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Synthesis and antioxidant properties of new chromone derivatives

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ABSTRACT

Nowadays, the recognition of the benefits of antioxidants is eliciting an increasingly interest in the search for new molecules with improved activity. The aim of the present work was to search for improved reactive oxygen species (ROS) and reactive nitrogen species (RNS) scavengers by testing new structures of 2-styrylchromones (2-SC) and 3-substituted flavones, which were synthesised by the Baker–Venkataraman approach. The new compounds were also tested for their metal chelating capacity and reducing activity. The obtained results showed that the methylation of hydroxyl groups decreases the scavenging of ROS and RNS by 2-SC. The decrease in the scavenging activities was, generally, more evident when the methylation occurred in B-ring, except for $O_2^{\cdot-}$ and $^1O_2$. On the other hand, the introduction of a substituent, either hydroxyl or methoxyl, in position 8 was sometimes favourable and others unfavourable to the scavenging activities, depending on the reactive species. In conclusion, the study of the antioxidant properties of the new 2-SC and flavones allowed establishing new structure–activity relationships and brought out, in some cases, pharmacophores with improved activity.

1. Introduction

The 2-styrylchromones (2-SC) designation presently represents a well recognized group of natural and synthetic chromones, vinylogues of flavones (2-phenylchromones). As it occurs for several flavones, several studies performed with 2-SC have disclosed interesting activities with potential therapeutic applications, possibly in the treatment of cancers,[1–5] allergies,[6] viral infections,[7,8] gout,[9] and oxidative stress related damage.[10] The antioxidant behaviour of these compounds is a matter of particular interest, as previously demonstrated by their strong protective effects against pro-oxidant agents observed in cellular systems[10] and in non-cellular systems.[11] Recently our group has tested several 2-SC and structural-similar flavonoids for their scavenging activities against reactive oxygen species (ROS) and reactive nitrogen species (RNS), providing outstanding results for several of the tested compounds, considering the nanomolar to micromolar range of the IC_{50} values found.[12] In that study, we demonstrated that the 3′,4′-dihydroxy substitution on the B-ring plays an important role in the scavenging activity of 2-SC for ROS and RNS. In addition, the number and position of the hydroxyl groups in the A-ring also seems to contribute to the scavenging effect of these compounds. It was also pointed out the likely contribution of the styryl moiety to the molecular stabilization, increasing the compound's antiradical activity.

The aim of the present work was to continue searching for improved ROS and RNS scavengers by testing new structures of 2-SC (**7a–7c**) and 3-substituted flavones (**8a–8c**, **9**) (Scheme 1 and Fig. 1). In what concerns to 2-SC we studied the effects resulting from the methylation of the 3′,4′-dihydroxy group (**7c**), the changes in the position of the hydroxyl groups in the A-ring as well as their methylation (**7a–7c**). Regarding the flavones, we tested new compounds with different length alkyl chains in the position 3 of the C-ring (**8a–8c**) and with a 3,4-dihydroxybenzoyl substituent in the same position (**9**).

Besides the scavenging of ROS and RNS the new compounds were also tested for their metal chelating activity and for their reducing capacity since these are indicators of antioxidant activity. A compound can act as antioxidant through the chelation of transition metals (especially iron and copper), preventing their participation in free radical generating reactions. In fact, iron and copper have a major role in the production of the very reactive hydroxyl radical ($HO^{\cdot}$), through the Fenton and Haber-Weiss reactions.[13–16] In addition, through a Fenton type reaction, preformed lipid hydroperoxides (ROOH) are decomposed to form the alkoxyl radicals ($RO^{\cdot}$), strong oxidants, which can propagate the chain reaction of lipid peroxidation.[6,17] On the other hand, the antioxidant power of certain substances, phenolic compounds in particular, can be assessed by methodologies that measure the reducing capacity employing ferric iron as oxidant.[18,19]

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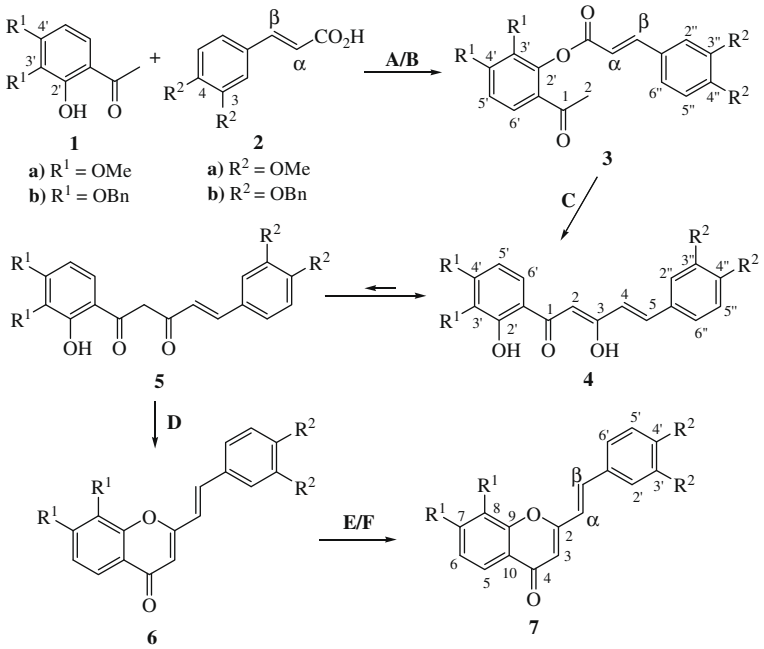

For compounds **3**, **4**, **5** and **6**
a) R^1 = OMe; R^2 = OBn
b) R^1 = OMe, R^2 = OMe
c) R^1 = OBn, R^2 = OMe

For compounds **7**
a) R^1 = OMe; R^2 = OH
b) R^1 = OH, R^2 = OH
c) R^1 = OH, R^2 = OMe

A: Py, $POCl_3$, room temp., N_2
B: CH_2Cl_2, DCC, 4-pyrrolidinopyridine, room temp., N_2
C: DMSO, KOH, 2 h, room temp.
D: *p*-toluenesulfonic acid, DMSO, 90ºC
E: HCl (37%)/AcOH glacial (1:10), 90ºC, 2-12 h.
F: BBr_3/CH_2Cl_2, -78ºC to room temp. (24 h)

Scheme 1. Synthesis of the studied 2-styrylchromones.

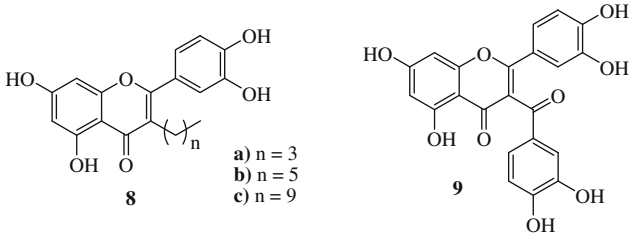

Figure 1. Chemical structures of the studied 3-substituted flavones.

2. Results and discussion

2.1. Chemistry

The 2-SC **7a–7c** and 3-substituted flavones **8a–8c** and **9** were synthesised by the Baker–Venkataraman method.[20–22] 3-Substituted flavones **8a–8c** and **9** have been recently published,[23,24] while 2-SC **7a–7c** are new compounds, reported for the first time in the present study. The first step of this synthesis involves the esterification of 2′-hydroxyacetophenones **1a**, **1b** with cinnamoyl chlorides **2a**, **2b**, prepared in situ from the corresponding cinnamic acids and phosphorous oxychloride in dry pyridine (Scheme 1).[25] The yield of compounds **3b** and **3c** is higher if the esterification reaction is conducted with *N*,*N*-dicyclohexylcarbodiimide (DCC), as coupling esterification agent, in the presence of a catalytic amount of 4-pyrrolidinopyridine.[26] Baker–Venkataraman rearrangement of esters **3a–3c** with potassium hydroxide in DMSO afforded β-diketones **5a–5c** in good yields (60–95%); these were submitted to a cyclodehydration process with a *p*-toluenesulfonic acid/DMSO mixture, leading to the synthesis of alkoxy-2-styrylchromones **6a–6c** in good yields (62–84%). The final step of our synthetic route consisted on the demethylation of alkoxy-2-styrylchromones **6a–6c**. The cleavage of the methyl groups of 3′,4′,7,8-tetramethoxy-2-styrylchromones **6b** was performed by treatment with boron tribromide (2.5 equiv per methyl group) in dichloromethane for 24 h (Scheme 1). The cleavage of the benzyl groups of methoxybenzyloxy-2-styrylchromones **6a**, **6c** was carried out by treatment with a mixture of chloridric acid in acetic acid, leading to the expected methoxy-2-styrylchromones **7a**, **7c**.

