Food Chemistry 120 (2010) 10–14

Contents lists available at ScienceDirect

Food Chemistry

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

Tyrosinase inhibitory activity of native plants from central Argentina: Isolation of an active principle from *Lithrea molleoides*

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ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 2 September 2009
Accepted 16 September 2009

In memory of Dr. J.J. Carpinella

Keywords:
Tyrosinase inhibitors
Lithrea molleoides
(Z,Z)-5-(trideca-4,7-dienyl)-resorcinol
Argentine plants

ABSTRACT

Screening of 91 native plants from central Argentina was carried out with the aim of finding new sources of anti-tyrosinase compounds. Extracts obtained from *Achyrocline satureioides, Artemisia verlotiorum, Cotoneaster glaucophylla, Dalea elegans, Flourensia campestris, Jodina rhombifolia, Kageneckia lanceolata, Lepechinia floribunda, Lepechinia meyenii, Lithrea molleoides, Porlieria microphylla, Pterocaulon alopecuroides, Ruprechtia apetala, Senna aphylla, Sida rhombifolia, Solanum argentinum, Tagetes minuta* and *Thalictrum decipiens* exhibited more than 90% inhibition of tyrosinase monophenolase activity at 1000 $\mu g\ ml^{-1}$. *D. elegans, L. meyenii* and *L. molleoides* were the most potent with IC_{50} values of 0.48, 10.43 and 3.77 $\mu g\ ml^{-1}$, respectively. *D. elegans, L. molleoides* and *T. decipiens* also showed more than 90% inhibition of diphenolase activity at 1000 $\mu g\ ml^{-1}$, with the first of these being the most effective (IC_{50} = 49.27 $\mu g\ ml^{-1}$). (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**) was isolated from *L. molleoides* as an effective tyrosinase inhibitor with L-tyrosine or L-DOPA as substrates (IC_{50} = 0.49 and 14.94 $\mu g\ ml^{-1}$, respectively). Compound **1** was 37 times more active in monophenolase inhibitory activity than kojic acid used as a reference. Effective extracts as well as (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol could prove to be promising preservative agents for use in the food industry.

1. Introduction

Tyrosinase (EC 1.14.18.1), a copper containing enzyme, also known as polyphenol oxidase (PPO), is involved in the initial step of melanin synthesis (Karioti, Protopappa, Megoulas, & Skaltsa, 2007). This protein, which catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) (monophenolase activity) and the consequent oxidation of L-DOPA to dopaquinone (diphenolase activity), is widespread in many organisms such as vertebrates, invertebrates, plants and microorganisms (Chen & Kubo, 2002).

As mentioned, tyrosinase catalyses the oxidation of phenolic compounds into highly reactive quinones, which can polymerise leading to the darkening of plant or shellfish products during processing or storage (Gawlik-Dziki, Złotek, & Świeca, 2008). Although enzymatic browning is beneficial for certain foods (He, Luo, & Chen, 2008), it produces a less attractive appearance and loss in nutritional quality of a variety of fruits, vegetables and crustaceans (Qiu et al., 2009). Hence, agents for inhibiting this phenomenon are needed in order to maintain the appearance, flavour, texture and nutritional value of many fresh-cut products.

Despite the large number of PPO inhibitors, only a few of these are used today, as many of them show side effects or low effectiveness (Yi, Wu, Cao, Song, & Ma, 2009). In the past, sulphites were widely used as tyrosinase inhibitors for their antibrowning effect. However, their use has been regulated in order to ensure consumer safety since these preservatives affect the nutritional quality of foods and can cause allergic reactions as well as gastrointestinal distress (Ruiz-Capillas & Jiménez-Colmenero, 2009). Another currently used preservative, ascorbic acid, could negatively affect the aroma of some beverages and its activity is temporary (Komthong, Igura, & Shimoda, 2007). The identification of new tyrosinase inhibitors for preservative use in food industry is thus of great concern to researchers.

