# Phenolic Antioxidants from the Whole Plant of *Phyllanthus urinaria*

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The 1,1-diphenyl-2-picrydydrazyl (DPPH) assay on the extract of *Phyllanthus urinaria* L. (Euphorbiaceae) displayed considerable radical-scavenging activity ($SC_{50}$ = 14.3 μg/ml). Further bio-assay-guided purification of the extract led to the isolation of a series of 15 phenolic compounds, including the ellagitannins **1**–**7**, the flavonoids **8**–**10**, and the simple hydroxylated (or glycosylated) aromatic acids **11**–**15**. Their structures were identified by spectroscopic analyses and comparison with authentic samples or literature data. The structure of repandinin B (**1**) was for the first time fully assigned by 1D- and 2D-NMR experiments. The phenolic compounds **1**, **3**, **4**, **6**, **9**, **11**, and **15** have not been isolated before from the title plant. The antioxidant activities and mushroom-tyrosinase-inhibitory activities of all compounds were determined by DPPH-radical-scavenging and mushroom-tyrosinase-inhibitory assays (*Table 2*).

**Introduction.** – It is well-known that antioxidants have a wide range of applications in human health care. There is evidence that free radicals are involved in chemical liver injury and implicated in the etiology of several diseases such as stroke, rheumatoid arthritis, diabetes, cancer, and aging processes [1]. Free radicals have also been reported to up-regulate the mRNA level for tyrosinase, a rate-limiting enzyme in melanin biosynthesis [2]. Accordingly, there is a great interest in plants containing rich natural antioxidants.

*Phyllanthus urinaria* L. (Euphorbiaceae) is a small tropical and subtropical annual herb widely growing in China, India, and South America. As a Chinese folk medicine, the whole plant has been used for the treatment of liver damage, hepatitis, jaundice, enteritis, diarrhea, and dropsy. The water extract of this plant was reported to have antiviral and anti-tumor activities [3–5]. Several traditional Chinese medicines (TCMs) are being produced as tablets and capsules from this herb, and used for liver protection.

In a preliminary experiment, we found that the 60% aqueous acetone extract of the whole plant of *P. urinaria* exhibits considerable free-radical-scavenging activity in the 1,1-diphenyl-2-picrydydrazyl (DPPH)-radical assay, with an $SC_{50}$ value of 14.3 μg/ml. As part of our continuing efforts to discover natural antioxidants from medicinal and edible plants [6–15], a detailed phytochemical investigation on *P. urinaria* was carried out, which led to the isolation of the phenolic compounds **1**–**15**, out of which **1**, **3**, **4**, **6**, **9**, **11**, and **15** were isolated for the first time from the title plant. All compounds were

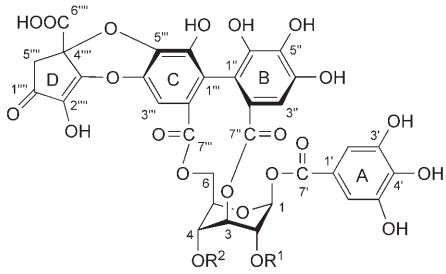
tested for their individual antioxidant activities as well as for their inhibitory action towards mushroom tyrosinase.

**Results and Discussion.** – 1. *Structure Elucidation*. The 60% aqueous acetone extract of air-dried P. urinaria (whole plant) was chromatographed repeatedly on *Diaion HP20SS*, *Sephadex LH-20*, *MCI* gel *CHP 20P*, and *Chromatorex ODS* supports, which afforded compounds **1**–**15**. By comparison of their spectroscopic data with reported literature values and authentic samples, the isolates were identified as repandinin B (**1**), furosin (**2**) [16], repandusinic acid A (**3**) [17], mallotinin (**4**) [18], geraniin (**5**) [19], acetonylgeraniin D (**6**) [20], corilagin (**7**) [21], rutin (**8**) [13], quercetin 3-*O*-*β*-D-glucoside (**9**) [22], kaempferol 7-methyl ether (**10**) [23], gentisic acid 4-*O*-*β*-D-glucopyranoside (**11**) [18], brevifolincarboxylic acid (**12**) [24], gallic acid (**13**) [13], protocatechuic acid (**14**) [25], and syringin (**15**) [26], respectively.

