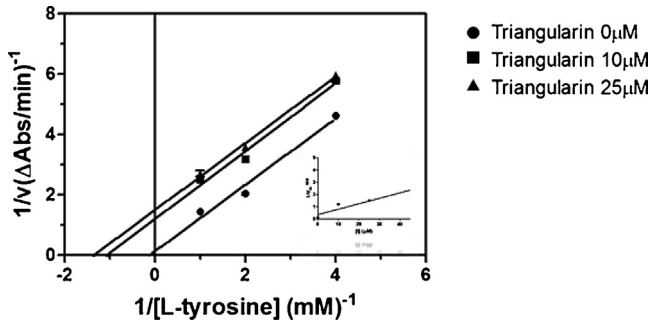

Fig. 7. Lineweaver–Burk plots for the monophenolase inhibitory activity of triangularin (**3**) using L-tyrosine as a substrate (250–1000 μM) at 25 °C, pH 6.8. The enzyme concentration was 200 U/mL. The inset represents the plot of apparent $1/V_m^{app}$ versus the concentration of **3** for determining the inhibition constant K_{IS}. Values are mean ± SD of three separate experiments.

The activity for **2** considerably diminishes in spite of its similarity to **1** structure in A ring. Another compound evaluated, **4**, showed decreasing activity as tyrosinase inhibitor. This reduction observed in **2** and **4** could be related to lack of resorcinol moiety in B ring and would reinforce the fact that the presence of the 4-substituted resorcinol moiety in B ring is the most important structural feature in relation to the tyrosinase inhibitory activity of compound as shown in compound **1**. Other structural considerations are important to point out. While **2** and **4** have presented low activity as tyrosinase inhibitors, **2** showed better inhibition than **4**. This observation could be by the presence of a prenyl moiety in C-8. Several prenylated flavonoids have been informed as tyrosinase inhibitors when this moiety is present in compounds with 4-substituted resorcinol moiety in B ring (Shimizu et al., 2000; Kim et al., 2003). But, Zheng et al. showed that an isoprenyl group at C-6 or/and C-8 in A ring without the presence of 4-substituted resorcinol moiety in B ring increased the antityrosinase activity (Zheng et al., 2013). In relation to this fact we demonstrated a similar behavior when two flavanones with a prenyl moiety in C-8 isolated from roots of *D. boliviana* showed moderate activity as tyrosinase inhibitors (Peralta et al., 2011).

Table 5
Kinetic and inhibition constants of compound 8PP (**1**) and triangularin (**3**) on mushroom tyrosinase.

	8PP	Triangularin
K_m	0.10 ± 0.02 μM	8.0 ± 0.9 μM
V_m	0.19 ± 0.01 μM	7.3 ± 2.2 μM
Inhibition	Reversible	Reversible
Inhibition type	Competitive	Uncompetitive
K_I	2.94 ± 0.01 μM	–
K_{IS}	–	7.8 ± 2.3 μM

Another consideration is related with the presence of free hydroxyl group at the C-5 position. It is observed that the activity decreased dramatically when the hydroxyl is substituted by methoxy group, this situation is observed between compound **4** and **6**. However, we consider that more studies about these structural factors are necessary to reveal inhibitory activity on tyrosinase of these compounds.

Triangularin (**3**) until moment is the unique chalcone obtained from this species and showed an interesting antityrosinase activity. The structure–activity relationship on the tyrosinase inhibitory activity of different chalcones has been studied. The 4-substituted resorcinol moiety in B ring is an important requirement in order to observe inhibitory activity on tyrosinase, but the hydroxylation in the A-ring also collaborates with this inhibitory activity specially in position 2′,4′ and 6′ of A-ring (Jun et al., 2007; Chang, 2009). Although both structural requirements are not present in **3**, the inhibitory activity on tyrosinase exerted by this compound is important. So, other structural factors could be relevant in order to showing this activity.

In conclusion, three C-methyl flavanones and a methyl chalcone have been isolated from aerial parts of *D. elegans* and informed for the first time in this species. Also, the prenylated flavanones previously reported as 6PP and 6P from *D. elegans*, have been chemically reassigned as 8PP (**1**) and 8P (**2**) on the bases of their 2D NMR spectral data. Thus, it was possible to unequivocally reassign the prenyl group position on A-ring for both flavanones. The tyrosinase inhibition activities of them were investigated. Among these compounds, **1** has demonstrated to be a strong inhibitor two times more active than Kojic acid. Triangularin (**3**) also has shown an important inhibitory activity. Their kinetics parameters were studied. So, the results obtained shown the potential of these compounds as promising agents for the treatment in disorders related with abnormal skin pigmentation.