2.2. Antioxidant activity

Free radical scavenging capacity of flavone derivatives, has been primarily attributed to the high reactivity of hydroxyl substituents that participate in the following reaction:[27]

$$\text{Flavone-OH} + R^{\cdot} \rightarrow \text{Flavone-O}^{\cdot} + RH$$

As reviewed by Heim et al.,[27] the B-ring 3′,4′-catechol structure is the most significant determinant of scavenging of ROS and RNS, leading to the formation of fairly stable *ortho*-semiquinone radicals through facilitating electron delocalization while the effects resulting from the hydroxylation pattern of ring A are more variable. This was only partially confirmed in our study.

All of the tested compounds were able to prevent $O_2^{\cdot -}$-dependent reduction of NBT in a concentration-dependent manner. The most effective were the 2-SC and flavone **9**. The order of potencies found was **7b** > **9** > **7a** > **7c** > **8a** > **8b** > **8c** (Table 1). We confirmed

Table 1
$O_2^{\cdot-}$, H_2O_2, $^1O_2$ and HOCl scavenging activities (IC_{50}, mean ± SE) of the tested 2-styrylchromones, flavones and positive controls

Compound	IC_{50} (μM)			
	$O_2^{\cdot-}$	H_2O_2	$^1O_2$	HOCl
2-Styrylchromones				
7a	20.8 ± 3.2	701.9 ± 58.2	7.97 ± 1.38	4.67 ± 0.64
7b	17.8 ± 3.8	799.3 ± 60.9	4.69 ± 0.64	67.2 ± 11.2
7c	26.0 ± 3.8	31%[a]	9.95 ± 2.27	47%[b]
Flavones				
8a	51.8 ± 3.3	501.8 ± 43.9	61.8 ± 7.0	1.45 ± 0.22
8b	62.8 ± 3.6	347.7 ± 28.2	58.6 ± 5.6	2.49 ± 0.38
8c	78.0 ± 4.9	235.7 ± 7.8	7.9 ± 1.0	1.70 ± 0.13
9	19.5 ± 2.6	366.9 ± 36.4	43.0 ± 7.6	2.29 ± 0.36
Positive controls				
Tiron	287.7 ± 32.0	—	—	—
Ascorbic acid	—	567.4 ± 33.0	10.2 ± 1.5	—
Lipoic acid	—	—	—	1.95 ± 0.06

Each study corresponds to five experiments, performed in triplicate.
[a] Scavenging effect (mean%) at the highest tested concentration (1 mM).
[b] Scavenging effect (mean%) at the highest tested concentration (200 μM).

that the methylation of the hydroxyl groups, especially those in the B-ring, seems to be disadvantageous to the $O_2^{\cdot-}$ scavenging activity (compound **7b** vs compounds **7a** and **7c**). However, the new 2-SC tested were shown to have improved $O_2^{\cdot-}$ scavenging activity when compared with those tested before, in spite of complete O-methylation of hydroxyl groups in compound **7c**.[12] This corresponds to a very interesting and new feature of 2-SC, implying a greater importance of the styrylchromone backbone, under the influence of electron-donor activation mediated by *O*-methoxy groups in the B-ring. Also, compound **7b** only differs from the previously tested compound **1A**[12] in the position of one hydroxyl in the A-ring, that is, 5-OH in **1A** became 8-OH in **7b**. Thus, it seems appropriate to infer that this modification improves the $O_2^{\cdot-}$ scavenging activity. In what concerns to flavones, the substitution of the position 3 in the C-ring by an alkyl chain seems to be more unfavourable to their scavenging activity as it becomes bigger. From previous studies, it is known that these 3-alkylpolyhydroxyflavones bearing an alkyl chain with increasing length allow the control of oxidation processes at variable depths in organized microstructures such as ionic or neutral micelles.[28] However, the structure–activity relationships drawn from antioxidant protection provided by these 3-alkylflavones strongly depends on the model studied,[29] supporting our present results. On the other hand, the C-3 3,4-dihydroxybenzoyl substituent, in compound **9**, improves the $O_2^{\cdot-}$ scavenging activity perhaps due to the catechol group included. The IC_{50} of the new 2-SC was remarkably lower than the positive control, tiron, (287.7 ± 32.0) (Table 1).

All of the tested compounds were able to scavenge H_2O_2 in a concentration-dependent manner. 2-SC were less effective than flavones. The order of potencies found was **8c** > **8b** > **9** > **8a** > **7a** > **7b** > **7c** (Table 1). The structural modifications introduced in the tested 2-SC are unfavourable for the H_2O_2 scavenging activity since these compounds are much weaker scavengers than the previously tested compounds **1A**–**1D** (3′,4′-dihydroxy-2-styrylchromone derivatives).[12] This suggests the importance of the catechol group in the B-ring, but also a possible critical contribution of the A-ring hydroxylation pattern. Results from the flavones **8a**, **8b**, **8c** show that the H_2O_2 scavenging activity increases with the growth of the alkyl chain. Moreover, all of the C-3 substituents introduced in the tested flavones where shown to improve the effect of the parent compound, luteolin.[12]

The tested flavones and compound **7a** were shown to be strong scavengers of HOCl. Compounds **7b** and **7c** were much weaker scavengers although both were effective in a concentration-dependent manner. The order of potencies found was **8a** > **8c** > **9** > **8b** > **7a** > **7b** > **7c** (Table 1). Considering what has been previously demonstrated for the flavonols quercetin and rutin[30] and for the isoflavones genistein, biochanin-A, and daidzein[31] the reaction of the tested flavones with HOCl is likely to occur via an electrophilic aromatic substitution consisting in the chlorination of C-6 and C-8 of the A-ring, where 5-OH and 7-OH groups are the most reactive. The presence of a substituent in C-3 is apparently favourable to the reaction with HOCl, since all of the tested flavones where shown to be more active than their parent compound, luteolin.[12]

All of the tested compounds were able to prevent the $^1O_2$-dependent oxidation of DHR. Some of them were even more effective than ascorbic acid. The order of potencies found was **7b** > **8c** > **7a** > **7c** > **9** > **8b**≈**8a** (Table 1). From the analysis of the results of 2-SC, it seems that the methylation of the hydroxyl groups is disadvantageous for the $^1O_2$ scavenging activity, since the compounds **7a** and **7c** where slightly less effective than compound **7b**. No meaningful differences were found between the new 2-SC and those tested before (3′,4′-dihydroxy-2-styrylchromone derivatives),[12] which indicates that the substitution in the position 8 does not bring important changes to the $^1O_2$ scavenging activity. The tested flavones where much less active then their parent compound, luteolin,[12] with the exception of compound **8c**. The high activity showed by this compound can be explained by the proposed mechanism of the chemical reactions of flavones with $^1O_2$. It has been previously suggested[32] that there is a cycloaddition of $^1O_2$ to the double bond of the C-ring, leading to the formation of a 1,2-dioxetane intermediate. This reaction is potentiated by electron-donating substituents in C-3, which activate the double bond. Following this rationale, the higher activity of compound **8c** over **8a** and **8b** is understandable because the alkyl chain C-3 substituent is bigger in **8c**, thus more electron-donating. The higher effect of 2-SC over flavones is probably due to the double bond in the styryl moiety, which is a possible target for $^1O_2$.[33]

The results from the ORAC assay are listed in Table 2. All the compounds were able to prevent the ROO·-dependent oxidation of fluorescein. All of them were considerably more active than ascorbic acid. The order of potencies found was **9** > **7a** > **8a** > **7b** > **8b** > **8c** > **7c** (Table 2). The results showed that compounds **7b** and **7a** achieved similar ORAC values while compound **7c** had a lower effect. This behaviour might be caused by the methylation of the hydroxyl substituents in B-ring. In fact, it was previously shown that methylation reduces the antioxidant capacity of polyphenols, tested by the same method.[34] All of the 2-SC were much less active than the previously tested 2-SC from group 1 (3′,4′-dihydroxy-2-styrylchromone derivatives).[12] This can also be explained by the methylation theory, but only for compounds **7a** and **7c**, not for compound **7b**. Thus, it is possible that the reduced

Table 2
ROO· scavenging activity of the tested 2-styrylchromones, flavones, and ascorbic acid expressed as ORAC values (mean ± SE)

Compound	$ORAC_{ROO\cdot}$ ± SE (μM trolox equiv/μM compound)	Concentration range (μM)
2-Styrylchromones		
7a	2.74 ± 0.12	0.25–2
7b	2.58 ± 0.13	0.5–4
7c	1.40 ± 0.08	0.5–4
Flavones		
8a	2.66 ± 0.16	0.5–4
8b	2.00 ± 0.14	1–8
8c	1.63 ± 0.14	0.5–4
9	4.32 ± 0.28	0.25–2
Positive control		
Ascorbic acid	0.405 ± 0.033	1–8

Each study corresponds to five experiments, performed in triplicate.