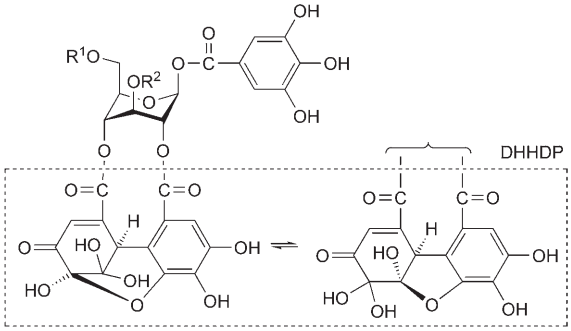
Repandinin B (**1**) had been previously isolated from the leaves of *Macaranga repandus*, and a preliminary structure was proposed in the Ph.D. thesis of *Saijo* [27], lacking assignment of the orientation of the ring-pertaining acyl groups at C(3) and C(6) of the glucosyl (Glc) unit [27]. Herein, we report the full characterization and NMR assignments of **1**.

The $^1$H- and $^{13}$C-NMR spectra of **1** (*Table 1*) were very similar to those of **7**, indicating a glucopyranosyl (Glc) moiety [$\delta$(H) 6.32 (*d*, $J = 2.6$), 3.99 (*s*-like), 4.82 (*s*-like), 4.36 (*d*-like, $J = 3.2$), 4.50 (*t*-like, $J = 11.0$), 4.85 (*t*, $J = 11.0$, $H_b$–C(6)), 4.02–4.04 (*m*, $H_a$–C(6))], a 'hexahydroxydiphenoyl' (HHDP) residue [$\delta$(H) 6.86 (*s*), 6.64 (*s*)], and a galloyl substituent [$\delta$(H) 7.06 (*s*)] [21]. The most-remarkable difference between **1** and **7** was due to two additional signals in **1**, ascribable to an isolated methylene group at $\delta$(H) 2.42, 2.82 (2*d*, $J = 16.9$ Hz each, $CH_2$(5'''')) in the $^1$H-NMR spectrum. Moreover, the $^{13}$C-NMR spectrum of **1** showed six extra signals due to a carboxy group [$\delta$(C) 173.0], a $CH_2$ moiety [$\delta$(C) 44.7], an oxygen-bearing quaternary C-atom [$\delta$(C) 77.5], and an $\alpha,\beta$-unsaturated C=O system [$\delta$(C) 196.5, 134.6, 151.9] in a five-membered ring. These data indicated the presence of an oxygenated brevifolincarboxylic acid moiety in **1**, whose structure was confirmed by the HMBC correlations of H–C(5'''') [$\delta$(H) 2.42, 2.82] with C(6'''') [$\delta$(C) 173.0], C(1'''') [$\delta$(C) 196.5], C(2'''') [$\delta$(C) 134.6], C(3'''') [$\delta$(C) 151.9], and C(4'''') [$\delta$(C) 77.5]. This specific acyl group (rings *B*–*D*) had been termed 'repandionoyl' by *Saijo* [27]. The $^{13}$C-NMR data of this moiety were in good agreement with those of repandinin A (**16**) [28], suggesting that the same ester group was linked at C(3) and C(6) of the Glc moiety in **1** and **16**. The difference between these two compounds was the absence of a 'dehydrohexahydroxydiphenoyl' (DHHDP) group at the Glc positions 2 and 4 of **1**.

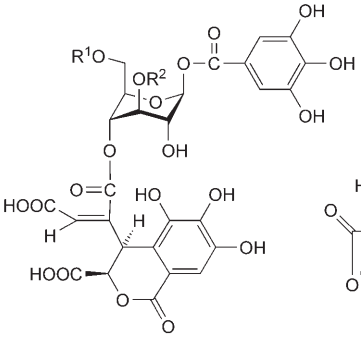
The structure of compound **1** was further confirmed by 2D-NMR experiments. In the HMBC spectrum of **1**, the long-range correlations of H–C(2',6') [$\delta$(H) 7.06] and H–C(1) [$\delta$(H) 6.32] with C(7') [$\delta$(C) 165.9], of H–C(3'') [$\delta$(H) 6.64] and H–C(3) [$\delta$(H) 4.82] with C(7'') [$\delta$(C) 168.6], and of H–C(3''') [$\delta$(H) 6.86] and H–C(6) [$\delta$(H) 4.85] with C(7''') [$\delta$(C) 166.5] indicated that rings *A* (galloyl group), *B*, and *C* were located at C(1), C(3), and C(6) of the Glc unit, respectively. The chemical shifts of C(4''') [$\delta$(C) 143.8] and C(5''') [$\delta$(C) 138.1], assigned to ring *C* because of the correlations with H–C(3''') through two- and three-bond couplings, were significantly different from those of the HHDP group in corilagin (**7**), and revealed the presence of



