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Inhibition of horseradish peroxidase catalytic activity by new 3-phenylcoumarin derivatives: Synthesis and structure–activity relationships

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Abstract—Twenty hydroxylated and acetoxylated 3-phenylcoumarins were synthesized, and the structure–activity relationships were investigated by evaluating the ability of these compounds to modulate horseradish peroxidase (HRP) catalytic activity and comparing the results to four flavonoids (quercetin, myricetin, kaempferol and galangin), previously reported as HRP inhibitors. It was observed that 3-phenylcoumarins bearing a catechol group were as active as quercetin and myricetin, which also show this substituent in the B-ring. The presence of 6,2′-dihydroxy group or 6,7,3′,4′-tetraacetoxy group in the 3-phenylcoumarin structure also contributed to a significant inhibitory effect on the HRP activity. The catechol-containing 3-phenylcoumarin derivatives also showed free radical scavenger activity. Molecular modeling studies by docking suggested that interactions between the heme group in the HRP active site and the catechol group linked to the flavonoid B-ring or to the 3-phenyl coumarin ring are important to inhibit enzyme catalytic activity.

1. Introduction

Heme peroxidases are widespread in eukaryotes and prokaryotes, and have a pivotal role in biology.[1,2] Despite the striking chemical diversity of these proteins, the biological oxidation reactions they catalyze involve similar high-oxidation state intermediates whose reactivity is modulated by the protein environment.[3–6] The following general mechanism was proposed for peroxidase-catalyzed reaction:

$$\text{peroxidase} + \text{ROOH} \leftrightarrow \text{compound I} + \text{ROH}$$

$$\text{compound I} + \text{AH}_2 \rightarrow \text{compound II} + \text{AH}^{\cdot}$$

$$\text{compound II} + \text{AH}_2 \rightarrow \text{peroxidase} + \text{AH}^{\cdot} + \text{H}_2\text{O},$$

where ROOH is a peroxide and ROH the corresponding two-electron reduction product. Compounds I and II are reactive redox intermediates, which are responsible for the one-electron oxidation of substrates (AH_2) to their corresponding radicals ($AH^{\cdot}$).[7,8]

Both mammalian and plant peroxidases have been reported to produce aromatic oxyl radicals from several aromatic substrates and reactive oxygen species as part of a defense mechanism against pathogens.[9] These organic and inorganic free radicals may also participate in plant physiological processes, such as lignification, cell wall biosynthesis, auxin catabolism, and wound healing,[10] and in mammalian cells as intracellular signaling, in pro-inflammatory eicosanoids biosynthesis, and microbial killing.[11–13]

Keywords: Catechol; Coumarin; Docking; Horseradish peroxidase.
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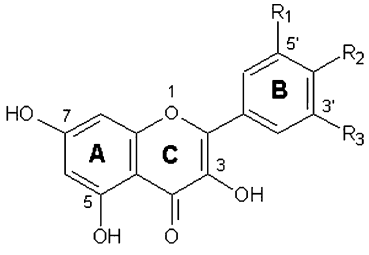
However, such reactive species generated by peroxidase-catalyzed reactions can also contribute to progressive human tissue damage in chronic inflammatory diseases, as atherosclerosis, asthma, and rheumatoid arthritis, which makes peroxidases interesting pharmacological targets.[13,14] The use of phenolic compounds, widely known as free radical scavengers, has been one of the therapeutic strategies against inflammatory diseases.[15] Suppression of prostaglandin synthesis by phenolic compounds has been reported to be mediated by the inhibition of cyclooxygenase peroxidative activity.[16,17]

Horseradish peroxidase (HRP) is a well-known and highly investigated member of the peroxidase superfamily I.[18] The HRP-catalyzed oxidation of luminol in the presence of H_2O_2 has been extensively used in the screening of new molecules with antioxidant activity, especially due to its high sensitivity, simple testing procedures, and relatively low cost. It has also been a useful tool in the evaluation of natural bioactive compounds, products of chemical synthesis, and even of compounds incorporated into pharmaceutical formulations.[19–21]

Although the antioxidant activity has been primarily attributed to the presence of free hydroxy groups, a significant antioxidant effect has also been reported for compounds where these groups are acetylated.[22,23] In previous works, our research group had reported some classes of natural compounds with antioxidant and anti-inflammatory activities, such as flavonoids, coumarins, and sesquiterpene lactones.[21,24,25] We also have identified hydroxy and acetoxy groups as the most promising structural features for the biological activities investigated. In order to understand better the relative contribution of these substituents to the antioxidant activity and to the modulation of peroxidase catalytic activity, twenty hydroxylated and acetoxylated 3-phenylcoumarins were synthesized having structural features of flavonoids previously reported as inhibitors of HRP activity (Fig. 1 and Scheme 1).[26] Their biological effects as well as structure–activity relationships are discussed. Moreover, molecular modeling studies by docking were performed in order to propose a binding model for the compounds and the HRP active site.