It is well known that plants are an important source of compounds with different activities such as insecticidal, herbicidal, antimicrobial, medicinal, antioxidant (Carpinella, Ferrayoli, & Palacios, 2005; Carpinella, Ferrayoli, Valladares, Defago, & Palacios, 2002; Carpinella & Rai, 2009; Carpinella et al., 2007; Rai & Carpinella, 2006) and enzyme inhibition including tyrosinase (Kim & Uyama, 2005). Many anti-tyrosinase compounds derived from plants are considered free of harmful side effects and can be obtained at low cost (Zheng, Cheng, Chao, Wu, & Wang, 2008). These observations and the need for new agents with tyrosinase

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doi:10.1016/j.foodchem.2009.09.061

inhibitory activity, led us to search for such chemicals among 91 extracts prepared from native plants of central Argentina that are generally used for beverages and/or for medicinal infusions.

The extracts were screened with the aim of selecting those with most activity. Subsequently, from the one obtained from *Lithrea molleoides* a compound was detected with a high anti-tyrosinase effect.

2. Materials and methods

2.1. Plant materials

Plants were collected in the hills of Córdoba Province, Argentina, from November 2005 to December 2007. Voucher specimens have been deposited in the "Marcelino Sayago" Herbarium of the School of Agricultural Science, Catholic University of Córdoba and were authenticated by the botanist, Gustavo Ruiz.

Plants were selected according to their availability, accessibility and especially the lack of scientific information about their activity and/or chemical pattern.

Crushed aerial plant material was extracted by 48 h maceration with ethanol. The yields of the most active extracts, obtained after solvent removal and expressed as percentage weight of air-dried crushed plant material, are shown in Table 1.

2.2. Chemicals, equipment and reagents

L-Tyrosine, 3,4-dihydroxy-L-phenylalanine (L-DOPA) and lyophilised mushroom tyrosinase were purchased from Sigma–Aldrich CO (St. Louis, MO). Kojic acid was obtained from Merck (Darmstadt, Germany). Silica gel (70–230 mesh) used for column chromatography was purchased from Sigma–Aldrich CO and all solvents were HPLC grade. ^{1}H- and ^{13}C-NMR spectra were recorded in Chloroform-d_3 with Bruker AVANCE II 400 spectrometer (Bruker Corporation, Ettlingen, Germany) operated at 400 MHz for ^{1}H and at 100 MHz for the ^{13}C nucleus. Chemical shifts (parts per million) are relative to internal tetramethylsilane used as a reference (δ = 0.00). MS spectra were measured with a ZAB SEQ (BeqQ) instrument (VG Analytical, Manchester). For quantifying the pure compound, HPLC was performed on a Phenomenex Prodigy 5 μ ODS (4.6 mm i.d. × 250 mm) reversed-phase column eluting with 90% acetonitrile in water with 1% trifluoracetic acid (TFA) as mobile phase and UV detection at 280 nm.

2.3. Isolation of the tyrosinase inhibitor compound

The resulting viscous extract from *L. molleoides* was dissolved in ethanol and subjected to a column chromatography with hexane/diethyl ether (Et_2O)/methanol (MeOH) gradient to yield ten fractions (F1–F10). F7–F10 were further purified by successive column chromatographies with the same solvent mixture at increasing polarity. Finally, five fractions were obtained (F1–F5) and F2 eluted with hexane/Et_2O (60:40, v/v) was subjected to another column chromatography and then radial preparative chromatography (solvent gradient methylene chloride (CH_2Cl_2)/Et_2O) affording with 100% CH_2Cl_2 a yellowish oil (yield 0.78 g/100 g of crushed plant material, by HPLC).

This compound was identified as the alkylresorcinol (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**) (Fig. 1) (Valcic, Wätcher, Eppler, & Timmermann, 2002).

(Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (***1***): $C_{19}H_{28}O_2$. t_R 4.46 min (by HPLC). EI-MS *m/z* (Int. rel.%) 288 [M$^+$] (3.3), 287 [M$^+$-1] (4.0), 217 [M-71] (2.7), 203 [M-85] (2.3), 163 [M-125] (14.6), 124 [M-164] (100), 81 (11.1), 77 (14.4). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.23 (1 H, d, J = 2.0 Hz, H-4,6), 6.17 (1 H, t, J = 2.0 Hz, H-2), 5.39 (1 H, m, H-4′), 5.38 (1 H, m, H-8′), 5.36 (1 H, m, H-5′), 5.34 (1 H, m, H-7′), 2.76 (2 H, t, J = 6.0 Hz, H-6′), 2.51 (2 H, t, J = 7.8 Hz, H-1′), 2.10 (2 H, q, J = 6.9 Hz, H-3′), 2.03 (2 H, q, J = 7.0 Hz, H-9′), 1.65 (2 H, q, J = 7.7 Hz, H-2′), 1.33 (2 H, m, H-10′), 1.30 (2 H, m, H-12′), 1.28 (2 H, m, H-11′), 0.91 (3 H, t, J = 6.8 Hz, Me). ^{13}C NMR (100.0 MHz, $CDCl_3$) δ 14.2 (C-13′), 22.6 (C-12′), 25.7 (C-6′), 26.7 (C-3′), 27.2 (C-9′), 29.3 (C-10′), 30.9 (C-2′), 31.5 (C-11′), 35.1 (C-1′), 100.2 (C-2), 107.9 (C-4,6), 127.8 (C-7′), 128.6 (C-5′), 129.1 (C-4′), 130.4 (C-8′), 145.6 (C-5), 156.8 (C-1,3).

2.4. Tyrosinase inhibitory assay

Tyrosinase inhibitory activity was determined spectrophotometrically. First, 2 μl of mushroom tyrosinase (2500 U ml^{-1} in

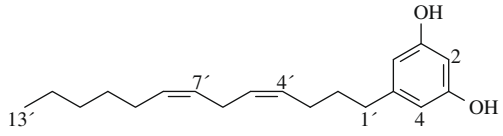

Fig. 1. Chemical structure of (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**).

Table 1
Native plants from central Argentina showing high tyrosinase inhibitory activity.

Plant species	Family	Common name	Yield (%)	Status[a]	Voucher: UCCOR number
Achyrocline satureioides (Lam.) DC.	Asteraceae	marcela hembra	4.5	N	140
Artemisia verlotiorum Lamotte	Asteraceae	yuyo de San Vicente	3.5	Adv.	230
Cotoneaster glaucophylla Franch.	Rosaceae	crategus	9.1	I	126
Dalea elegans Hook. and Arn.	Fabaceae		11.8	N	254
Flourensia campestris Griseb.	Asteraceae	chilca	11.8	N	221
Jodina rhombifolia (Hook. and Arn.) Reissek	Santalaceae	sombra de toro	5.0	N	153
Kageneckia lanceolata Ruiz and Pav.	Rosaceae	durazno de la sierra	14.7	N	264
Lepechinia floribunda (Benth.) Epling	Lamiaceae	salvia blanca	3.7	N	195
Lepechinia meyenii (Walp.) Epling	Lamiaceae		3.8	N	233
Lithrea molleoides (Vell.) Engl.	Anacardiaceae	molle de beber	7.2	N	183
Porlieria microphylla (Baill.) Descole, O'Donell and Lourteig	Zygophyllaceae	cucharero	1.1	N	154
Pterocaulon alopecuroides (Lam.) DC.	Asteraceae		5.5	N	217
Ruprechtia apetala Wedd.	Polygonaceae	manzano del campo	1.9	N	151
Senna aphylla (Cav.) H.S. Irwin et Barneby	Fabaceae	pichana	5.1	N	174
Sida rhombifolia L.	Malvaceae	escoba dura	2.2	N	141
Solanum argentinum Bitter and Lillo	Solanaceae	duraznillo blanco	5.4	N	34
Tagetes minuta L.	Asteraceae	suico	2.5	N	138
Thalictrum decipiens Boivin	Ranunculaceae	albaquilla	4.7	N	287

[a] Adv.: adventive; I: introduced; N: native.

