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New Phenolic Compounds and Antioxidant Potential of *Catharanthus roseus*

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Screening of the phenolic compounds from seeds, stems, leaves and petals of *Catharanthus roseus* (L.) G. Don (cv. Little Bright Eye) was achieved by HPLC-DAD-ESI-MS/MS. This is the first detailed study of noncolored phenolics in *C. roseus*, which allowed the characterization of three caffeoylquinic acids and fifteen flavonol glycosides (di- and trisaccharides of kaempferol, quercetin and isorhamnetin). Fifteen compounds are reported for the first time in this species. The scavenging ability of the different plant matrices was assessed against DPPH$^{\bullet}$ radical and against reactive oxygen (superoxide radical) and a reactive nitrogen (nitric oxide) species. A concentration-dependent protective effect was observed for seeds and tissues, with petals shown to be the most active.

KEYWORDS: ***Catharanthus roseus*; phenolic compounds; HPLC-DAD-ESI-MS/MS; antioxidant activity**

INTRODUCTION

Catharanthus roseus (L.) G. Don (formerly *Vinca rosea* L., Apocynaceae) is commonly known as the Madagascar periwinkle and was originally an endemic subshrub species of Madagascar, having now acquired a pantropical distribution. The leaves of *C. roseus* were used in traditional medicine as an oral hypoglycemic agent and the study of this activity led to the discovery of two terpenoid indole alkaloids (TIA), vinblastine and vincristine (*1*), the first natural anticancer agents to be clinically used and, since they are present in very low levels on *C. roseus* leaves, the TIA pathway has been intensively investigated (*2, 3*).

Although *C. roseus* has become one of the best studied medicinal plants, primarily at the alkaloids level and the use of cell suspension cultures for production of natural products, the characterization of natural products other than alkaloids in this plant remains extremely scarce. Water extracts of this plant are consumed for various applications, such as bleeding arresting, diabetes, fever or rheumatism (*4*). In addition, the leaves of the plants were chewed to suppress the sensations of hunger and fatigue (*3*).

As part of an in-depth phytochemical characterization of *C. roseus* aiming to discover new bioactive principles, we decided to investigate the phenolic compounds accumulated by this plant. Phenolics represent the most abundant and widely spread class of plant natural products, playing important functions for the plant, including support of the plant body, protection against biotic and abiotic stresses, herbivore deterrence, and signaling in plant-plant and plant-microbe interactions. For humans, plant phenolics are the basis of several plant-derived drugs and recently they have attracted much attention due to their implication in protection against cancer, cardiovascular and neurodegenerative diseases, associated to their antioxidant activity (*5, 6*).

What is known about the occurrence of phenolic compounds in *C. roseus* has been recently reviewed (*7, 8*). These reviews show that only few phenolic compounds have been reported for this genus, possibly due to the fact that many of the phenolics characterization has been done in cell cultures, where natural products metabolism is poorly expressed. In fact, no detailed study of noncolored phenolics in *C. roseus* has been done. Little information is also available about the antioxidant potential of *C. roseus.* Previous studies concerned the evaluation of the influence of environmental factors on the antioxidant defense system of the plant, namely antioxidant enzymes and indole alkaloids (*9, 10*), and one study has characterized the oxygen radical absorbance capacity (ORAC) of crude extracts of several culinary and medicinal herbs including *C. roseus*, which displayed one of the highest capacities among the medicinal species (*11*).

The aim of this work was to achieve further knowledge on the phenolics composition of the plant, specifically from the

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distinct plant parts, and to evaluate their antioxidant capacity. For these purposes, the main phenolic compounds accumulated by the organs of the plant were screened by HPLC-DAD-ESI-MS/MS and antioxidant potential was assessed against DPPH$^{\bullet}$, a reactive oxygen species (superoxide radical) and a reactive nitrogen species (nitric oxide).

MATERIALS AND METHODS

Reagents. Acetic acid, acetonitrile, methanol and *N*-(1-naphthyl)-ethylenediamine dihydrochloride were obtained from Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH$^{\bullet}$), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate, nitroblue tetrazolium chloride and sulfanilamide were from Sigma-Aldrich (St. Louis, MO) and sodium nitroprussiate dehydrate from Riedel-de-Haën (Seelze, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Plant Material. Plants of *Catharanthus roseus* (L.) G. Don cv. Little Bright Eye were grown at 25 °C in a growth chamber, under a 16 h photoperiod, using white fluorescent light at a photon flux density of 70 μmol/m^2/s. Seeds were acquired from AustraHort (Australia) and voucher specimens were deposited at the Herbarium of the Department of Botany of the Faculty of Sciences of the University of Porto (PO 61912).

Stems, leaves and petals were separated, frozen and lyophilized. The dried materials were powdered and kept in a desiccator, in the dark.

Samples Preparation. For the identification of phenolic compounds ca. 0.15 g of leaves and stems and ca. 0.01 g of petals were thoroughly mixed with 1.5 and 1 mL of methanol:water (1:1), respectively. To check for possible anthocyanins in petals, a methanol:acetic acid:water (25:4:21) extract was also prepared. About 0.5 g of seeds was powdered using a mortar and pestle and extracted with 1.5 mL of methanol:water (1:1). These mixtures were then ultrasonicated (1 h), macerated for 15 h and ultrasonicated again (1 h). Suspensions obtained were centrifuged (12000 rpm, 5 min), and the supernatant was collected and filtrated trough a 0.45 μm size pore membrane. The complete extraction procedure was performed at room temperature.