3. Experimental

3.1. General experimental procedures

^1H and ^{13}C NMR spectra were acquired in $CDCl_3$ on a Brüker Avance II 400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer using TMS as internal standard. CD spectra were obtained with a Jasco J-810 spectropolarimeter. UV spectral data were recorded on a Shimadzu 160A spectrophotometer. HRMS were obtained in a QTOF mass spectrometer (micrOTOF-Q11 Series, Bruker) equipped with an electrospray ionization (ESI) interface. Column chromatography was performed on silica gel Merck. TLC was carried out using 20 cm × 20 cm plates with 0.5 mm layer of silica gel GF_{254} (Merck). Spots were visualized by UV illumination (254 nm).

3.2. Chemicals

Tyrosinase (EC 1.14.18.1) from mushroom (3933 U/mg), Kojic acid (purity: 99%) and L-tyrosine (purity: 99%) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

3.3. Plant material

D. elegans Gillies ex Hook. et Arn. (Fabaceae) was collected in February 2012, during the flowering period, in its natural habitat in hills near Cabalango (Córdoba, Argentina, GPS coordinates: latitude: 31°24′04.62″ South; longitude: 64°34′19.21″ West; height: 763 m). Plant material was identified by Prof. Dr. Gloria Barboza of the Botanical Museum, Universidad Nacional de Córdoba, Córdoba, Argentina (CORD). Roots and aerial parts were

separated for further processing. A representative voucher specimen is on deposit as CORD Peralta 2 in the herbarium at the Botanical Museum (UNC).

3.4. Extraction and isolation

D. elegans was dried at room temperature and the roots were separated from the aerial parts, and both parts were powdered. Roots and aerial parts of *D. elegans* were processed for extraction and isolation of 8PP (**1**) and 8P (**2**), respectively, according to Cafaratti et al. (1994).

The flavonoids (**3**–**6**) were obtained from the aerial parts of *D. elegans*. Briefly, 60.25 g of plant material was extracted in soxhlet with benzene. The solvent was subsequently removed to yield crude extract (5.98 g), which was subjected to column chromatography (CC) using silica gel chromatography as stationary phase and eluted with *n*-hexane/ethyl acetate (100:0 to 0:100) and giving twenty fractions. Fraction 2 was purified again over silica gel using *n*-hexane/acetone/ethyl acetate (8:1:1) to yield compound demethoxymatteucinol (**4**, 5.6 mg). Compound triangularin (**3**, 9.1 mg) was obtained by purification of the fraction 3 using preparative TLC (*n*-hexane/ethyl acetate; 7:3). Fraction 7 gave compounds comptonin (**5**, 13.6 mg) and 7-hydroxy-5-methoxy-6,8-dimethylflavanone (**6**, 11.3 mg) by purification using preparative TLC ($CHCl_3$/ethanol 97:3).

3.4.1. (2S)-2′,4′-dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenylpinocembrin (8PP, **1**)

UV (MeOH) λ_{max} (log ε) 294 (3.49), 334 (sh) (2.91) nm; CD (*c* 0.162, MeOH) $[\theta]_{294}$ −0.31, $[\theta]_{334}$ +0.18; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz), see Table 1; HRMS *m*/*z* 423.1834 $[M-H]^-$ (calcd. for $C_{25}H_{27}O_6$, 423.1802).

3.4.2. (2S)-8-prenylpinocembrin (8P, **2**)

UV (MeOH) λ_{max} (log ε) 294 (4.91), 334 (sh) (4.33) nm; CD (*c* 0.006, MeOH) $[\theta]_{294}$ −2.67, $[\theta]_{334}$ +0.35; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz), see Table 1; HRMS *m*/*z* 323.1292 $[M-H]^-$ (calcd. for $C_{20}H_{19}O_4$, 323.1278).

3.4.3. 2′,6′-Dihydroxy-4′-methoxy-3′-methylchalcone (triangularin, **3**)

UV (MeOH) λ_{max} (log ε) 325 (4.35) nm; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) (see Table 2) were coincident with those previously reported (Mustafa et al., 2005). HRMS *m*/*z* 283.0984 $[M-H]^-$ (calcd. for $C_{17}H_{15}O_4$, 283.0965).

3.4.4. (2S)-6,8-dimethylpinocembrin (demethoxymatteucinol, **4**)

UV (MeOH) λ_{max} (log ε) 296 (4.05), 342 (sh) (3.51) nm; CD (*c* 0.002, MeOH) $[\theta]_{296}$ −14.70; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) (see Table 1) were according to those previously reported (Basnet et al., 1993). HRMS *m*/*z* 283.0963 [M−H] − (calcd. for $C_{17}H_{15}O_4$, 283.0965).