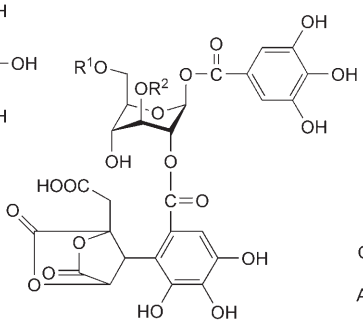
**1** $R^1 = R^2 = H$
**16** $R^{1,2}$ = DHHDP



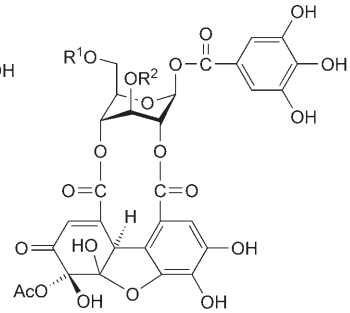

**2** $R^1 = R^2 = H$
**5** $R^{1,2}$ = DHHDP



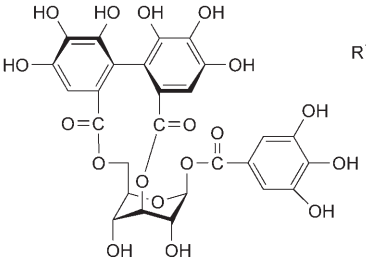
**3** $R^{1,2}$ = HHDP



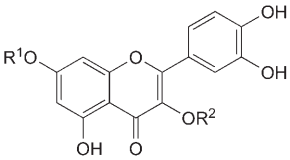
**4** $R^{1,2}$ = HHDP



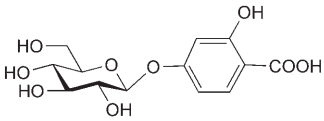
**6** $R^{1,2}$ = HHDP



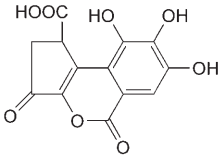
**7**



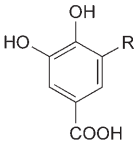
**8** $R^1 = H$, $R^2$ = Rha-(1→6)-Glc
**9** $R^1 = H$, $R^2$ = Glc
**10** $R^1$ = Me, $R^2 = H$



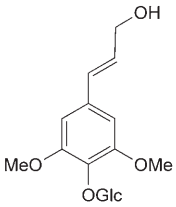
**11**



**12**



**13** R = OH
**14** R = H



**15**

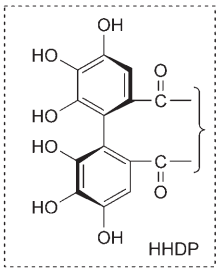


Table 1. *$^{13}$C- and $^{1}$H-NMR Data of* **1** *and* **7**. At 125/500 MHz, resp., in ($D_6$)acetone/$D_2O$; $\delta$ in ppm, $J$ in Hz. Arbitrary atom numbering.