As methanol interferes with some assays, and attending to the usual consumption of the species, for the antioxidant capacity screening aqueous extracts were prepared by boiling 1.5 g of dried material for 20 min in 300 mL of water and filtering through a Büchner funnel. The resulting extracts were then lyophilized. The lyophilized extracts were kept in a desiccator, in the dark.

HPLC-DAD-ESI-MS/MS Analyses. Chromatographic separations were carried out on a 250 × 4 mm, 5 μm, RP-18 LiChroCART column (Merck, Darmstadt, Germany) protected with a 4 × 4 mm LiChroCART guard column, with 1% acetic acid (A) and acetonitrile (B) as solvents, starting with 12% B and using a gradient to obtain 20% B at 30 min. The flow rate was 1 mL/min and the injection volume 20 μL. The HPLC system was equipped with an Agilent 1100 series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and a G1315B photodiode array detector, controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range 240−400 nm, and chromatograms were recorded at 340 nm. For the study of possible anthocyanins spectroscopic data were accumulated between 240 and 600 nm and chromatograms were recorded at 530 nm. The mass detector was a G2445A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as nebulizing gas at a pressure of 65 psi and the flow was adjusted to 11 L/min. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. The full scan mass covered the range from *m*/*z* 100 to1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionization mode, and in positive mode for the study of anthocyanins in flowers. The classical nomenclature (*12*) for glycoconjugates was adopted to designate the fragment ions, with some modifications. This nomenclature was modified, in order to be adapted to our compounds. The ions $^{k,l}\mathbf{X}^{n}_{j}$, $\mathbf{Y}^{n}_{j}$ and $\mathbf{Z}^{n}_{j}$ represent those fragments still containing the flavonoid aglycon, where **j** is the number of the interglycosidic bonds broken, counted from the aglycon (**j** = 0 indicates the interglycosidic bonds with phenolic hydroxyl of the aglycon and **j** = 2″ the interglycosidic bonds on hydroxyl in 2″), **n** represents the position where the oligosaccharide is attached to the aglycon, and **k** and **l** denote the cleavage within the carbohydrate rings (**Figure 2**). In this context, the ions obtained by MS3 scan have been labeled starting with the ion produced and followed by the resultant ion, e.g. the ion $[\mathbf{Y}^{7}_{0}\mathbf{Y}^{3}_{2''}]^{-}$ (MS3 of compound **2**) (**Table 1**) denotes that arising from the fragmentation of ion $\mathbf{Y}^{7}_{0}{}^{-}$ (loss of glycosylation in the 7 position, MS3[(M − H)→Y^{7}_{0}]$^{-}$) by loss of a sugar (rhamnose) bond to the hydroxyl at 2″ position (−146). The losses indicated in the MS3 scan of the fragment come from trapped and fragmented ions and not from the deprotonated molecular ion. This terminology completes the nomenclature provided by Domon and Costello (*12*), by addition of specific pathways of the ions obtained from the MS3 event.

Table 1 shows the most frequent ions which characterize the fragmentation of the compounds. Other ions were found, but they have not been included due to the lack of space and their low relevance to the MS ions. MS3 data were derived from the fragmentation of the main ion. The UV spectra of some compounds were not observed properly because they were hidden by others or present in traces. Compounds were numbered according to increasing retention time (t_R) and **Table 1** was designed taking into account the type of glycosylation.

DPPH$^{\bullet}$ Scavenging Activity. The disappearance of DPPH$^{\bullet}$ was monitored spectrophotometrically at 515 nm on a Multiskan Ascent plate reader (Thermo-Electron Corporation), following a described procedure (*13*). For each extract, a dilution series (five different concentrations) was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μL of lyophilized extract dissolved in water and 200 μL of 150 μM DPPH$^{\bullet}$ dissolved in methanol. The plate was incubated for 20 min at room temperature after addition of DPPH$^{\bullet}$. Three experiments were executed, each one of them consisting in three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

Superoxide Radical ($O_2^{\bullet-}$) Scavenging Activity. The effect of the lyophilized extracts on the superoxide radical-induced reduction of NBT was monitored spectrophotometrically in a Multiskan Ascent plate reader in kinetic mode, at 562 nm. Superoxide radicals were generated by the NADH/PMS system according to a described procedure (*5*). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were executed, each one of them consisting in three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

Nitric Oxide ($^{\bullet}$NO) Scavenging Activity. The ability of the aqueous lyophilized extract to scavenge nitric oxide radical was evaluated spectrophotometrically in a Multiskan Ascent plate reader according to a previously described method (*6*). A dilution series (five different concentrations) was prepared in a 96-well plate. The reaction mixtures in the sample wells consisted of dissolved lyophilized extract and SNP. The plates were incubated at 25 °C for 60 min under light. Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H_3PO_4) was then added and the absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was determined at 540 nm. Three experiments were executed, each one of them consisting in three determinations. IC values were estimated by interpolation of the nonlinear regression curve built using the mean values of the three experiments.