50 mM phosphate buffer, pH 6.5) was mixed with 138 μl of 50 mM phosphate buffer. Then, 20 μl of tested solution dissolved in DMSO at the concentrations needed or DMSO (control), were added. All samples were first tested at 1000 μg ml^{-1} and those showing 90% inhibition or higher in three different repetitions were further evaluated for the concentration necessary for 50% inhibition (IC_{50}). The assay mixture was then incubated at 37 °C for 90 min with gentle agitation. At this stage a stable absorbance was reached. Finally, 40 μl of 2.5 mM L-tyrosine or L-DOPA in phosphate buffer were added and immediately monitored ($t = 0$) at 450 nm for dopachrome formation in the reaction mixture. Measurements were repeated up to 15 min for monophenolase activity and up to 5 min for diphenolase activity. Differences in absorbance between each time measured and time zero were calculated and the inhibition percentage was determined respect to control.

Kojic acid, dissolved in 50 mM phosphate buffer was used as a positive control. Each measurement was made at least in duplicate.

2.5. Statistical analysis

The results are expressed as mean ± standard error (SD). The inhibitory concentration (IC_{50}) was calculated by log-Probit analysis.

3. Results

Because of the urgent need for new agents with inhibitory activity on tyrosinase, we performed a screening of extracts obtained from native plants from central Argentina with the aim of finding these.

The results of the screening showed that 18 of the 91 extracts tested showed high monophenolase inhibitory activity on tyrosinase with percentages above 90% at 1000 μg ml^{-1} (see Table 2).

Dalea elegans was the most potent plant showing an IC_{50} of 0.48 μg ml^{-1} followed by *L. molleoides* (IC_{50} = 3.77 μg ml^{-1}) and *Lepechinia meyenii* (IC_{50} = 10.43 μg ml^{-1}) (Table 2).

D. elegans, L. molleoides and *Thalictrum decipiens* showed more than 90% inhibition of diphenolase activity at 1000 μg ml^{-1}, with the first of these being the most effective (IC_{50} = 49.27 μg ml^{-1}) followed by *L. molleoides* (IC_{50} = 79.44 μg ml^{-1}) and finally *T. decipiens* (IC_{50} = 158.03 μg ml^{-1}) (Table 3). Because of these results and due to its high availability and accessibility in the hill area of Córdoba, *L. molleoides* was selected as a first potential source of new anti-tyrosinase compounds.

Table 2
Most effective extracts of native plants from central Argentina on mushroom tyrosinase. Monophenolase activity.