| Position | **1** | | **7** | |
|---|---|---|---|---|
| | $\delta$(C) | $\delta$(H) | $\delta$(C) | $\delta$(H) |
| Glc: | | | | |
| 1 | 93.9 | 6.32 (*d*, $J = 2.6$) | 94.2 | 6.26 (*d*, $J = 2.3$) |
| 2 | 68.5 | 3.99 (*s*-like) | 68.7 | 4.03 (br. *s*) |
| 3 | 71.3 | 4.82 (*s*-like) | 70.8 | 4.74 (br. *s*) |
| 4 | 62.0 | 4.36 (*d*-like, $J = 3.2$) | 62.0 | 4.07 (br. *s*) |
| 5 | 75.3 | 4.50 (*t*-like, $J = 11.0$) | 75.3 | 4.37 (br. *s*) |
| 6 | 64.1 | 4.85 (*t*, $J = 11.0$, $H_b$–C(6)), 4.02–4.04 (*m*, $H_a$–C(6)) | 64.2 | 4.45 (br. *d*, $J = 10.9$, $H_a$–C(6)), 4.69 (br. *d*, $J = 10.9$, $H_b$–C(6)) |
| Ring *A*: | | | | |
| 1′ | 120.1 | | 120.3 | |
| 2′,6′ | 110.3 | 7.06 (*s*) | 110.5 | 7.03 (*s*) |
| 3′,5′ | 145.7 | | 145.7 | |
| 4′ | 139.5 | | 139.4 | |
| 7′ | 165.9 | | 165.6 | |
| Ring *B*: | | | | |
| 1″ | 115.4 | | 115.7 | |
| 2″ | 129.0 | | 125.2 | |
| 3″ | 108.2 | 6.64 (*s*) | 109.9 | 6.61 (*s*) |
| 4″ | 145.6 | | 144.6 | |
| 5″ | 133.8 | | 136.4 | |
| 6″ | 144.6 | | 144.7 | |
| 7″ | 168.6 | | 167.4 | |
| Ring *C*: | | | | |
| 1‴ | 117.6 | | 116.6 | |
| 2‴ | 124.6 | | 125.2 | |
| 3‴ | 111.5 | 6.86 (*s*) | 107.7 | 6.74 (*s*) |
| 4‴ | 143.8 | | 145.2 | |
| 5‴ | 138.1 | | 133.0 | |
| 6‴ | 145.1 | | 145.7 | |
| 7‴ | 166.5 | | 168.8 | |
| Ring *D*: | | | | |
| 1⁗ | 196.5 | | | |
| 2⁗ | 134.6 | | | |
| 3⁗ | 151.9 | | | |
| 4⁗ | 77.5 | | | |
| 5⁗ | 44.7 | 2.42 (*d*, $J = 16.9$, $H_a$–C(5⁗)), 2.82 (*d*, $J = 16.9$, $H_b$–C(5⁗)) | | |
| 6⁗ | 173.0 | | | |

two ether linkages between rings *C* and *D*. Thus, full NMR assignment of compound **1** was achieved, and the structure was solved as '1-*O*-galloyl-3,6-repandionoyl-$\alpha$-D-glucose'[1]).

[1]) For a systematic name, see *Exper. Part*.

2. *Biological Studies.* The isolated phenolic compounds **1**–**15** can be structurally placed into three major groups: the ellagitannins **1**–**7**, the flavonoids **8**–**10**, and the simple hydroxylated (or glycosylated) aromatic acids **11**–**15**. Their yields are listed in *Table 2*. As can be seen, geraniin (**5**) and corilagin (**7**) were the major phenolic constituents in the title plant. The high yield of gallic acid (**13**) is most likely due to the hydrolysis of tannins having galloyl substituents.

Table 2. *Yields and Biological Properties of* **1**–**15** *from* P. urinaria. Activity data represent averages ± S. D. for $n = 3$. For details, see *Exper. Part*.

| No. | Name (Class) | Yield [%] | $SC_{50}$ [μM][a]) | $IC_{50}$ [μM][b]) |
|---|---|---|---|---|
| Ellagitannins tannins: | | | | |
| **1** | Repandinin B | 0.0012 | 25.6 ± 0.7 | n.t.[c]) |
| **2** | Furosin | 0.0082 | 30.3 ± 0.2 | 158 ± 2 |
| **3** | Repandusinic acid A | 0.014 | 23.9 ± 0.2 | 134 ± 8 |
| **4** | Mallotinin | 0.008 | 14.7 ± 0.1 | 60.0 ± 0.5 |
| **5** | Geraniin | 1.38 | 18.7 ± 0.1 | 106 ± 3 |
| **6** | Acetonylgeraniin D | 0.0016 | 19.1 ± 0.2 | 266 ± 3 |
| **7** | Corilagin | 0.14 | 27.4 ± 0.1 | 101 ± 11 |
| | Flavonoids: | | | |
| **8** | Rutin | 0.036 | 42.3 ± 0.1 | 795 ± 2 |
| **9** | Quercetin 3-*O*-*β*-D-glucoside | 0.0052 | 21.8 ± 0.2 | 607 ± 2 |
| **10** | Kaempferol 7-methyl ether | 0.013 | n.a.[d]) | 365 ± 6 |
| | Phenolic acids and their glycosides: | | | |
| **11** | Gentisic acid 4-*O*-*β*-D-glucopyranoside | 0.0048 | 191 ± 1 | 31.9 ± 0.6 |
| **12** | Brevifolincarboxylic acid | 0.086 | 49.5 ± 0.2 | 240 ± 4 |
| **13** | Gallic acid | 0.41 | 14.0 ± 0.1 | 152 ± 7 |
| **14** | Protocatechuic acid | 0.001 | 48.8 ± 0.2 | 186 ± 4 |
| **15** | Syringin | 0.00028 | n.a. | n.a. |
| | Ascorbic acid | | 32.4 ± 0.2 | 795 ± 1 |
| | Arbutin | | | 272 ± 2 |