RESULTS AND DISCUSSION

Characterization of Phenolic Compounds. The HPLC-DAD-ESI-MS/MS screening of the hydroalcoholic extracts of *C. roseus* material (seeds, stems, leaves and flowers) revealed the presence of numerous flavonoids (compounds **2** and **5**−**18**) (**Figure 1**), whose UV spectra were typical of flavonol-3-*O*-

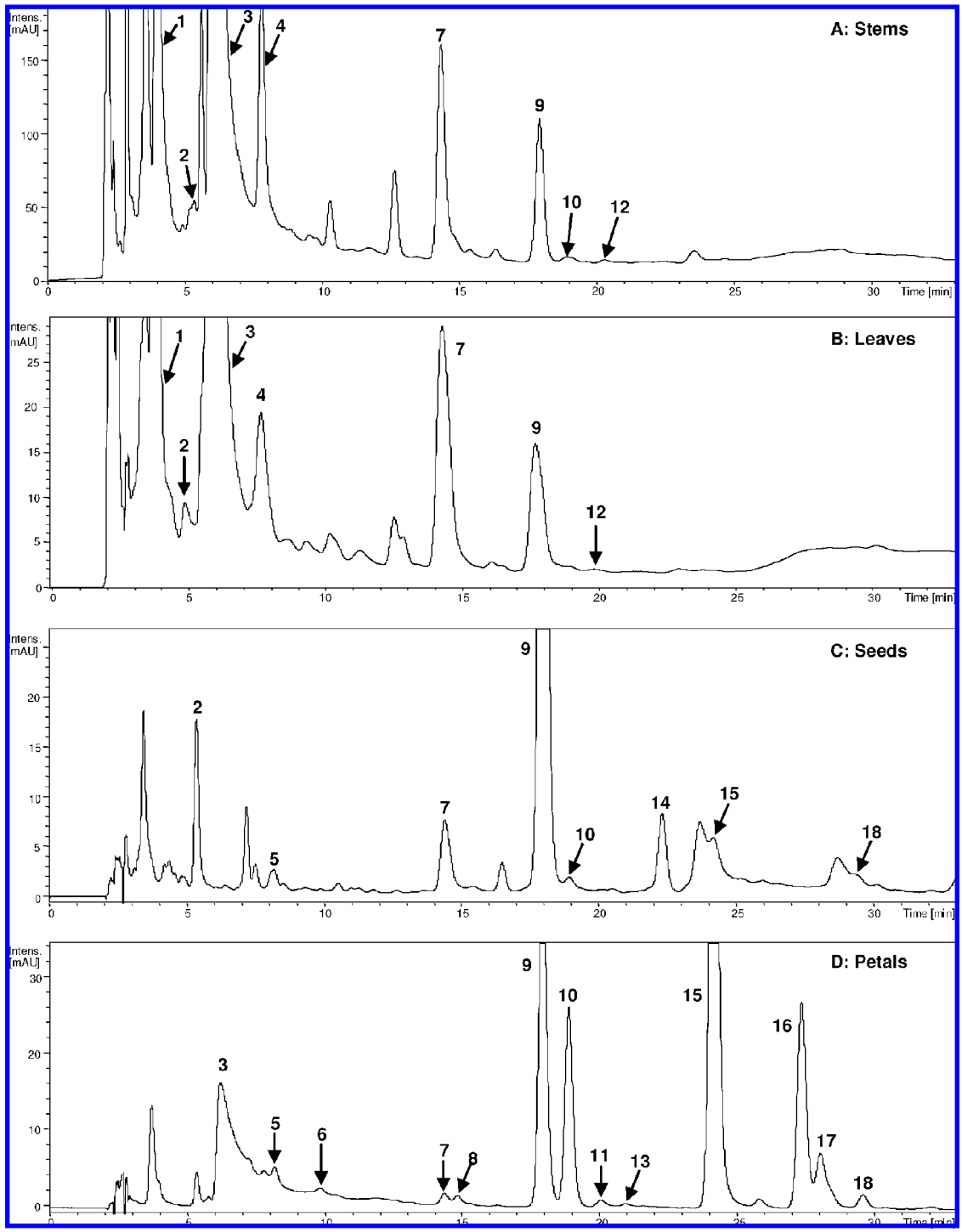

Figure 1. HPLC-DAD chromatogram of *Catharanthus roseus* extracts: (**A**) stems; (**B**) leaves; (**C**) seeds; (**D**) petals. Detection at 340 nm. (**1**) 3-*O*-Caffeoylquinic acid; (**2**) kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)-7-*O*-hexoside; (**3**) 4-*O*-caffeoylquinic acid; (**4**) 5-*O*-caffeoylquinic acid; (**5**) kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-galactoside; (**6**) kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-glucoside; (**7**) quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside); (**8**) quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside); (**9**) kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside); (**10**) kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside); (**11**) quercetin-3-*O*-(6-*O*-rhamnosyl-galactoside); (**12**) isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside); (**13**) quercetin-3-*O*-(6-*O*-rhamnosyl-glucoside); (**14**) isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside); (**15**) kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside); (**16**) kaempferol-3-*O*-(6-*O*-rhamnosyl-glucoside); (**17**) isorhamnetin-3-*O*-(6-*O*-rhamnosyl-galactoside); (**18**) isorhamnetin-3-*O*-(6-*O*-rhamnosyl-glucoside).

glycosyl derivatives (*14*) (**Table 1**). In their MS (MS2 or MS3) fragmentations ions were observed at *m/z* 285, 300/301 or 315 with high abundance (base peak), corresponding to the deprotonated aglycon ions of kaempferol, quercetin or isorhamnetin, respectively (**Table 1**).