ORAC value is caused by the hydroxyl or methoxyl substitution in position 8, which is, apparently, unfavourable to the scavenging activity against ROO·. In what concerns to flavones, the analysis of the results shows that the ORAC decreases along with the increase of the length of the alkyl chain in C-3 (compounds **8a**, **8b**, and **8c**). Because the increase of the length of the alkyl chain implies a decrease in the hydrophilicity, the obtained results are likely due to solubility issues, since the reaction occurs in aqueous solution. On the other hand, compound **9** was shown to be the most active of the tested flavones, which indicates that the 3,4-dihydroxybenzoyl substituent in C-3 is more favourable to the scavenging activity against ROO· than the alkyl chain. Still, a hydroxyl substituent (quercetin) or no substituent at all (luteolin) caused stronger effects.[12]

The $ONOO^-$-induced oxidation of DHR was efficiently prevented by all of the tested compounds. In general, the compounds were shown to be more effective in the absence of bicarbonate than in its presence, with the exception of compound **7a**. The order of potencies found was **7c** > **8a** > **9** > **8c** > **7b** > **7a** > **8b**, without bicarbonate and **7a** > **8a** > **7b** > **8b** > **8c** > **9** > **7c**, with bicarbonate (Table 3). Compounds **7b**, **7a**, and **7c** were all less active than the previously tested 3′,4′-dihydroxy-2-styrylchromone derivatives[12] either with or without the addition of bicarbonate, to mimic physiological conditions of CO_2. This means that the presence of a substituent in position 8 and the methylation of the hydroxyl substituents are detrimental to the scavenging effect against $ONOO^-$. The decrease of this effect upon methylation of the hydroxyl groups has previously been shown by others for quercetin and phenol.[35] The C-3 substituents in the tested flavones could not favour the $ONOO^-$ scavenging activity. In fact, all of these compounds were much less potent than their unsubstituted counterpart, luteolin.[12] It is possible that the C-3 substituents cause steric hindrance to the catechol moiety, which is accepted to be of major importance to the scavenging activity of flavonoids and other phenolic compounds.[36–38] All of the tested compounds, except compound **7a**, were less effective scavengers of $ONOO^-$ in the presence of bicarbonate. The extremely fast reaction between $ONOO^-$ and CO_2 ($K = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$)[39,40] originates the nitrosoperoxycarbonate anion ($ONOOCO_2^-$), whose decomposition leads to the formation of different species including the highly reactive nitrogen dioxide ($^{\cdot}NO_2$) and carbonate ($CO_3^{\cdot-}$) radicals.[41] The presence of these radicals in solution affects the reactivity of the $ONOO^-$ scavengers, either increasing or decreasing their effects. Nevertheless, the results of the assays in which bicarbonate was added give us, theoretically, a better approach to the developments in a biological system. In fact it is likely that many of the reactions of $ONOO^-$ in vivo are promoted by $ONOOCO_2^-$ derivatives, which are more efficient nitrating and oxidizing species than $ONOO^-$.[41,42]

Table 3
·NO and $ONOO^-$ (with and without 25 mM $NaHCO_3$) scavenging effects (IC_{50}, mean ± SE) of the tested 2-styrylchromones, flavones and positive controls

Compound	IC_{50} (μM)		
	·NO	$ONOO^-$ without $NaHCO_3$	$ONOO^-$ with $NaHCO_3$
2-Styrylchromones			
7a	0.368 ± 0.039	1.21 ± 0.14	0.93 ± 0.21
7b	0.193 ± 0.025	0.85 ± 0.13	1.59 ± 0.05
7c	0.254 ± 0.012	0.59 ± 0.08	2.08 ± 0.16
Flavones			
8a	2.72 ± 0.46	0.60 ± 0.13	1.24 ± 0.19
8b	3.29 ± 0.39	1.48 ± 0.36	1.80 ± 0.29
8c	4.85 ± 0.87	0.83 ± 0.09	1.82 ± 0.22
9	3.62 ± 0.70	0.66 ± 0.09	1.97 ± 0.21
Positive controls			
Rutin	1.17 ± 0.05	—	—
Ebselen	—	1.00 ± 0.11	5.27 ± 0.32

Each study corresponds to five experiments, performed in triplicate.

All of the tested compounds where shown to efficiently inhibit ·NO-induced oxidation of DAF-2, in a concentration-dependent manner. 2-SC showed to be better scavengers of ·NO than flavones. The order of potencies found was **7b** > **7c** > **7a** > **8a** > **8b** > **9** > **8c** (Table 3). The scavenging activity against ·NO in 2-SC was improved by the introduction of a hydroxyl substituent in the position 8 (compound **7b** vs compounds **1A** and **1B**).[12] On the other hand, the methylation of the hydroxyl groups in rings A or B provoked a slight decrease in the activity (compound **7b** vs compounds **7a** and **7c**). The new flavones were considerably less active than luteolin,[12] which means than none of the substituents in C-3 is favourable to the ·NO scavenging activity.

All the tested compounds were able to significantly reduce ferric ion, except **8c**. 2-SC showed to be better reducers than flavones. The order of potencies found was **7b** > **7c** > **7a** > **9** > **8b** > **8a** (Fig. 2). The reducing capacity is an indicator of the hydrogen-donating ability. From the results obtained one can infer that the methylation of the hydroxyl groups in 2-SC increases the reducing capacity (Fig. 2 **7b** vs **7a** and **7c**). Compound **9** was considerably more active than the other flavones, probably because of the presence of a catechol group in the C-3 substituent. Despite phenolic compounds exert their antioxidant effects by redox mechanisms, the compounds with high ferric reducing capacity may exert pro-oxidant effects resulting from the increased production of ferrous iron and its consequent participation in Fenton reactions.[43,44] On the other hand, several flavones are also endowed with metal chelation effects, which is considered a mechanism of antioxidant activity. The spectral changes of the tested 2-SC upon addition of Fe(II) indicate the formation of iron complexes. For compounds **7a** and **7b** the spectrums show bathochromic shifts at 380 to about 420 nm and at 370 nm to about 390 nm, respectively, which, according to the existent knowledge about structural-similar flavonoids, are probably related to changes in B-ring absorption.[45] These shifts were associated with decreases in absorbance (Fig. 3). As expected, compound **7c**, gave rise to spectral changes very different from those that occurred in compounds **7a** and **7b** (Fig. 3). In this compound, the 'iron binding motif' of the B-ring, that is, the 3′,4′-dyhydroxy substituent, is methylated, hindering the complexation with Fe(II). On the other hand, the spectrum shows a small bathochromic shift (10 nm) at a shorter wavelength (270 nm), which is likely to be related to changes in the A-ring,[45] associated with a small absorbance decrease. According to Guo et al.,[46] the tested flavones have two 'iron binding motifs', the 3′,4′-dyhydroxy substituent and the 5,7-dihydroxy substituent. However, the substituents in the position C-3 seem to affect the capacity of flavones to complex ferrous iron since the expected bathochromic shifts were not observed in the spectrum of these compounds (data not shown). This may be due to steric hindrance to the iron binding groups caused by the alkyl and 3,4-dihydroxybenzoyl substituents.

From this study it became clear that the methylation of hydroxyl groups decreases the scavenging of ROS and RNS by 2-SC, which was somehow expected because it is widely known that hydroxyl groups are important for this type of effect. The decrease in the scavenging activities was, generally, more evident when the methylation occurred in B-ring, except for $O_2^{\cdot-}$ and $^1O_2$. On the other hand, the introduction of a substituent, either hydroxyl or methoxyl, in position 8 was sometimes favourable and others unfavourable to the scavenging activities, depending on the reactive species. The effect of the C-3 substituents in flavones was also variable according to the reactive species. In conclusion, the study of the antioxidant properties of the new 2-SC and flavones allowed establishing new structure–activity relationships and brought out, in some cases, pharmacophores with improved activity.