Species	Inhibition (%)[a,b]	IC_{50} (μg ml^{-1}) values and 95% confidence limits (lower, upper)[b]
Achyrocline satureioides	95.42 ± 2.15	26.72 (10.39, 68.71)
Artemisia verlotiorum	94.88 ± 1.94	79.69 (17.11, 37.11)
Cotoneaster glaucophylla	94.67 ± 0.25	19.17 (9.55, 38.48)
Dalea elegans	90.75 ± 1.36	0.48 (0.17, 1.36)
Flourensia campestris	100.00 ± 0	107.91 (65.28, 178.37)
Jodina rhombifolia	95.90 ± 0.55	31.35 (6.67, 147.35)
Kageneckia lanceolata	93.15 ± 1.97	26.87 (11.66, 61.94)
Lepechinia floribunda	95.91 ± 0.37	266.19 (115.42, 613.91)
Lepechinia meyenii	96.99 ± 2.33	10.43 (4.45, 24.42)
Lithrea molleoides	99.56 ± 1.07	3.77 (2.40, 5.92)
Porlieria microphylla	97.40 ± 2.83	45.03 (21.36, 94.95)
Pterocaulon alopecuroides	99.36 ± 1.57	14.28 (5.79, 35.23)
Ruprechtia apetala	91.54 ± 0.43	118.97 (54.29, 260.67)
Senna aphylla	97.52 ± 0.57	76.39 (38.66, 150.92)
Sida rhombifolia	94.99 ± 1.17	103.36 (58.72, 181.94)
Solanum argentinum	98.04 ± 0.67	19.97 (3.10, 128.88)
Tagetes minuta	97.20 ± 0.58	41.58 (14.94, 115.74)
Thalictrum decipiens	92.21 ± 2.66	21.90 (8.12, 59.23)
(Z.Z)-5-(trideca-4,7-dienyl)-resorcinol	100 ± 0	0.49 (0.22, 1.09)
Kojic acid	100 ± 0	18.25 (9.37, 35.53)

[a] Data represent the mean ± standard error media of the evaluated parameter.
[b] Value at 10 min from the beginning.

Table 3
Most effective extracts of native plants from central Argentina on mushroom tyrosinase. Diphenolase activity.

Species	Inhibition (%)[a,b]	IC_{50} (μg ml^{-1}) values and 95% confidence limits (lower, upper)[b]
Dalea elegans	99.34 ± 1.11	49.27 (23.16, 104.80)
Lithrea molleoides	92.33 ± 0.55	79.44 (27.48, 229.67)
Thalictrum decipiens	93.02 ± 0.12	158.03 (76.37, 326.99)
(Z.Z)-5-(trideca-4.7-dienyl)-resorcinol	98.35 ± 1.17	14.94 (5.85, 38.09)
Kojic acid	98.66 ± 0.48	2.64 (1.06, 6.57)

[a] Data represent the mean ± SD of the parameter evaluated.
[b] Value at 2 min from the beginning.

From the ethanolic extract of this plant, a compound containing a C-13 unsaturated hydrocarbon side chain attached at position five to a dihydroxyphenyl moiety was obtained. It was identified as (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**) (Fig. 1).

Compound **1** inhibited the monophenolase activity of tyrosinase showing an IC_{50} value of 0.49 μg ml^{-1} (Table 2), while 50% inhibition of the diphenolase activity was reached at 14.94 μg ml^{-1} (Table 3). It inhibited tyrosinase activity when using L-tyrosine or L-DOPA as substrates in a dose-dependent manner (Fig. 2a and b). It exhibited significant inhibitory effect on monophenolase activity, with 32% inhibition at 0.24 μg ml^{-1}, 93% at 3.91 μg ml^{-1} and total inhibition at 7.81 μg ml^{-1}. At the latter concentration, kojic acid showed a weak inhibition (22%) and just reached 96% inhibition at 125 μg ml^{-1}. Compound **1** inhibited 21% of diphenolase enzyme activity at 3.91 μg ml^{-1}, 81% at 62.50 μg ml^{-1} and 98% at 1000 μg ml^{-1}. In regard to this activity, kojic acid was more effective than the alkyl resorcinol, reaching 98% inhibition at 250 μg ml^{-1}.

Monophenolase activity is typically characterised by a lag time, which is the time required to reach the steady-state concentration of *o*-diphenols which reduce the *met* form of tyrosinase to the *deoxy* form which then converts to the *oxy* form. The kinetic course of the oxidation of L-tyrosine observed with the formation of dopachrome in the presence of different concentrations of compound **1** is shown in Fig. 3. At concentrations of 1.95 and 3.91 μg ml^{-1}, the lag period was extended till 10 min. Furthermore, compound **1** reduced the reaction rate in a dose-dependent manner.

4. Discussion

Much effort has been spent in the search for new effective, stable, low-toxic anti-tyrosinase compounds.