The two main flavonoids in extracts of stems and leaves (**Figures 1A** and **1B**) showed deprotonated molecular ions at *m/z* 755 (compound **7**) and 739 (compound **9**) and in their MS2 base peak ions occurred at *m/z* 300 and 285 respectively, pointing to quercetin and kaempferol triglycosides with two rhamnoses and one hexose. This MS2 fragmentation type, in which the base peak corresponds to the deprotonated aglycon ion, indicates that the triglycoside is linked to only one phenolic hydroxyl (*15*). Thus, these compounds are identified as quer-

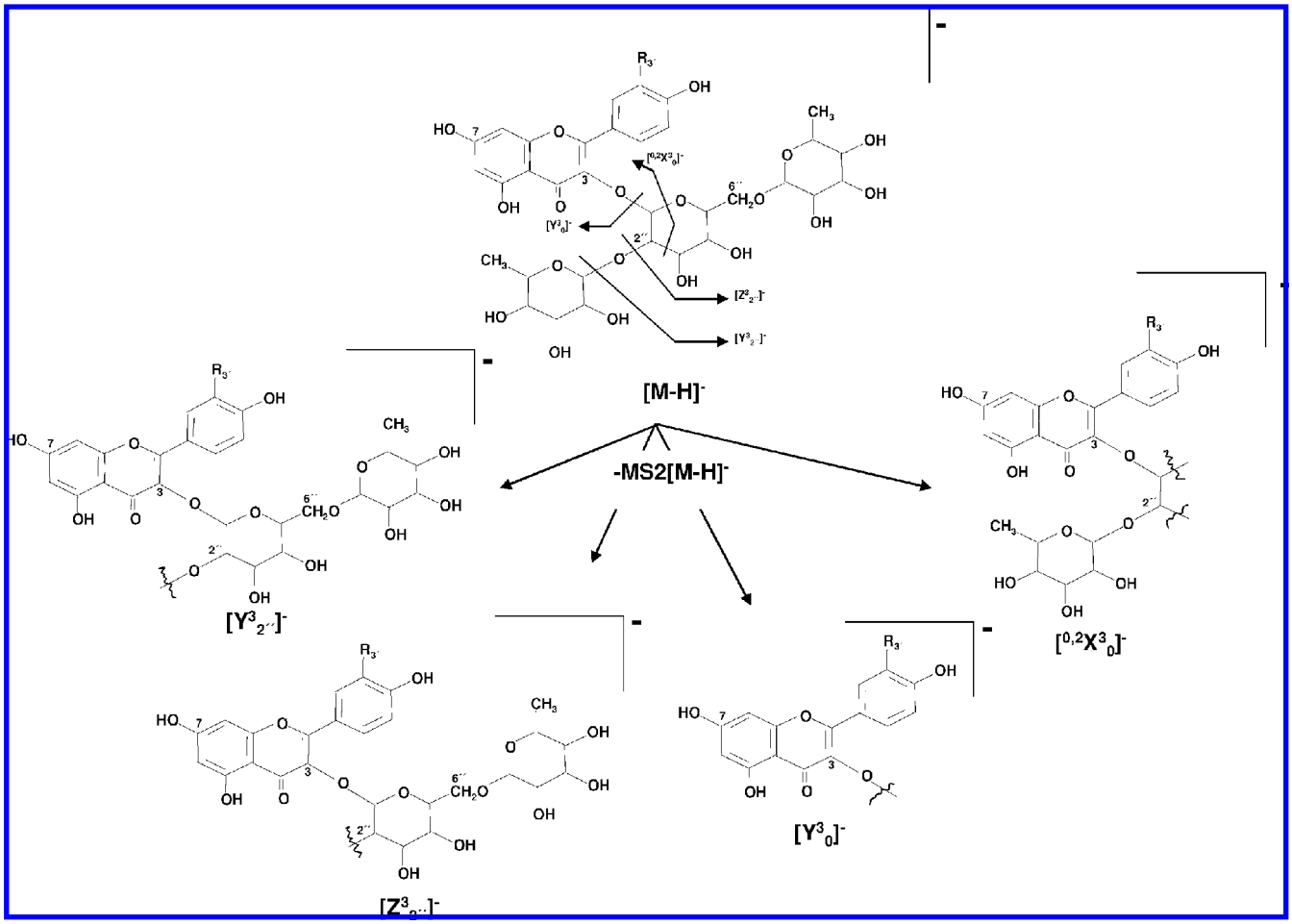

Figure 2. MS2[M − H]$^-$ fragmentation of flavonol-3-*O*-(2,6-di-*O*-rhamnosyl-hexoside). Compounds: **7** and **8** ($R_{3'}$: OH); **9** and **10** ($R_{3'}$: H); **12** and **14** ($R_{3'}$: OCH_3).