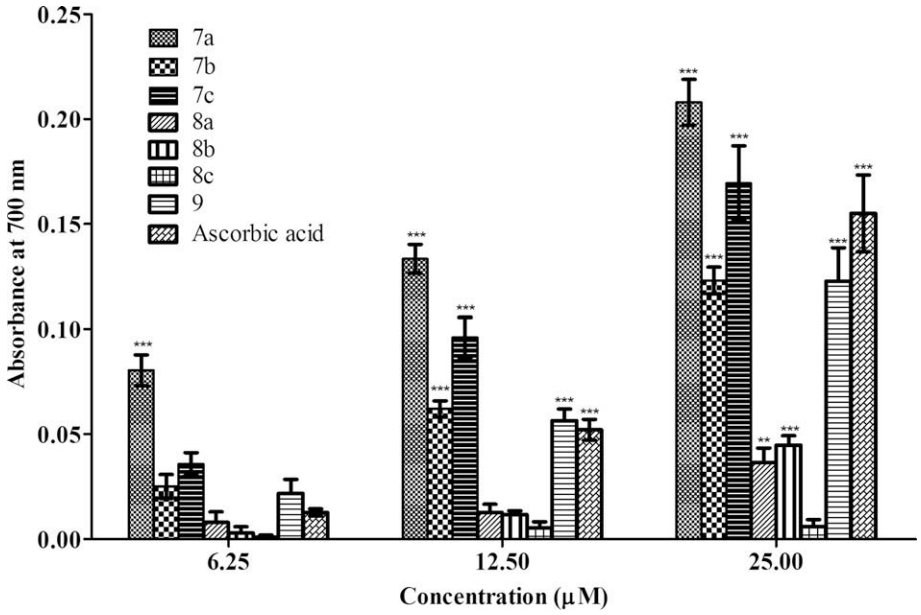

Figure 2. Reducing activity of the tested compounds and ascorbic acid. Each value represents mean ± SE of triplicate measurements. $^{***}P$ <0.001, $^{**}P$ <0.01, $^{*}P$ <0.05, significantly different from control.

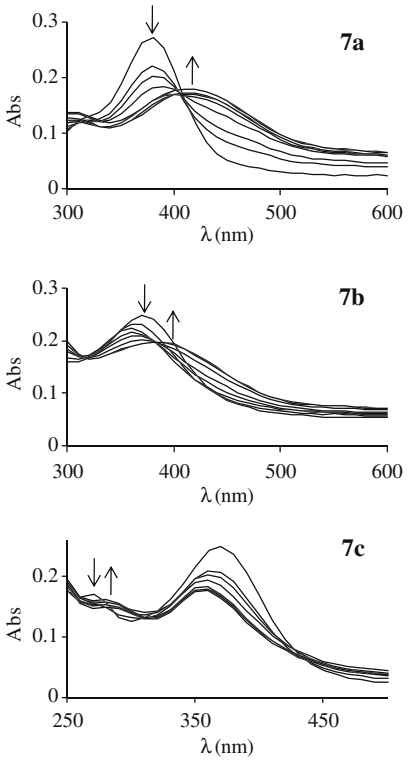

Figure 3. UV–vis absorption spectrum of the tested compounds, at the concentration of 10 μM, in the presence of 0, 2, 4, 6, 8, 10, 12, 14, and 16 μM of Fe(II), in 20 mM phosphate buffer, pH 7.2. The direction of the arrows indicates crescent amounts of Fe(II).

3. Experimental

3.1. General methods

Melting points were measured in a Büchi Melting Point B-540 apparatus and are uncorrected. NMR spectra were recorded on a Bruker Avance 300 spectrometer (300.13 for ^{1}H and 70.47 MHz for ^{13}C), with $CDCl_3$ as solvent, unless otherwise stated. Chemical shifts (δ) are reported in ppm, and coupling constants (J) in hertz. The internal standard was TMS. Unequivocal ^{13}C assignments were made with the aid of 2D *g*HSQC and *g*HMBC (delays for one bond and long-range J C/H couplings were optimised for 145 and 7 Hz, respectively) experiments. Electron impact (EI, 70 eV) MS were recorded on VG Autospec Q spectrometer. Electrospray (ESI, 3000 V, 30 V, source and desolvatation temperatures of 80 °C and 150 °C, respectively) MS were recorded on Micromass Q-Tof-2™ spectrometer. Microanalysis were recorded on CHNS CARLO ERBA 1108 analyser (University of Vigo). Elemental analyses were obtained with a CHNS 932 LECO analyser (University of Aveiro). Preparative thin-layer chromatography was performed with Merck Silica Gel 60 DGF_{254}.

A microplate reader (Synergy HT, BIO-TEK), for fluorescence, absorbance in UV–vis and luminescence measurements, plus temperature control capacity, was used for all the assays.

3.2. Chemicals

All the chemicals and reagents were of analytical grade. Dihydrorhodamine 123 (DHR), 4,5-diaminofluorescein (DAF-2), 30% hydrogen peroxide, ascorbic acid, sodium hypochlorite solution, with 4% available chlorine, lipoic acid, diethylenetriaminepentaacetic acid (DTPA), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC-5), β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), rutin, lucigenin, tiron, and ebselen, were obtained from Sigma–Aldrich (St. Louis, USA). α,α′-Azodiisobutyramidine dihydrochloride (AAPH), histidine and trolox were obtained from Fluka Chemie GmbH (Steinheim, Germany). Fluorescein sodium salt was obtained from Aldrich (Milwaukee, USA).

Column chromatography was performed with Merck Silica Gel 60, 70–230 mesh. All other chemicals and solvents used were obtained from commercial sources and used as received or dried using standard procedures.

3.3. Synthesis of (*E*)-2′-cinnamoyloxyacetophenones 3a–c

Method 1: The appropriate cinnamic acid **2a,b** (9.46 mmol) and phosphorous oxychloride (4.0 mL, 43 mmol) were added to a solution of the appropriate acetophenone **1a,b** (8.6 mmol) in dry pyridine (170 mL). The solution was stirred under different conditions of time and temperature according to the substitution of the compound: **3a**, 2 h at room temperature; **3b**, 12 h at room temperature and **3c**, initially 4 h at room temperature and then 2 h at 60 °C. After that, the solution was poured into ice and water (200 mL), and the pH adjusted to 4 with hydrochloric acid. The obtained solid was filtered and taken in chloroform (50 mL). The organic layer was washed with water (2 × 60 mL), and dried through Na_2SO_4. The solvent was evaporated and the residue was purified by silica gel column chromatography using a 3:7 mixture of light petroleum/dichloromethane as eluent. The solvent was evaporated to dryness in each case, and the residue was crystallized from ethanol to give the expected (*E*)-2′-cinnamoyloxyacetophenones **3a–c** in moderate to good yields: **3a** (86%, 3.98 g), **3b** (40%, 1.33 g) and **3c** (50%, 2.3 g).

Method 2: 3,4-Dimethoxycinnamic acid **2a** (686.5 mg, 3.3 mmol), 4-pyrrolidinopyridine (49 mg, 0.33 mmol) and *N,N*′-dicyclohexylcarbodiimide (680 mg, 3.3 mmol) were added to a solution of the appropriate acetophenone **1a,b** (3.3 mmol) in dichloromethane (20 mL). The solution was stirred, at room temperature, for 18 h. After that, the solid was filtered and washed abundantly with dichloromethane (2 × 20 mL). The filtrate was evaporated and the residue was purified by silica gel column chromatography using a (3:7) mixture of light petroleum/dichloromethane as eluent. The solvent was evaporated to dryness in each case, and the residue was crystallized from ethanol to give the expected (*E*)-2′-cinnamoyloxyacetophenones **3b,c** in good yields: **3b** (88%, 1.12 g), **3c** (67%, 1.19 g).