According to IC_{50} values, using L-tyrosine as substrate, we were surprised to find that ethanolic extract from *D. elegans* (IC_{50} = 0.48 μg ml^{-1}) was 38 times more effective than that of kojic acid, the IC_{50} of which was 18.25 μg ml^{-1}. *L. meyenii* and *L. molleoides* extracts (IC_{50} = 10.43 and 3.77 μg ml^{-1}, respectively) were also highly active showing almost two and five times, respectively, more potency than that of the reference. *Cotoneaster glaucophylla, Pterocaulon alopecuroides, Solanum argentinum* and *T. decipiens* (IC_{50} = 14.28–21.90 μg ml^{-1}), in turn, exhibited similar levels of effectiveness to that of kojic acid.

Among plants with inhibition of the oxidation of L-DOPA catalysed by tyrosinase, *D. elegans*, *L. molleoides* and *T. decipiens*

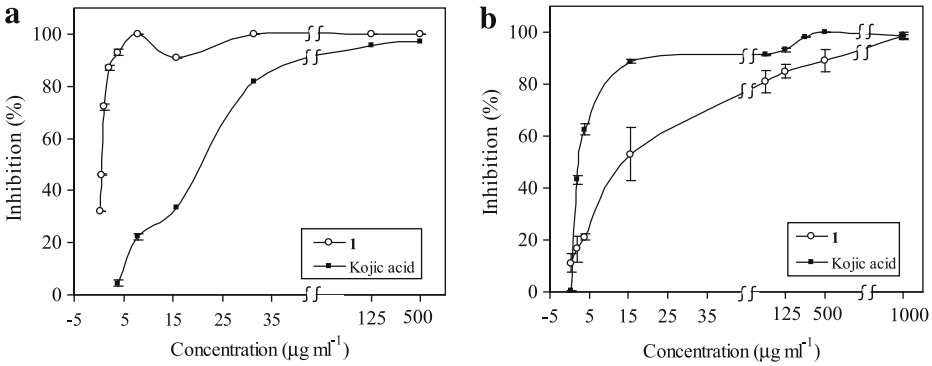

Fig. 2. (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**) inhibits tyrosinase activity in a dose-dependent manner. Compound **1** or kojic acid were incubated with tyrosinase at 37 °C with (a) L-tyrosine or (b) L-DOPA as substrates. Data represent the mean ± SD for duplicate within one experiment as a percentage of the control mean measured at 10 and 2 min from beginning of experiment for mono- and diphenolase activity, respectively.

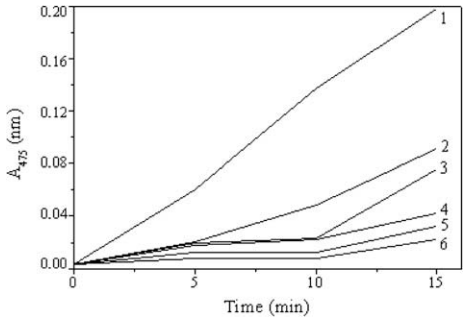

Fig. 3. Course of the oxidation of L-tyrosine by tyrosinase in the presence of different concentrations of (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol (**1**). Concentrations of **1** for curves 1–6 were 0, 0.24, 0.49, 0.98, 1.95 and 3.91 μg ml^{-1}, respectively. Compound **1** was incubated with tyrosinase at 37 °C with L-tyrosine as substrate. Dopachrome formation was determined at different times till 15 min.

(IC_{50} = 49.27–158.03 μg ml^{-1}) showed themselves less effective than that of the positive control (IC_{50} = 2.64 μg ml^{-1}).

A literature survey appears to show no information of anti-tyrosinase properties in any of the 18 plant species with high inhibitory effectiveness.