Table 1. t_R, UV, and −MS Data for Flavonol Glycosides from Hydroalcoholic Extracts of *Catharanthus roseus*[a]

	Compounds[b]	t_R (min)	UV (nm)	[M-H]$^-$ (*m/z*)	-MS2[M-H]$^-$ (*m/z*) (%)	-MS3[(M-H)→(M-H-162)]$^-$ (*m/z*) (%)				
					Flavonol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)-7-*O*-hexoside					
					Y^7_0 (-162)	$Y^7_0Y^3_{2''}$ (-146)	$Y^7_0Z^3_{2''}$ (-164)	$Y^7_0{}^{0,2}X^3_0$ (-266)	$[Y^7_0{}^{0,2}X^3_0$-146]$^-$ (-412)	$Y^7_0Y^3_0$ Aglc-H/2H
2	K-3-(2,6-Rh-Gal)-7-Hx	5.3	265,219sh,347	901	739(100)	593(30)	575(40)	473(20)		285(100)
5	K-3-(6-Rh-Gal)-7-Gal	8.2	265,347	755	593(100)				327(16)	285(100)
6	K-3-(6-Rh-Gal)-7-Glc	9.8	---[c]	755	593(100)					285(100)
					Flavonol-3-*O*-(2,6-di-*O*-rhamnosyl-hexoside)					
					-MS2[M-H]$^-$, (*m/z*) (%)					
					$Y^3_{2''}$ (-146)	$Z^3_{2''}$ (-164)	$^{0,2}X^3_0$ (-266)	$[^{0,2}X^3_0$-146]$^-$ (-412)	Y^3_0 Aglc-H/2H	
7	Q-3-(2,6-Rh-Gal)	14.2	255,267sh,299sh,353	755	609(17)	591(36)	489(18)	343(35)	300(100)	
8	Q-3-(2,6-Rh-Glc)	14.8	255,267sh,299sh,354	755	609(17)	591(44)	489(14)	343(10)	300(100)	
9	K-3-(2,6-Rh-Gal)	17.9	265,296sh,347	739	593(15)	575(44)	473(10)	327(23)	285(100)	
10	K-3-(2,6-Rh-Glc)	18.9	265,299sh,348	739	593(20)	575(60)	473(18)	327(10)	284(100)	
12	I-3-(2,6-Rh-Gal)	20.2	---[c]	769	623(22)	605(26)	502(5)	357(6)	315(100)	
14	I-3-(2,6-Rh-Glc)	22.2	255,267sh,300sh,354	769	623(12)	605(9)	502(1)	357(12)	315(100)	
					Flavonol-3-*O*-(6-*O*-rhamnosyl-hexoside)					
					-MS2[M-H]$^-$, (*m/z*) (%)					
					$^{0,2}X^3_0$ (-266)	Y^3_0 Aglc-H/2H				
11	Q-3-(6-Rh-Gal)	20.0	---[c]	609	343(10)	300(100)				
13	Q-3-(6-Rh-Glc)	20.9	---[c]	609		301(100)				
15	K-3-(6-Rh-Gal)	24.1	265,295sh,347	593	327(10)	285(100)				
16	K-3-(6-Rh-Glc)	27.3	265,295sh,347	593		285(100)				
17	I-3-(6-Rh-Gal)	27.9	255,266sh,301sh,355	623		315(100)				
18	I-3-(6-Rh-Glc)	29.6	255,266sh,301sh,355	623		315(100)				

[a] Main observed fragments. Other ions were found but they have not been included. [b] Q: quercetin. K: kaempferol. I: isorhamnetin. Hx: hexoside. Gal: galactoside. Glc: glucoside. Rh: rhamnoside. [c] Compounds hidden by others or in traces. Their UV spectra have not been properly observed.

cetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside) (**7**) and kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside) (**9**) already detected in *C. roseus* leaves (*16*) and stems (*17*). Their structures had been previously determined. Compound **7** was found in *Lysimachia fortunei* (*18*) and compound **9** in *L. mauritiana* (*19*). The MS2 (**Table 1**) fragmentation of these compounds is in accordance with the proposed structures. As discussed above, the base peak corresponds to the deprotonated aglycon ion ($[\mathbf{Y^3_0}]^-$), as expected for flavonoid glycosides with one substitution. On the other hand, it can be observed that the fragmentation of the rhamnose in the 2″ position gives rise to the ions $[\mathbf{Y^3_{2''}}]^-$ ([(M − H) − 146]$^-$) and $[\mathbf{Z^3_{2''}}]^-$ ([$Y^3_{2''}$-18]$^-$, [(M − H) − 164]$^-$) (*20*) (**Figure 2**). In some cases the $[\mathbf{Z^3_{2''}}]^-$ ion exhibits a very high abundance, even being the base peak, but the simultaneous loss of rhamnosyl radical and water indicates an interglycosidic bond, and not a link to phenolic hydroxyl (*21, 22*). Other obvious peaks resulted from the internal cleavage of galactose to originate the ion $[\mathbf{^{0,2}X^3_0}]^-$, a fragment that preserves the rhamnose linked at 2″ position (**Figure 2**), and $[\mathbf{^{0,2}X^3_0\text{-}146}]^-$ ion, which lost the rhamnose at the 2″ position (**Table 1**). These compounds are also present in seeds and petals: compound **9** is the main compound of the seeds and very abundant in petals, while compound **7** is important in the seeds but vestigial in the petals (**Figure 1**).