[a]) DPPH-Radical-scavenging activity. [b]) Tyrosinase-inhibitory activity. [c]) Not tested. [d]) Not active.

The antioxidant activities of **1**–**15** were evaluated by DPPH-radical-scavenging and mushroom-tyrosinase-inhibitory assays (*Table 2*). Most of the isolated compounds showed significant radical-scavenging activity as well as inhibition of tyrosinase, often better than those of the positive controls. Among them, all of the ellagitannins **1**–**7** with catechol and/or pyrogallol groups showed stronger activities than the other types of compounds. Flavonoid **9**, with a smaller group at C(3), exhibited higher activities than **8**. Our results are consistent with previous reports [13][14]. However, two major compounds, geraniin (**5**) and corilagin (**7**), were found to exhibit higher activities.

Interestingly, gentisic acid 4-*O*-*β*-D-glucopyranoside (**11**) showed a nearly tenfold stronger tyrosinase-inhibitory activity than arbutin, but a reduced DPPH-radical-scavenging activity; these results indicate that activity is not solely due to the phenyl group, but also to the other substituents. Finally, gallic acid (**13**) also showed high activities in both the DPPH and tyrosinase-inhibitory assays. Though eventually (or at

least partly) derived from the tannins after harvesting and during plant extraction, **13** may, nevertheless, play an important role for the antioxidant activity of this herb.

We hope that these results will aid in the evaluation of the phytotherapeutic properties of *P. urinaria* and promote reasonable utilization of this important medicinal herb in health care.

## Experimental Part

*General.* TLC: Pre-coated silica-gel-*G* plates (*Qingdao Haiyang Chemical Co.*), with benzene/AcOEt/HCOOH 3 : 6 : 1; spots were detected by spraying with $FeCl_3$ or 10% $H_2SO_4$, followed by heating. Column chromatography (CC): silica gel (200–300 mesh; *Qingdao Marine Chemical Factory*), *Diaion HP20SS* (*Mitsubishi Chemical Industry, Ltd*), *MCI-gel CHP 20P* (75–150 μm; *Mitsubishi*), and *Chromatorex ODS* (100–200 mesh; *Fuji Silysia Chemical Co., Ltd.*). Optical rotations: *SEPA-3000* automatic digital polarimeter. IR Spectra: *Bio-Rad FTS-135* spectrophotometer; in $cm^{-1}$. 1D- and 2D-NMR spectra: *Bruker DRX-500* instrument at 500/125 MHz, resp.; $\delta$ in ppm, $J$ in Hz. Mass spectra were recorded on a *VG Autospec-3000* mass spectrometer; in $m/z$.

*Plant Material. Phyllanthus urinaria* L. was collected at Xishuangbanna, Yunnan Province, China, and identified by Prof. *C. R. Yang*. A voucher specimen was deposited at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, P. R. China.