3.3.1. (*E*)-2′-(3,4-Dibenzyloxycinnamoyloxy)-3′,4′-dimethoxyacetophenone (3a)

Mp 111–112 °C; ^{1}H NMR: δ 7.81 (d, *J* 15.9 Hz, 1H, H-β), 7.66 (d, *J* 8.9 Hz, 1H, H-6′), 7.31–7.48 (m, 10H, 3″,4″-OCH$_2$C$_6$*H*$_5$), 7.21 (d, *J* 2.0 Hz, 1H, H-2″), 7.15 (dd, *J* 8.4 and 2.0 Hz, 1H, H-6″), 6.95 (d, *J* 8.4 Hz, 1H, H-5″), 6.87 (d, *J* 8.9 Hz, 1H, H-5′), 6.53 (d, *J* 15.9 Hz, 1H, H-α), 5.23 (s, 2H, 4″-OC*H*$_2$C$_6$H$_5$), 5.21 (s, 2H, 3″-OC*H*$_2$C$_6$H$_5$), 3.94 (s, 3H, 4′-OC*H*$_3$), 3.83 (s, 3H, 3′-OC*H*$_3$), 2.51 (s, 3H, H-2); ^{13}C NMR: δ 196.0 (C-1), 165.0 (C═O ester), 157.1 (C-4′), 151.4 (C-4″), 148.9 (C-3″), 147.1 (C-β), 144.2 (C-2′), 141.4 (C-3′), 136.7 and 136.5 (C-1 of 3″,4″-OCH$_2$C$_6$H$_5$), 128.5 (C-3,5 of 3″,4″-OCH$_2$C$_6$H$_5$), 127.9 (C-4 of 3″,4″-OCH$_2$C$_6$H$_5$), 127.4 (C-1″), 127.2 and 127.1 (C-2,6 of 3″,4″-OCH$_2$C$_6$H$_5$), 125.8 (C-6′), 124.7 (C-1′), 123.5 (C-6″), 114.3 (C-α), 114.0 (C-5″), 113.7 (C-2′), 109.0 (C-5′), 71.2 (C-3″), 70.8 (C-4″), 60.8 (C-3′), 56.0 (C-4′), 29.8 (C-2); MS (EI) *m/z* (rel. int.): 538 (M$^{+\cdot}$, 4), 360 (3), 343 (25), 253 (3), 196 (4), 181 (10), 91 (100), 65 (7); Anal. Calcd for $C_{33}H_{30}O_7$ (538.59): C, 73.59; H, 5.61. Found: C, 73.76; H, 5.53.

3.3.2. (*E*)-3′,4′-Dimethoxy-2′-(3,4-dimethoxycinnamoyloxy)-acetophenone (3b)

Mp 124–125 C; ^{1}H NMR: δ 7.88 (d, *J* 15.9 Hz, 1H, H-β), 7.67 (d, *J* 8.9 Hz, 1H, H-6′), 7.20 (dd, *J* 8.2 and 2.0 Hz, 1H, H-6″), 7.15 (d, *J* 2.0 Hz, 1H, H-2″), 6.91 (d, *J* 8.2 Hz, 1H, H-5″), 6.89 (d, *J* 8.9 Hz, 1H, H-5′), 6.62 (d, *J* 15.9 Hz, 1H, H-α), 3.96 and 3.95 (2 s, 3H + 6H, 4′,3″,4″-OC*H*$_3$), 3.86 (s, 3H, 3′-OC*H*$_3$), 2.54 (s, 3H, H-2); ^{13}C NMR: δ 196.0 (C-1), 165.1 (C═O ester), 157.1 (C-4′), 151.5 (C-4″), 149.2 (C-3″), 147.3 (C-β), 144.3 (C-2′), 141.5 (C-3′), 127.0 (C-1″), 125.9 (C-6′), 124.8 (C-1′), 123.3 (C-6″), 114.2 (C-α), 110.9 (C-5″), 109.7 (C-2″), 109.0 (C-5′), 60.9 (3′-OC*H*$_3$), 56.1, 56.0 and 55.9 (3″,4′,4″-OC*H*$_3$), 29.8 (C-2); MS (ESI+) *m/z* (rel. int.): 409 ([M+Na]$^+$, 100), 425 ([M+K]$^+$, 20), 795 ([2 M+Na]$^+$, 72). Anal. Calcd for $C_{21}H_{22}O_7$ (386.40): C, 65.28; H, 5.74. Found: C, 65.10; H, 5.68.

3.3.3. (*E*)-3′,4′-Dibenzyloxy-2′-(3,4-dimethoxycinnamoyloxy)-acetophenone (3c)

Mp 92–93 °C. ^{1}H NMR: δ 7.77 (d, *J* 15.9 Hz, 1H, H-β), 7.65 (d, *J* 8.9 Hz, 1H, H-6′), 7.31–7.46 (m, 7H, 3″,4″-OCH$_2$C$_6$*H*$_5$), 7.20–7.23 (m, 3H, 3″,4″-OCH$_2$C$_6$*H*$_5$), 7.15 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6″), 7.09 (d, *J* 1.9 Hz, 1H, H-2″), 6.96 (d, *J* 8.9 Hz, 1H, H-5′), 6.90 (d, *J* 8.3 Hz, 1H, H-5″), 6.50 (d, *J* 15.9 Hz, 1H, H-α), 5.21 (s, 2H, 4′-OC*H*$_2$C$_6$H$_5$), 5.06 (s, 2H, 3′-OC*H*$_2$C$_6$H$_5$), 3.945 (s, 3H, 4″-OC*H*$_3$), 3.943 (s, 3H, 3″-OC*H*$_3$), 2.51 (s, 3H, H-2), ^{13}C NMR: δ 196.0 (C-1), 165.0 (C═O ester), 156.3 (C-4′), 151.5 (C-4″), 149.2 (C-3″), 147.2 (C-β), 144.7 (C-2′), 140.8 (C-3′), 137.0 (C-1 of 3′-OCH$_2$C$_6$H$_5$), 135.9 (C-1 of 4′-OCH$_2$C$_6$H$_5$), 128.7 (C-3,5 of 3′-OCH$_2$C$_6$H$_5$), 128.4 (C-3,5 of 4′-OCH$_2$C$_6$H$_5$), 128.3 (C-4 of 3′-OCH$_2$C$_6$H$_5$), 128.2 (C-2,6 of 3′-OCH$_2$C$_6$H$_5$), 127.9 (C-4 of 4′-OCH$_2$C$_6$H$_5$), 127.4 (C-2,6 of 4′-OCH$_2$C$_6$H$_5$), 127.1 (C-1″), 125.9 (C-6′), 125.1 (C-1′), 123.2 (C-6″), 114.2 (C- α), 110.9 (C-5″), 110.5 (C-5′), 109.7 (C-2″), 75.1 (3′-OCH$_2$C$_6$H$_5$), 71.0 (4′-OCH$_2$C$_6$H$_5$), 56.0 (4″-OCH$_3$), 55.9 (3″-OCH$_3$), 29.9 (C-2); MS (ESI) *m/z* (rel. int.): 539 ([M+H]$^+$, 5), 561 ([M+Na]$^+$, 100), 577 ([M+K]$^+$, 20). Anal. Calcd for $C_{33}H_{30}O_7$ (538.59): C, 73.59; H, 5.61. Found: C, 73.35; H, 5.61.

3.4. Synthesis of 5-aryl-3-hydroxy-1-(2-hydroxyaryl)penta-2,4-dien-1-ones (4a–c)

Potassium hydroxide (powder, 1.4 g, 25 mmol) was added to a solution of the appropriate 2′-cinnamoyloxyacetophenone **3a–c** (5 mmol) in dimethyl sulfoxide (30 mL). The solution was stirred under nitrogen at room temperature for 2 h. After that period, the solution was poured into ice and water (150 mL), and the pH adjusted to 4 with diluted hydrochloric acid. The obtained solid was removed by filtration, taken in chloroform (80 mL) and washed with water (2 × 80 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated to dryness in each case. The residue was crystallized from ethanol to give the expected 5-aryl-3-hydroxy-1-(2-hydroxyaryl)penta-2,4-dien-1-ones **4a–c**: **4a** (93%, 2.5 g), **4b** (62%, 1.2 g) and **4c** (96%, 2.6 g).