Another compound with a similar fragmentation is isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside) (compound **12**), detected only in trace amounts in the stems and leaves (**Figure 1**, **Table 1**). In addition, compounds **8**, **10** and **14**, isomers of **7**, **9** and **12**, respectively, and displaying the same MS2 fragmentation were detected. The longer t_R in reversed phase HPLC of **8**, **10** and **14** relative to their isomers (**Figure 1**, **Table 1**) indicates that glucose could be the hexose, derivatives of which elute after those of galactose (*23*). These compounds were therefore tentatively characterized as quercetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside) (**8**), kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside) (**10**) and isorhamnetin-3-*O*-(2,6-di-*O*-rhamnosyl-glucoside) (**14**). Compound **8** was detected solely in the petals and in trace amounts. Compound **10** was found in the stems, seeds and petals, in higher amounts in the later. Compound **14** was present just in the seeds.

Six diglycosides, rhamnohexoside derivatives of quercetin, kaempferol and isorhamnetin (compounds **11**, **13**, **15**−**18**), were also detected. In their MS2 fragmentation (**Table 1**) mainly the deprotonated aglycon ion was observed, which indicates a 1→6 interglycosidic linkage (*20*). These data, in conjunction with the reversed phase HPLC chromatographic behavior, suggest these compounds to be similar to the triglycosides without rhamnose in the 2″ position previously mentioned. Thus, quercetin-3-*O*-(6-*O*-rhamnosyl-galactoside) (**11**), quercetin-3-*O*-(6-*O*-rhamnosyl-glucoside) (**13**), kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside) (**15**), kaempferol-3-*O*-(6-*O*-rhamnosyl-glucoside) (**16**), isorhamnetin-3-*O*-(6-*O*-rhamnosyl-galactoside) (**17**) and isorhamnetin-3-*O*-(6-*O*-rhamnosyl-glucoside) (**18**) were tentatively identified. Compounds **15** and **18** were found in small amounts in the seeds, while petals contained compounds **11**, **13**, **15**-**18**, compound **15** being the most abundant in this material (**Figure 1**).

Three compounds (**2**, **5** and **6**) exhibited a MS2 fragmentation in which the ion corresponding to the loss of 162 u from the [M − H]$^-$ was only seen. In the MS3[(M − H) → (M − H − 162)]$^-$ event of compound **2** the fragmentation was similar to that of the triglycosides referred to above, whereas the MS3 of compounds **5** and **6** resembles that of the diglycosides group. These data indicate that these compounds are derivatives of the previous ones, containing an additional glycosylation at the 7 position with hexose (*15*). For biosynthetic reasons, kaempferol-3-*O*-(2,6-di-*O*-rhamnosyl-galactoside)-7-*O*-hexoside (**2**) must proceed from **9**, which is the most abundant in seeds. Compounds **5** and **6** can derive from **15** and **16**, respectively, the main phenolics in petals, although in *Vinca minor* kaempferol-3-*O*-(6-*O*-rhamnosyl-glucoside)-7-*O*-glucoside was found (*16*). Thus, compounds **5** and **6** are isomers of kaempferol-3-*O*-(6-*O*-rhamnosyl-hexoside)-7-*O*-hexoside, tentatively kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-galactoside (**5**) and kaempferol-3-*O*-(6-*O*-rhamnosyl-galactoside)-7-*O*-glucoside (**6**).

Syringetin-3-*O*-robinoside (3,5,7,4′-tetrahydroxy-3′,5′-dimethoxyflavone-3-*O*-(6-*O*-rhamnosyl-galactoside)), a flavonoid isolated before from *C. roseus* stems (*17*), was not detected in the analyzed samples; neither was any other syringetin derivative. For it we had extracted from the HPLC−MS the ion at *m*/*z* 653 [M − H]$^-$, as well as those ions for which the fragmentation led to the ion at *m*/*z* 345 (deprotonated syringetin ion) ("extracted ion chromatogram"). However, no syringetin-3-*O*-robinoside was found.

The presence of anthocyanins in the petals was also checked, since this class of compounds has already been detected in cell cultures and in flowers (*7, 24*−*27*), but no metabolite was observed. In fact, the flowers of the *C. roseus* variety used in this study (cv. Little Bright Eye) have white petals. Thus, it is not surprising that no anthocyanin compounds were detected in the analyzed sample.

In addition to the flavonoids described above, we also detected 3-*O*-caffeoylquinic acid (**1**) (t_R, 3.9 min; UV, 299sh, 325 nm; MS, 353 [M − H]$^-$; MS2[M − H]$^-$, 191 (100), 179 (48)), 4-*O*-caffeoylquinic acid (**3**) (t_R, 5.9 min; UV, 299sh, 325 nm; MS, 353 [M − H]$^-$; MS2[M − H]$^-$, 191 (80), 179 (60), 173 (100)) and 5-*O*-caffeoylquinic acid (**4**) (t_R, 7.7 min; UV, 299sh, 325 nm; MS, 353 [M − H]$^-$; MS2[M − H]$^-$, 191 (100), 179 (2)) in stems and leaves and compound **3** in petals (**Figure 1**), according to the method of Clifford et al. (*28*). 5-*O*-Caffeoylquinic acid was detected in *C. roseus* leaves (*29*). The structures of the identified compounds are shown in **Figure 3**.

Our results show that leaves and stems are particularly rich in caffeoylquinic acids in comparison with seeds and petals, while these later mainly present a great variety of flavonoids. This may relate with the proposed functions of caffeoylquinic acids as protectors against herbivorism and infection (*30*), a function particularly relevant for the organs involved in vegetative growth (stems and leaves). On the other hand, the abundance of flavonoids in petals and seeds may be associated with the frequently reported function of this group of compounds in the attraction of pollinators and seed dispersers (*31*).