3.4.5. (2S)-7-hydroxy-5-methoxy-6-methylflavanone (comptonin, **5**)

UV (MeOH) λ_{max} (log ε) 283 (4.22), 324 (sh) (3.80) nm; CD (*c* 0.001, MeOH) $[\theta]_{283}$ −5.87, $[\theta]_{324}$ +0.24; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) (see Table 3) were according to those previously reported (Kenneth et al., 1987). HRMS *m*/*z* 283.0983 $[M-H]^-$ (calcd. for $C_{17}H_{15}O_4$, 283.0965).

3.4.6. (2S)-7-hydroxy-5-methoxy-6,8-dimethylflavanone (**6**) (Amor et al., 2005)

UV (MeOH) λ_{max} (log ε) 287 (4.08), 340 (sh) (3.59) nm; CD (*c* 0.0005, MeOH) $[\theta]_{287}$ −0.18, $[\theta]_{340}$ +0.15; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$,100 MHz) (see Table 3). HRMS *m*/*z* 297.1122 $[M-H]^-$ (calcd. for $C_{18}H_{17}O_4$, 297.1121).

3.5. Tyrosinase inhibition assay

The assay was performed as previously described (Peralta et al., 2011). 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 flavonoids **1**–**6** dissolved in DMSO (final concentration 0.1, v/v) and subsequently diluted to the appropriate concentrations with the above buffer] was mixed and preincubated at 25 °C for 10 min. Then, 0.50 mL of L-tyrosine (1.7 mM, Sigma) solution 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 = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$, where $Abs_{control}$ is the absorbance of the control solution and Abs_{sample} is the absorbance of the sample solution.

3.6. Kinetic analysis of tyrosinase activity inhibition

The kinetic parameters of tyrosinase inhibition in the absence or presence of 8PP and triangularin were obtained. For this reason, the same protocol described above was carried out, except for varying concentrations of L-tyrosine (250, 500, and 1000 μM) and **1** (0, 1 and 5 μM) or **3** (0, 10 and 25 μM). The inhibition type was assayed by the Lineweaver–Burk plot, and the inhibition constant (K_I or K_{IS}) were determined by the second plots of the apparent K_m or V_m versus the concentration of the inhibitor, respectively.

3.7. Statistical analysis

All assays were independently performed in triplicate, and results were expressed as mean ± SD of three separate experiments. The IC_{50} values and the kinetics parameters were estimated using the GraphPad Prism 5.0 software on a PC compatible computer.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

Authors wish to acknowledge the assistance of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and the Universidad Nacional de Córdoba, both of which support facilities used in this investigation. This work was supported by ANPCyT (FONCyT PICT 2010-1576), CONICET D32/10, SeCyT-Universidad Nacional de Córdoba (05/C375) and MINCyT Cba PID 2010. M.D.S. is a doctoral fellow of SECyT-UNC. M.A.P, M.G.O. and J.L.C. are members of the Research Career of CONICET. We are indebted to Dr. Gloria Bonetto for her help with the NMR experiments, Dr. Romina Di Paola Naranjo for her assistance with the HRMS experiments and Prof. Dr. Gloria Barboza for the identification of the species under study.

References

Amor, E., Villaseñor, I., Nawaz, S., Hussain, M.S., Choudhary, I., 2005. A dihydrochalcone from *Syzygium samarangense* with anticholinesterase activity. Philipp. J. Sci. 134 (2) 105–111.

Basnet, P., Kadota, S., Shimizu, M., Xu, H.X., Namba, T., 1993. 2′-Hydroxymatteucinol, a new C-methyl flavanone derivative from *Matteccia orientalis*; potent hypoglycemic activity in streptozotocin (STZ)-induced diabetic rat. Chem. Pharm. Bull. 41 (10) 1790–1795.

Belofsky, G., Aronica, M., Foss, E., Diamond, J., Santana, F., Darley, J., Dowd, P., Coleman, C., Ferreira, D., 2014. Antimicrobial and antiinsectan phenolic metabolites of *Dalea searlsiae*. J. Nat. Prod. 77, 1140–1149.

Braz Filho, R., Gottlieb, O., Mourao, A., 1975. A stilbene and two flavanones from *Derris rariflora*. Phytochemistry 14, 261–263.