3.4.1. 5-(3,4-Dibenzyloxyphenyl)-3-hydroxy-1-(2-hydroxy-3,4-dimethoxyphenyl)penta-2,4-dien-1-one (4a)

Mp 179–180 °C; ^{1}H NMR: δ 14.56 (s, 1H, 3-O*H*), 12.55 (s, 1H, 2′-O*H*), 7.52 (d, *J* 15.9 Hz, 1H, H-5), 7.46 (d, *J* 9.2 Hz, 1H, H-6′), 7.32–7.49 (m, 10H, 3″,4″-OCH$_2$C$_6$*H*$_5$), 7.15 (d, *J* 1.9 Hz, 1H, H-2″), 7.11 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6″), 6.94 (d, *J* 8.3 Hz, 1H, H-5″), 6.50 (d, *J* 9.2 Hz, 1H, H-5′), 6.38 (d, *J* 15.9 Hz, 1H, H-4), 6.18 (s, 1H, H-2), 5.21 (s, 2H, 4″-OC*H*$_2$C$_6$H$_5$), 5.20 (s, 2H, 3″-OC*H*$_2$C$_6$H$_5$), 3.94 (s, 3H, 4′-OC*H*$_3$), 3.91 (s, 3H, 3′-OC*H*$_3$); ^{13}C NMR: δ 194.7 (C-1), 173.8 (C-3), 158.1 (C-4′), 157.2 (C-2′), 150.9 (C-4″), 149.0 (C-3″), 139.2 (C-5), 136.9 (C-3′), 136.8 and 136.7 (C-1 of 3″,4″-OCH$_2$C$_6$H$_5$), 128.6 (C-3,5 of 3″,4″-OCH$_2$C$_6$H$_5$), 128.0 (C-4 of 3″,4″-OCH$_2$C$_6$H$_5$), 127.3 and 127.1 (C-2,6 of 3″,4″-OCH$_2$C$_6$H$_5$), 127.3 (C-1″), 124.6 (C-6′), 122.7 (C-6″), 120.2 (C-4), 114.4 (C-5″), 114.3 (C-1′), 113.7 (C-2″), 103.0 (C-5′), 96.4 (C-2), 71.4 (3″-OCH$_2$C$_6$H$_5$), 70.9 (4″-OCH$_2$C$_6$H$_5$), 60.7 (3′-OCH$_3$), 56.1 (4′-OCH$_3$); MS (FAB$^+$), *m/z* (rel. int.): 539 [M+H]$^+$. Anal. Calcd for $C_{33}H_{30}O_7$ (538.59): C, 73.59; H, 5.61. Found: C, 73.35; H, 5.63.

3.4.2. 3-Hydroxy-1-(2-hydroxy-3,4-dimethoxyphenyl)-5-(3,4-dimethoxyphenyl)penta-2,4-dien-1-one (4b)

Mp 159–160 °C; ^{1}H NMR: δ 14.58 (d, *J* 0.7 Hz, 1H, 3-O*H*), 12.58 (s, 1H, 2′-O*H*), 7.59 (d, *J* 15.9 Hz, 1H, H-5), 7.48 (d, *J* 9.2 Hz, 1H, H-6′), 7.15 (d, *J* 8.3 and 1.9 Hz, 1H, H-6″), 7.08 (d, *J* 1.9 Hz, 1H, H-2″), 6.90 (d, *J* 8.3 Hz, 1H, H-5″), 6.51 (d, *J* 9.2 Hz, 1H, H-5′), 6.46 (d, *J* 15.9 Hz, 1H, H-4), 6.21 (s, 1H, H-2), 3.954 (s, 3H, 3″-OC*H*$_3$), 3.946 (s, 3H, 4′-OC*H*$_3$), 3.94 (s, 3H, 4″-OC*H*$_3$), 3.92 (s, 3H, 3′-OC*H*$_3$); ^{13}C NMR: δ 194.7 (C-1), 173.9 (C-3), 158.0 (C-4′), 157.2 (C-2′), 150.9 (C-4″), 149.2 (C-3″), 139.4 (C-5), 136.8 (C-3′), 128.1 (C-1″), 124.6

(C-6′), 122.4 (C-6″), 120.0 (C-4), 114.4 (C-1′), 111.1 (C-5), 109.5 (C-2″), 103.0 (C-5′), 96.4 (C-2), 60.7 (3′-OCH_3), 56.1, 56.0 and 55.9 (4′,3″,4″-OCH_3); MS (ESI) *m/z* (rel. int.): 387 ($[M+H]^+$, 100), 409 ($[M+Na]^+$, 35), 425 ($[M+K]^+$, 5), 795 ($[2\,M+Na]^+$, 40). $C_{21}H_{22}O_7$ (386.40): C, 65.28; H, 5.74. Found: C, 65.45; H, 5.71.

3.4.3. 1-(3,4-Dibenzyloxy-2-hydroxyphenyl)-3-hydroxy-5-(3,4-dimethoxyphenyl)penta-2,4-dien-1-one (4c)

Mp 147–148 °C; ^{1}H NMR: δ 14.60 (s, 1H, 3-O*H*), 12.59 (s, 1H, 2′-O*H*), 7.57 (d, *J* 15.7 Hz, 1H, H-5), 7.25–7.51 (m, 11H, H-6′ and 3′,4′-$OCH_2C_6H_5$), 7.13 (d, *J* 8.3 Hz, 1H, H-6″), 7.06 (s, 1H, H-2″), 6.88 (d, *J* 8.3 Hz, 1H, H-5″), 6.49 (d, *J* 9.1 Hz, 1H, H-5′), 6.43 (d, *J* 15.7 Hz, 1H, H-4), 6.17 (s, 1H, H-2), 5.15 (s, 2H, 4′-$OCH_2C_6H_5$), 5.11 (s, 2H, 3′-$OCH_2C_6H_5$), 3.94 and 3.92 (s, 6H, 3″,4″-OCH_3); ^{13}C NMR: δ 194.6 (C-1), 173.9 (C-3), 157.6 and 157.4 (C-2′,4′), 150.9 (C-4″), 149.2 (C-3″), 139.3 (C-5), 137.6 (C-1 of 3′-$OCH_2C_6H_5$), 136.3 (C-1 of 4′-$OCH_2C_6H_5$), 136.1 (C-3′), 128.6 (C-3,5 of 3′,4′-$OCH_2C_6H_5$), 128.2 (C-2,6 of 3′-$OCH_2C_6H_5$), 128.0 (C-4 of 3′,4′-$OCH_2C_6H_5$), 127.8 (C-1″), 127.2 (C-2,6 of 4′-$OCH_2C_6H_5$), 124.5 (C-6′), 122.4 (C-6″), 120.0 (C-4), 114.4 (C-1′), 111.1 (C-5″), 109.5 (C-2″), 104.6 (C-5′), 96.4 (C-2), 74.7 (3′-$OCH_2C_6H_5$), 70.7 (4′-$OCH_2C_6H_5$), 55.95 and 55.86 (3″,4″-OCH_3); MS (ESI) *m/z* (rel. int.): 539 ($[M+H]^+$, 100), 561 ($[M+Na]^+$, 35), 577 ($[M+K]^+$, 10). HRMS (ESI), *m/z*: $C_{33}H_{31}O_7$: 539.2070 $[M+H]^+$. Found: 539.2076.

3.5. Synthesis of alkoxy-2-styrylchromones (6a–c)

p-Toluenesulfonic acid monohydrate (0.48 g, 2.5 mmol) was added to a solution of the appropriate 5-aryl-3-hydroxy-1-(2-hydroxyaryl)penta-2,4-dien-1-one **4a–c** (5.0 mmol) in dimethyl sulfoxide (30 mL). The solution was heated under nitrogen at 90 °C, until the complete consumption of the starting material was observed (2–8 h). After that period, the solution was poured into ice and water (150 mL), and the obtained solid was removed by filtration, taken in dichloromethane (100 mL) and the organic layer washed with water (2 × 100 mL). After evaporation of the solvent, the residue was purified by silica gel column chromatography using dichloromethane as eluent. The solvent was evaporated to dryness in each case, and the residue was crystallized from ethanol to give the alkoxy-2-styrylchromones **6a–c**: **6a** (84%, 2.2 g), **6b** (62%, 1.1 g) and **6c** (77%, 2.0 g).

3.5.1. (*E*)-3′,4′-Dibenzyloxy-7,8-dimethoxy-2-styrylchromone (6a)

Mp 165–168 °C; ^{1}H NMR: δ 7.92 (d, *J* 9.0 Hz, 1H, H-5), 7.50–7.32 (m, 10H, 3′,4′-$CH_2C_6H_5$), 7.52 (d, *J* 16.0 Hz, 1H, H-β), 7.19 (d, *J* 1.9 Hz, 1H, H-2′), 7.13 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6′), 7.02 (d, *J* 9.0 Hz, 1H, H-6), 6.95 (d, *J* 8.4 Hz, 1H, H-5′), 6.58 (d, *J* 16.0 Hz, 1H, H-α), 6.23 (s, 1H, H-3), 5.23 (s, 2H, 3′-$OCH_2C_6H_5$), 5.22 (s, 2H, 4′-$OCH_2C_6H_5$), 4.04 (s, 3H, 8-O*CH*$_3$), 4.00 (s, 3H, 7-O*CH*$_3$); ^{13}C NMR: δ 178.0 (C-4), 161.8 (C-2), 156.6 (C-7), 150.7 (C-4′), 150.3 (C-9), 149.1 (C-3′), 136.9 and 136.6 (C-1′ of 3′,4′-$OCH_2C_6H_5$), 136.7 (C-8), 136.59 (C-β), 128.6 (C-3′,5′ of 3′,4′-$OCH_2C_6H_5$), 128.4 (C-1′); 127.99 and 127.96 (C-4′ of 3′,4′-$OCH_2C_6H_5$), 127.4 and 127.2 (C-2′,6′ of 3′,4′-$OCH_2C_6H_5$), 122.5 (C-6′), 121.0 (C-5), 118.8 (C-10), 118.4 (C-α), 114.4 (C-5′), 113.4 (C-2′), 109.49 and 109.44 (C-3 and C-6), 71.5 (3′-$OCH_2C_6H_5$), 71.0 (4′-$OCH_2C_6H_5$), 61.6 (8-O*CH*$_3$), 56.4 (7-O*CH*$_3$); MS (ESI) *m/z* (rel. int.): 521 ($[M+H]^+$, 100), 543 ($[M+Na]^+$, 15), 559 ($[M+K]^+$, 5). Anal. Calcd for $C_{33}H_{28}O_6$ (520.57): C, 76.14; H, 5.42. Found: C, 75.83; H, 5.13.