Screening of Antioxidant Capacity. Basic knowledge on the efficacy of compounds in extracts to quench free radicals can be inferred from the DPPH$^\bullet$ assay. The screening of the lyophilized aqueous extracts of the several *C. roseus* materials revealed a concentration-dependent antioxidant capacity for all of them, with the petals presenting the strongest effects (IC_{50} at 197 μg/mL), followed by seeds (IC_{50} at 265 μg/mL) and leaves (IC_{50} at 447 μg/mL). Stems displayed the weakest activity (IC_{50} at 476 μg/mL) (**Figure 4A**).

In the present work, extracts from *C. roseus* tissues were tested for their ability to scavenge superoxide radical and nitric oxide, reactive oxygen and nitrogen species, respectively. Superoxide radical is the first product of oxygen univalent reduction. Its biological significance is related with its capacity to generate other more reactive species, like hydroxyl radical and peroxynitrite, despite its participation in the bactericidal

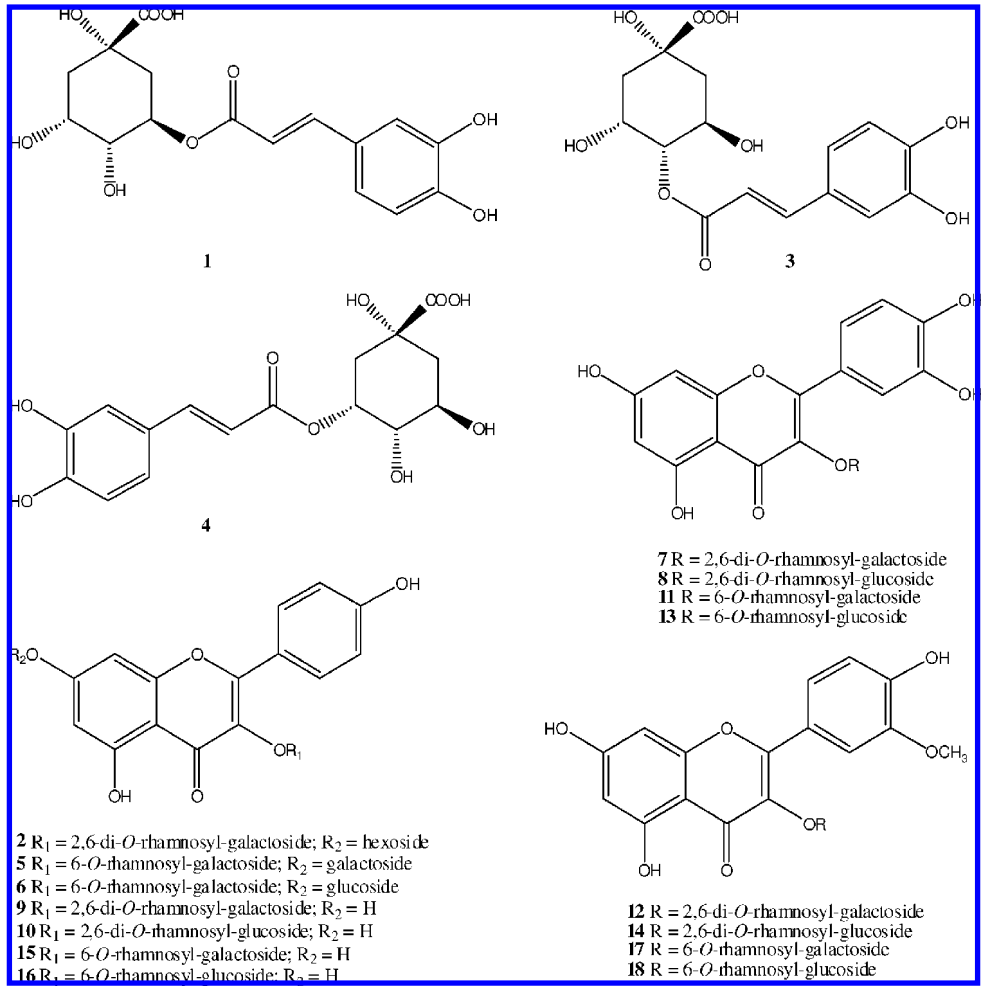

Figure 3. Chemical structures of the phenolic compounds identified in *Catharanthus roseus* material. Identity of compounds as in **Figure 1**.

activity of phagocytic cells (*32*). Extracts from the four tissues exhibited a concentration-dependent superoxide radical scavenging capacity: the seeds were the most effective material (IC_{50} at 74 μg/mL), followed by leaves (IC_{50} at 90 μg/mL) and stems (IC_{50} at 202 μg/mL), while petals showed the lower activity (IC_{50} at 260 μg/mL) (**Figure 4**B).