Briganti, S., Camera, E., Picardo, M., 2003. Chemical and instrumental approaches to treat hyperpigmentation. Pigm. Cell Res. 16, 101–110.
Burkart, A., 1952. Las leguminosas Argentinas Silvestres y Cultivadas, second ed. Acme Agency, Buenos Aires.
Cafaratti, M., Ortega, M.G., Scarafia, M.E., Ariza Espinar, L., Juliani, H., 1994. Prenylated flavanones from *Dalea elegans*. Phytochemistry 36, 1083–1084.
Chang, T.S., 2009. An updated review of tyrosinase inhibitors. Int. J. Mol. Sci. 10, 2440–2475.
Elingold, I., Isollabella, M.P., Casanova, M.B., Celentano, A.M., Perez, C., Cabrera, J.L., Diez, R.A., Dubin, M., 2008. Mitochondrial toxicity and antioxidant activity of a prenylated flavonoid isolated from *Dalea elegans*. Chem. Biol. Interact. 171, 294–305.
Harbone, J.B., Mabry, T.J., 1982. The Flavonoids: Advances in Research, first ed. New York, London.
Jun, N., Hong, G., Jun, K., 2007. Synthesis and evaluation of 2′,4′,6′-trihydroxychalcones as a new class of tyrosinase inhibitors. Bioorg. Med. Chem. 15, 2396–2402.
Kenneth, R., Eckhard, W., Gerhard, S., 1987. Structure revision for two C-methyl flavanones from *Pityrogramma pallid*. J. Plant Physiol. 131, 45–48.
Kim, S.J., Son, K.H., Chang, H.W., Kang, S.S., Kim, H.P., 2003. Tyrosinase inhibitory prenylated flavonoids from *Sophora flavescens*. Biol. Pharm. Bull. 26, 1348–1350.
Lee, N., Son, K., Chang, H., Kang, S., Park, H., Heo, M., Kim, H., 2004. Prenylated flavonoids as tyrosinase inhibitors. Arch. Pharm. Res. 27, 1132–1135.
Mabry, T.J., Markhan, K.R., Thomas, M.B., 1970. The Systematic Identification of Flavonoids, first ed. Springer-Verlag, Nueva York.
Mitscher, L.A., Raghav Rao, G.S., Khanna, I., Veysoglu, T., Drake, S., 1983. Antimicrobial agents from higher plants: prenylated flavonoids and other phenols from *Glycyrrhiza lepidota*. Phytochemistry 22, 573–576.
Mustafa, K., Perry, N., Weavers, R., 2005. Lipophilic C-methylflavonoids with no B-ring oxygenation in *Metrosideros* species (Myrtaceae). Biochem. Syst. Ecol. 33, 1049–1059.
Nanayakkara, N.P., Burandt, C.L., Jacob, M.R., 2002. Flavonoids with activity against methicillin-resistant *Staphylococcus aureus* from *Dalea scandens* var. paucifolia. Planta Med. 68, 519–522.
Ortega, M.G., Scarafia, M.E., Juliani, H., 1996. Antimicrobial agents in *Dalea elegans*. Fitoterapia 67, 81.
Peralta, M.A., Ortega, M.G., Agnese, A.M., Cabrera, J.L., 2011. Prenylated flavanones with anti-tyrosinase activity from *Dalea boliviana*. J. Nat. Prod. 74, 158–162.
Peralta, M.A., Calise, M., Fornari, M.C., Ortega, M.G., Diez, R.A., Cabrera, J.L., Pérez, C., 2012. A prenylated flavanone from *Dalea elegans* inhibits rhodamine 6 G efflux and reverses fluconazole-resistance in *Candida albicans*. Planta Med. 78, 981–987.
Pérez, C., Tiraboschi, I.N., Ortega, M.G., Agnese, A.M., Cabrera, J.L., 2003. Further antimicrobial studies of 2′4′-dihidroxy-5′-(1‴,1‴-dimethylallyl)-6-prenylpinocembrin from *Dalea elegans*. Pharm. Biol. 41, 171–174.
Seo, S.Y., Sharma, V.K., Sharma, N., 2003. Mushroom tyrosinase: recent prospects. J. Agric. Food Chem. 51, 2837–2853.
Shimizu, K., Kondo, R., Sakai, K., 2000. Inhibition of tyrosinase by flavonoids, stilbenes and related 4-substituted resorcinols: structure–activity investigations. Planta Med. 66, 11–15.
Slade, D., Ferreira, D., Marais, J.P., 2005. Circular dichroism, a powerful tool for the assessment of absolute configuration of flavonoids. Phytochemistry 66, 2177–2215.
Tropicos.org, 2013, December 10. Missouri Botanical Garden, http://www.tropicos.org/Name/40021469.
Zuloaga, F.O., Morrone, O., 1999. Catálogo de las Plantas Vasculares de la República Argentina II. In: Hollowell, V.C. (Ed.), Monographs in Systematic Botany from the Missouri Botanical Garden. Botanical Garden Press, St. Luis, pp. 670–671.
Zheng, Z.-P., Tan, H.-Y., Chen, J., Wang, M., 2013. Characterization of tyrosinase inhibitors in the twigs of *Cudrania tricuspidata* and their structure–activity relationship study. Fitoterapia 84, 242–247.