3.5.2. (*E*)-3′,4′,7,8-Tetramethoxy-2-styrylchromone (6b)

Mp 171–172 °C; ^{1}H NMR: δ 7.93 (d, *J* 9.0 Hz, 1H, H-5), 7.59 (d, *J* 15.9 Hz, 1H, H-β), 7.18 (dd, *J* 8.3 and 2.0 Hz, 1H, H-6′), 7.12 (d, *J* 2.0 Hz, 1H, H-2′), 7.03 (d, *J* 9.0 Hz, 1H, H-6), 6.91 (d, *J* 8.3 Hz, 1H, H-5′), 6.68 (d, *J* 15.9 Hz, 1H, H-α), 6.26 (s, 1H, H-3), 4.06 (s, 3H, 8-O*CH*$_3$), 4.01 (s, 3H, 7-O*CH*$_3$), 3.98 (s, 3H, 3′-O*CH*$_3$), 3.94 (s, 3H, 4′-O*CH*$_3$); ^{13}C NMR: δ 178.0 (C-4), 161.8 (C-2), 156.6 (C-7), 150.7 (C-4′), 150.3 (C-9), 149.3 (C-3′), 136.7 (C-β), 136.6 (C-8), 128.1 (C-1′), 122.2 (C-6′), 121.0 (C-5), 118.8 (C-10), 118.2 (C-α), 111.1 (C-5′), 109.5 (C-6), 109.4 (C-3), 109.2 (C-2′), 61.6 (8-O*CH*$_3$), 56.4 (7-O*CH*$_3$), 56.0 (3′,4′-OCH_3); MS (ESI+) *m/z* (rel. int.): 396 ($[M+H]^+$, 100), 391 ($[M+Na]^+$, 10), 759 ($[2\,M+Na]^+$, 8). Anal. Calcd for $C_{21}H_{20}O_6$ (368.38): C, 68.47; H, 5.47. Found: C, 68.74; H, 5.52.

3.5.3. (*E*)-7,8-Dibenzyloxy-3′,4′-dimethoxy-2-styrylchromone (6c)

Mp 176–177 °C; ^{1}H NMR: δ 7.90 (d, *J* 9.0 Hz, 1H, H-5), 7.32–7.54 (m, 10H, 7,8-$OCH_2C_6H_5$), 7.44 (d, *J* 15.9 Hz, 1H, H-β), 7.07 (d, *J* 9.0 Hz, 1H, H-6), 7.03 (dd, *J* 8.3 and 1.9 Hz, 1H, H-6′), 6.98 (d, *J* 1.9 Hz, 1H, H-2′), 6.88 (d, *J* 8.3 Hz, 1H, H-5′), 6.60 (d, *J* 15.9 Hz, 1H, H-α), 6.20 (s, 1H, H-3), 5.28 (s, 2H, 7-$OCH_2C_6H_5$), 5.22 (s, 2H, 8-$OCH_2C_6H_5$), 3.94 (s, 3H, 3′-O*CH*$_3$), 3.93 (s, 3H, 4′-O*CH*$_3$); ^{13}C NMR: δ 178.0 (C-4), 161.7 (C-2), 155.9 (C-7), 150.7 (C-4′), 150.5 (C-9), 149.2 (C-3′), 137.3 (C-8), 136.9 (C-β), 136.12 and 136.06 (C-1′ of 7,8-$OCH_2C_6H_5$), 128.7 and 128.4 (C-3′,5′ of 7,8-$OCH_2C_6H_5$), 128.4 (C-2′,6′ of 8-$OCH_2C_6H_5$), 128.3 and 128.2 (C-4′ of 7,8-$OCH_2C_6H_5$), 128.0 (C-1′), 127.4 (C-2′,6′ of 7-$OCH_2C_6H_5$), 122.0 (C-6′), 121.0 (C-5), 119.1 (C-10), 117.9 (C-α), 111.2 (C-6), 111.1 (C-5'), 109.5 (C-3), 109.3 (C-2′), 75.8 (8-$OCH_2C_6H_5$), 71.2 (7-$OCH_2C_6H_5$), 56.0 and 55.9 (3′,4′-OCH_3); MS (ESI) *m/z* (rel. int.): 521 ($[M+H]^+$, 100), 543 ($[M+Na]^+$, 10). Anal. Calcd for $C_{33}H_{28}O_6$ (520.57): C, 76.14; H, 5.42. Found: C, 76.08; H, 5.40.

3.6. Synthesis of dihydroxy-2-styrylchromones (7a,c)

The appropriate benzyloxy-2-styrylchromone **6a**, **6c** (0.54 mmol) was added to a solution of chloridric acid (37%, 2 mL) in glacial acetic acid (20 mL). The mixture was refluxed at 110 °C for 2:30 h for **7a** and 12 h for **7c**. The solution was carefully poured into ice and water (30 mL), and the obtained solid was removed by filtration, abundantly washed with water (4 × 50 mL) and then dried affording the expected hydroxy-2-styrylchromones in good yields: **7a** (82%, 0.15 g) and **7c** (82%, 0.15 g).

3.6.1. (*E*)-3′,4′-Dihydroxy-7,8-dimethoxy-2-styrylchromone (7a)

$Mp_{dec.}$ 233–234 °C; ^{1}H NMR (DMSO-d_6): δ 9.64 (br s, 1H, 4′-O*H*), 9.20 (br s, 1H, 3′-O*H*), 7.74 (d, *J* 8.9 Hz, 1H, H-5), 7.44 (d, *J* 16.0 Hz, 1H, H-β), 7.25 (d, *J* 8.9 Hz, 1H, H-6), 7.13 (d, *J* 1.6 Hz, 1H, H-2′), 7.04 (dd, *J* 8.2 and 1.6 Hz, 1H, H-6′), 6.90 (d, *J* 16.0 Hz, 1H, H-α), 6.81 (d, *J* 8.2 Hz, 1H, H-5′), 6.35 (s, 1H, H-3), 3.96 (s, 6H, 7.8-O*CH*$_3$); ^{13}C NMR (DMSO-d_6): δ 176.5 (C-4), 161.9 (C-2), 156.4 (C-7), 149.7 (C-9), 148.0 (C-4′), 145.8 (C-3′), 136.7 (C-β), 136.2 (C-8), 126.5 (C-1′), 121.0 (C-6′), 120.1 (C-5), 118.2 (C-10), 117.0 (C-α), 115.9 (C-5′), 114.3 (C-2′), 110.3 (C-6), 108.5 (C-3), 61.2 (8-O*CH*$_3$), 56.5 (7-O*CH*$_3$); MS (EI) *m/z* (rel. int.): 340 ($M^{+\cdot}$, 100), 323 (9), 310 (19), 255 (21), 228 (34), 214 (15), 181 (37), 167 (8), 152 (17), 126 (26), 91 (10), 69 (18). Anal. Calcd for $C_{19}H_{16}O_6$ (340.33): C, 67.05; H, 4.74. Found: C, 67.46; H, 4.76.