Nitric oxide is involved in several physiological processes, like blood pressure control, neural signal transduction, platelet function and antimicrobial defense (*33, 34*). Despite the beneficial effects, an overproduction of this reactive nitrogen species is associated with several types of biological damage, such as lipid peroxidation, protein oxidation and nitration, enzymes inactivation and DNA damage. In addition, it reacts rapidly with superoxide radical to form peroxynitrite, a major damaging oxidant produced in vivo (*35*). The analyzed extracts displayed protective activity against nitric oxide, which was concentration dependent. Among the distinct materials the scavenging ability followed the order petals (IC_{25} at 232 μg/mL) > seeds (IC_{25} at 320 μg/mL) > leaves (IC_{25} at 505 μg/mL) > stems (IC_{25} at 546 μg/mL), as shown in **Figure 4C**. Thus, besides the scavenging capacity observed for both superoxide radical and nitric oxide, *C. roseus* may also prevent the formation of other biologically important oxidative species resultant from the reaction of those two, like peroxynitrite and hydroxyl radical.

Total antioxidant capacity in plants and their derived products results from the interactions between the several constituents, which may include synergistic or additive effects. Therefore, it is important to consider the extracts as a whole, because their effects are not due to a single compound, but to a large number of structurally related and unrelated compounds contributing to that effect (*36*). Herbal water extracts, namely infusions, are an important source of antioxidant phenolic compounds in the human diet. So, according to the folk use of this species referred above, it seems more realistic to evaluate the potential of an aqueous extract. The phenolics composition of the aqueous lyophilized extracts of *C. roseus* material used for the antioxidant activity screening were similar to the hydromethanolic ones used for phenolics characterization. This allows us to suggest that the identified compounds might contribute to the observed properties. In fact, both 5-*O*-caffeoylquinic acid (*37*) and quercetin, kaempferol and isorhamnetin glycosides (*38, 39*) have already revealed antioxidant capacity, in several systems. When comparing the results obtained with those found for the extracts of some edible matrices, such as *Brassica oleracea* var. *costata* or *Cydonia oblonga*, assayed under the same conditions and already reported as antioxidants, it can be noticed that, in a general way, *C. roseus* materials are more effective (*6, 13, 40*−*42*), although less active than reference compounds like kaempferol or rutin (*5, 6*).

The antioxidant activity of phenolics is ascribed to the number of substituted hydroxyl or methoxyl groups and glycosylation around the flavonoid skeleton (*37*), molecules bearing *ortho*-dihydroxyls being more active than those not possessing such functionalities. It is known that the *ortho*-dihydroxyl substitution on phenol renders the oxidation intermediate, *ortho*-hydroxyphenoxyl radical, more stable due to intramolecular hydrogen bonding interaction. The *ortho*-hydroxyphenoxyl radical and/

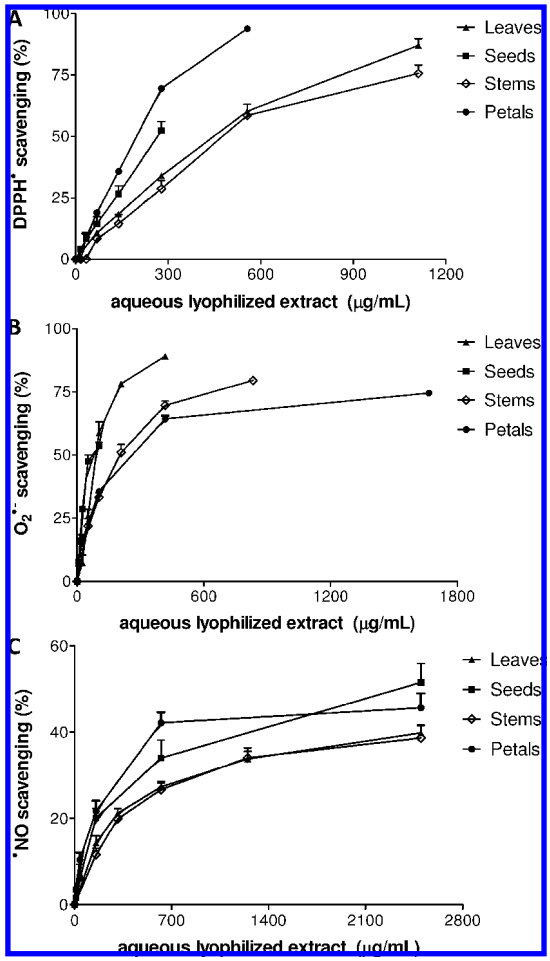

Figure 4. Effect of *Catharanthus roseus* aqueous extracts on the scavenging of (**A**) DPPH•; (**B**) superoxide radical; (**C**) nitric oxide. Values show mean ± SE from 3 experiments performed in triplicate.

or *ortho*-semiquinone radical anion can be easily further oxidized to form the final *ortho*-quinone. In addition, the presence of a hydroxyl in 5- position of the flavonoid contributes to the scavenging potential by increasing resonance in the presence of a 2,3 double bond and 3-hydroxyl and 4-oxo groups (*43*).

In conclusion, this is the first detailed study about noncolored phenolics in *C. roseus* and, as far as we know, with the exceptions of compounds **4**, **7** and **9**, fifteen are described for the first time in this species. The different plant materials displayed antioxidant capacity, for which the identified compounds are, at least partially, contributors. In a general way, petals and seeds revealed the strongest potential, which may turn them interesting matrices for food, pharmaceutical or cosmetic industries. So, as this species is already cultivated in large scale for the extraction of its alkaloids, and in addition to its ornamental features, it could be used for the preparation of antioxidant extracts to be used as preservatives, extending the shelf life of oxidizable formulations and foodstuffs, or to be included as additives with health benefits.

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