*Extraction and Isolation.* The air-dried plant material (2.5 kg) was extracted with 60% aq. acetone (3 × 10 l) at r.t. After removal of the org. solvent, the aq. layer was filtered, and purified directly by CC (*Diaion HP20SS*; $H_2O$/MeOH 1 : 0 → 0 : 1) to afford six fractions (*Fr. 1–6*). *Fr. 1* (16 g) was subjected to repeated CC (1. *Sephadex LH-20*, $H_2O$/MeOH 1 : 0 → 0 : 1; 2. *CHP 20P*, $H_2O$/MeOH 1 : 0 → 0 : 1; 3. *Chromatorex ODS*, $H_2O$/MeOH 6 : 4 → 0 : 10) to yield **3** (354 mg), **11** (120 mg), **12** (293 mg), and **13** (60 mg). *Fr. 3* (113 g) was first purified by reverse-phase CC (1. *Chromatorex ODS*, $H_2O$/MeOH 6 : 4 → 0 : 10; 2. *Sephadex LH-20*, $H_2O$/MeOH 1 : 0 → 0 : 1; 3. *MCI-gel CHP 20P*, $H_2O$/MeOH 1 : 0 → 0 : 1) and then by regular-phase CC ($SiO_2$; $CH_3Cl$/MeOH/$H_2O$ 90 : 10 : 1) to afford **1** (29 mg), **2** (205 mg), **4** (200 mg), **5** (16.8 g), **6** (39 mg), **7** (3.331 g), **13** (10.117 g), **12** (1.867 g), and **15** (7 mg). *Fr. 4* (55 g) was subjected to repeated CC (*MCI-gel CHP 20P, Chromatorex ODS, Sephadex LH-20*; eluting with $H_2O$/MeOH 1 : 0 → 0 : 1 each) to provide **5** (17.2 g), **7** (48 mg), and **8** (908 mg). *Fr. 5* (21 g) was repeatedly subjected to CC (1. *Sephadex LH-20*, then *MCI-gel CHP 20P*, each $H_2O$/MeOH 1 : 0 → 0 : 1; 2. *Chromatorex ODS*, $H_2O$/MeOH 1 : 1 → 0 : 1) to afford **5** (199 mg), **9** (129 mg), **10** (329 mg), and **14** (25 mg).

*Repandinin B* (*=(a*R*)-(1*S*,26*R*,28*S*,29*R*,30*R*)-6,7,8,11,17,29,30-Heptahydroxy-3,16,23-trioxo-28-{[(3,4,5-trihydroxyphenyl)carbonyl]oxy}-2,13,19,24,27-pentaoxahexacyclo[24.3.1.0$^{4,9}$.0$^{10,22}$.0$^{12,20}$.0$^{14,18}$]triaconta-4,6,8,10(22),11,17,20-heptaene-14-carboxylic Acid*; **1**). Hazel-colored, amorphous powder. $[\alpha]^{24}_{D} = -56.0$ ($c = 0.033$, MeOH). $^1$H- and $^{13}$C-NMR: see *Table 1*. FAB-MS (neg.): 771 ($[M-H]^-$), 726 ($[M-H-COOH]^-$). HR-FAB-MS (neg.): 771.0662 ($[M-H]^-$, $C_{33}H_{23}O^-_{22}$; calc. 771.0680).

*Radical-Scavenging Assay.* The DPPH (1,1-diphenyl-2-picrydydrazyl)-radical assay was performed as described in a previous paper [13], ascorbic acid being used as pos. control. Scavenging activity (in %) was determined as $100 \times (A_{control} - A_{sample})/A_{control}$. $SC_{50}$ Values were obtained through extrapolation of linear-regression data, and refer to the concentration of sample required to scavenge 50% of the DPPH radicals.

*Tyrosinase-Inhibitory Assay.* Tyrosinase-inhibitory activity was determined spectrophotometrically according to the method of *Kittisak* [29], with minor modifications. Assays were conducted in a 96-well microtiter plate, an *E-max* plate reader (*Molecular Devices*) being used to determine the absorbance at 490 nm. Ascorbic acid was used as pos. control. The test substance was dissolved in 20% aq. DMSO, and incubated with 25 U/ml of mushroom tyrosinase in *PBS* buffer (pH 6.8) at r.t. for 10 min. Then, L-DOPA (2.5 mg/ml) in the same buffer was added, and the mixture was incubated at r.t. for 30 min. Scavenging activity was determined at 490 nm by the following equation: % inhibition $= 100 \times \{[(A-B)-(C-D)]/(A-B)\}$, where $A$ is the optical density (OD) without test substance; $B$ is the OD without test substance,

but with tyrosinase; *C* is the OD with test substance; and *D* is the OD with test substance, but without tyrosinase. $IC_{50}$ Values were obtained through extrapolation from linear-regression analysis, and correspond to the concentration of sample required for 50% inhibition.

*Statistics.* The data are presented as means ± S.D. for three determinations. Correlation analysis of antioxidant activity *vs.* total phenolic content was analyzed with the correlation and regression tools in EXCEL.

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