A compound belonging to the alkylresorcinol family was isolated from *L. molleoides* and identified as (Z,Z)-5-(trideca-4,7-dienyl)-resorcinol **1**. Surprisingly, compound **1** (IC_{50} = 0.49 μg ml^{-1}) was 37 times more active than kojic acid when L-tyrosine was used as substrate. Its activity was 81 times stronger than the well-known tyrosinase inhibitor arbutin, the IC_{50} of which corresponded to 39.71 μg ml^{-1} (Zheng et al., 2008) and 44 times more active than the extensively used sodium bisulphite (IC_{50} = 21.84 μg ml^{-1}) (Friedman & Bautista, 1995).

The well-known *trans*-stilbene resveratrol, showed an IC_{50} of 12.46 μg ml^{-1} (Kim, Yun, Lee, Lee, & Min, 2002) which is higher than that observed for compound **1**. Oxyresveratrol isolated from *Morus alba* and considered one of the most potent tyrosinase inhibitors in nature (Kim, Son, Chang, Kang, & Kim, 2003), showed an IC_{50} of 0.29 μg ml^{-1} (Kim et al., 2002), very close to that obtained for the isolated alkylresorcinol when inhibiting the hydroxylation of L-tyrosine.

When L-DOPA was used as substrate, compound **1** showed significant inhibition of diphenolase activity (IC_{50} of 14.94 μg ml^{-1}) but weaker in comparison to that of kojic acid. On the contrary, the alkylresorcinol was 1.6 times more active than ascorbic acid (IC_{50} = 24.6 μg ml^{-1}) (Yi et al., 2009).

Compounds with 4-substituted resorcinol skeleton have been reported as potent inhibitors of tyrosinase (Shimizu, Kondo, & Sakai, 2000). Among these, 4-hexylresorcinol has been recognised as a safe and effective tyrosinase inhibitor for use in the food industry (Zheng et al., 2008) and its use as a preservative is permitted in many countries (Martínez-Alvarez, López-Caballero, Montero, & Gómez-Guillén, 2007). According to Kim and Uyama (2005), the effectiveness of these molecules is due to substitution in 4-position, mainly with hydrophobic substituents, since resorcinol without substitution is a poor inhibitor of the phenoloxidase (Kim & Uyama, 2005). The same seems to happen with resorcinols substituted in 5-position, as is demonstrated by compound **1** activity and for other 5-alkyl resorcinols (Miura et al., 1995). The long hydrophobic alkyl chain may associate with the hydrophobic binding pocket close to the binuclear copper active site, as happened with other hydrophobic inhibitors (Conrad, Dawso, Hubbard, Meyers, & Strothkamp, 1994).

Despite *L. molleoides* shows medicinal properties (Araujo, Bela, Bueno, Rodrigues, & Shimizu, 2006; Fernández et al., 2002; Kott et al., 1999; Muschietti et al., 2005), its effect in inhibiting tyrosinase has not yet been explored. It has been reported that compound **1** produces paralysis on nematodes (Valcic et al., 2002) and exerts a cytotoxic effect on human tumoral cell lines (López et al., 2005), but its tyrosinase activity inhibition has not yet been described. This means that this is the first time that a compound showing this property has been isolated from *L. molleoides*.

Although toxic effects of compound **1** have not yet been determined, other alkyl resorcinols can be found in many edible plants, fruits and grains (Seitz, 1992), thus denoting its low toxicity. Besides, *L. molleoides* is used in traditional medicine as an infusion (López et al., 2005), and thus few or no side effects are expected. In this sense, it is expected that compound **1** could present few side effects, thus ensuring its safe use as an anti-tyrosinase compound.

From the more than satisfactory results obtained from this work and taking into account their potential applications in food industry, the tyrosinase inhibitors found could arise as promising agents for preserving many food products.

Acknowledgements

This work was supported by the Catholic University of Córdoba, FONCyT PICT 2005 and PICTO CRUP 2005. MBJ gratefully acknowledges receipt of a fellowship from CONICET. We thank Joss Heywood for revising the English language.

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