3.6.2. (*E*)-7,8-Dihydroxy-3′,4′-dimethoxy-2-styrylchromone (7c)

$Mp_{dec.}$ 287–291 °C; ^{1}H NMR (DMSO-d_6): δ 10.18 (br s, 1H, 7-O*H*), 9.52 (br s, 1H, 8-O*H*), 7.78 (d, *J* 15.8 Hz, 1H, H-β), 7.35 (d, *J* 8.7 Hz, 1H, H-5), 7.32 (d, *J* 1.8 Hz, 1H, H-2′), 7.22 (dd, *J* 8.2 and 1.8 Hz, 1H, H-6′), 7.07 (d, *J* 15.8 Hz, 1H, H-α), 7.03 (d, *J* 8.2 Hz, 1H, H-5′), 6.91 (d, *J* 8.7 Hz, 1H, H-6), 6.23 (s, 1H, H-3), 3.84 (s, 3H, 4′-O*CH*$_3$), 3.81 (s, 3H, 3′-O*CH*$_3$); ^{13}C NMR (DMSO-d_6): δ 176.8 (C-4), 161.5 (C-2), 150.41 (C-4′), 150.38 (C-7), 149.1 (C-3′), 146.2 (C-9), 136.4 (C-β), 132.9 (C-8), 128.1 (C-1′), 122.1 (C-6′), 118.5 (C-α), 117.1 (C-10), 115.0 (C-5), 113.7 (C-6), 111.7 (C-5′), 109.7 (C-2′), 108.7 (C-3), 55.6 (3′,4′-OCH_3); MS (ESI+) *m/z* (rel. int.): 341 ($[M+H]^+$, 17), HRMS (ESI+), *m/z*: $C_{19}H_{17}O_6$: calcd 341.1025 $[M+H]^+$. Found: 341.1023.

3.7. Synthesis of 3′,4′,7,8-tetrahydroxy-2-styrylchromone (7b)

A solution of 1 M boron tribromide in dichloromethane (0.54 mL, 0.54 mmol) was slowly added to a cool solution (2-propanol cryostat bath at −78 C) of 3′,4′,7,8-tetramethoxy-2-styrylchromone **6b** (49.3 mg, 0.134 mmol) in freshly distilled dichloromethane (5 mL). The solution was stirred under nitrogen at room temperature for 24 h. After that period, ice and water (20 mL) was slowly added to the solution and then vigorously stirred. The obtained solid was removed by filtration and abundantly washed with water (4 × 50 mL) and then dried affording the expected 3′,4′,7,8-tetrahydroxy-2-styrylchromone **7b** (76%, 32 mg).

3.7.1. (*E*)-3′,4′,7,8-Tetrahydroxy-2-styrylchromone (7b)

$Mp_{dec.}$ 254–255 °C; ^{1}H NMR (DMSO-d_6): δ 10.20 (s, 1H, 7-O*H*), 9.57 (s, 1H, 4′-O*H*), 9.48 (s, 1H, 8-O*H*), 9.20 (s, 1H, 3′-O*H*), 7.71 (d, *J* 16.3 Hz, 1H, H-β), 7.35 (d, *J* 8.7 Hz, 1H, H-5), 7.09 (d, *J* 1.8 Hz, 1H, H-2′), 7.01 (dd, *J* 7.8 and 1.8 Hz, 1H, H-6′), 6.91 (d, *J* 8.7 Hz, 1H, H-6), 6.83 (d, *J* 16.3 Hz, 1H, H-α), 6.81 (d, *J* 7.8 Hz, 1H, H-5′), 6.25 (s, 1H, H-3); ^{13}C NMR (DMSO-d_6): δ 176.8 (C-4), 161.6 (C-2), 150.3 (C-7), 147.7 (C-4′), 146.2 (C-9), 145.7 (C-3′), 136.9 (C-β), 132.8 (C-8), 126.9 (C-1′), 120.6 (C-6′), 117.0 (C-α and C-10), 115.9 (C-5′), 115.0 (C-5), 114.2 (C-2′), 113.7 (C-6), 108.4 (C-3); MS (EI) *m/z* (rel. int.): 312 ($M^{+\cdot}$, 66), 311 (8), 295 (2), 160 (22), 153 (50), 129 (8), 115 (15), 105 (27), 101 (34), 91 (47), 83 (90), 77 (44), 69 (30), 59 (100); HRMS (ESI), *m/z*: $C_{17}H_{13}O_6$: calcd 313.0712 $[M+H]^+$. Found: 313.0722.

3.8. Synthesis of 3-alkyl-3′,4′,5,7-tetrahydroxyflavones (8a–c)

3-Alkyl-3′,4′,5,7-tetrahydroxyflavones **8a–c** were synthesised according to procedures previously described in the literature.[23]

3.9. Synthesis of 3-(3,4-dihydroxybenzoyl)-3′,4′,5,7-tetrahydroxyflavone (9)

3-(3,4-Dihydroxybenzoyl)-3′,4′,5,7-tetrahydroxyflavone **9** was synthesised according to procedures previously described in the literature.[24]

3.10. ROS and RNS scavenging assays

3.10.1. Superoxide radical scavenging assay

Superoxide radical was generated by the NADH/PMS system and the $O_2^{\cdot-}$ scavenging activity was determined by monitoring the effect of the tested compound on the $O_2^{\cdot-}$-induced reduction of NBT at 560 nm as previously described.[12] The antioxidant tiron was used as positive control. The results were expressed as the inhibition (in percentage) of the NBT reduction to diformazan. Each study corresponds to five experiments, performed in triplicate.

3.10.2. Hydrogen peroxide scavenging assay

The H_2O_2 scavenging activity was measured by monitoring the H_2O_2-induced oxidation of lucigenin as previously described.[12] The endogenous antioxidant ascorbic acid was used as positive control. The results were expressed as the inhibition (in percentage) of the H_2O_2-induced oxidation of lucigenin. Each study corresponds to five experiments, performed in triplicate.

3.10.3. Hypochlorous acid scavenging assay

The HOCl was measured by using a previously described fluorescent methodology[12] based on the HOCl-induced oxidation of DHR to rhodamine. HOCl was prepared by adjusting the pH of a 1% (m/v) solution of NaOCl to 6.2 with dropwise addition of 10% H_2SO_4. Lipoic acid was used as positive control. The results were expressed as the inhibition (in percentage) of HOCl-induced oxidation of DHR. Each study corresponds to five experiments, performed in triplicate.

3.10.4. Singlet oxygen scavenging assay

The $^1O_2$ scavenging activity was measured by monitoring the oxidation of non-fluorescent DHR to fluorescent rhodamine by this ROS, as previously described.[12] Ascorbic acid was used as positive control. The results were expressed as the inhibition (in percentage) of $^1O_2$-induced oxidation of DHR. Each study corresponds to five experiments, performed in triplicate.

3.10.5. Peroxyl radical scavenging assay

The ROO$^{\cdot}$ scavenging activity was measured by monitoring the fluorescence decay resulting from ROO$^{\cdot}$-induced oxidation of fluorescein and expressed as the 'Oxygen Radical Absorbance Capacity' (ORAC), as previously described.[12] Trolox was used as the standard control in each assay. Ascorbic acid was used as positive control. The results were expressed as ORAC values. Each study corresponds to four experiments, performed in triplicate.

3.10.6. Peroxynitrite scavenging assay

The $ONOO^-$ scavenging activity was measured by monitoring the $ONOO^-$-induced oxidation of non-fluorescent DHR to fluorescent rhodamine, as previously described.[12] Ebselen was used as positive control. In a parallel set of experiments, the assays were performed in the presence of 25 mM $NaHCO_3$ in order to simulate the physiological CO_2 concentrations. This evaluation is important because, under physiological conditions, the reaction between $ONOO^-$ and bicarbonate is predominant, with a very fast rate constant ($k_2 = 3–5.8 \times 10^4\ M^{-1}\ s^{-1}$).[47] The results were expressed as the inhibition (in percentage) of $ONOO^-$-induced oxidation of DHR. Each study corresponds to five experiments, performed in triplicate.

3.10.7. Nitric oxide scavenging assay

The ·NO scavenging activity was measured by monitoring the ·NO-induced oxidation of non-fluorescent DAF-2 to the fluorescent triazolofluorescein (DAF-2T), as previously described.[12] Rutin was used as positive control. The results were expressed as the inhibition (in percentage) of ·NO-induced oxidation of DAF-2. Each study corresponds to five experiments, performed in triplicate.

3.10.8. Reducing power assay

The reducing power was determined according to a previously described method.[48] Test compounds, in 1 mL of a ultrapure water/DMSO 1:4 solution, were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10% w/v) was added to the mixture, which was then centrifuged for 10 min at 3000 *g*. The upper layer of the solution (2.5 mL) was mixed with ultrapure water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1% w/v), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as positive control.

3.10.9. Determination of metal chelating activity

The metal chelating activity was assessed by monitoring the changes in the UV–vis spectrum of the test compounds after addition of Fe(II).[46] Experiments were performed by sequential additions of 2 μL of $FeCl_2$ solution (300 μM stock solution) to 300 μL of a 10 μM (final concentration) compound solution. The reactions were performed in phosphate buffer 20 mM, pH 7.2.

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