Myricetin	$R_1 = R_2 = R_3 = OH$
Quercetin	$R_1 = R_2 = OH$, $R_3 = H$
Kaempferol	$R_1 = R_3 = H$, $R_2 = OH$
Galangin	$R_1 = R_2 = R_3 = H$

Figure 1. Chemical structure of the flavonoids included in the molecular modeling analysis by docking.[26]

2. Results

The acetoxylated 3-phenylcoumarin derivatives were synthesized by the Perkin–Oglialoro reaction of phenylacetic acid derivatives with salicylaldehyde derivatives, in Ac_2O and CH_3CO_2K (Scheme 1).[27] The corresponding hydroxylated 3-phenylcoumarins were obtained by deacetylation in the presence of aqueous HCl solution and MeOH. Structures of the synthesized compounds were established on the basis of their spectral data (see Section 4.2 for details).

2.1. Effect in the HRP catalytic activity

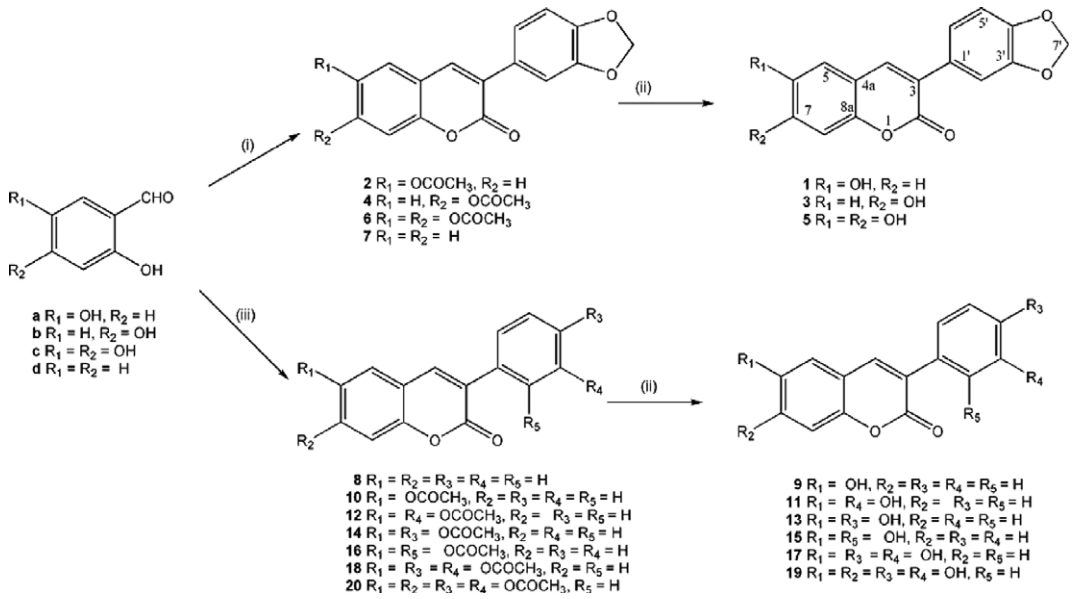
The modulatory effect of all 3-phenylcoumarins synthesized on the HRP catalytic activity was first screened at 50 μM (Fig. 2A). Most acetoxylated compounds were not tested in higher concentrations, due to low solubility in the reaction medium. Non-substituted coumarins (**7**, **8**), the acetoxylated derivatives (except for compound **20**), and those compounds not bearing a vicinal dihydroxy group (except for compound **15**) were not significantly active, under the assessed conditions. The other compounds (**5**, **15**, **17**, **19**, **20**) were further evaluated at six concentrations (0–50 μM) and showed concentration-dependent inhibitory effects on HRP catalytic activity (Fig. 2B).

The calculated IC_{50} values for the 3-phenylcoumarins (Table 1) were compared to four flavonoids, previously reported as HRP inhibitors.[26] The results revealed similar structure–activity relationships for both classes of compounds. For example, the most active compounds within each class bear a catechol group. Moreover, removal of hydroxy groups in the quercetin B-ring (yielding kaempferol and galangin) or in the 3-phenyl ring of the coumarin structure (compare **17** to **11** and **13**, Fig. 2A) reduced their inhibitory effects on the HRP catalytic activity.

Hydroxy group locations on the 3-phenyl ring influenced the coumarin activity (see compounds **11**, **13**, and **15**, Fig. 2A). Coumarin **15**, bearing one hydroxy group at position C-2′, inhibited enzyme activity more than its analogues **11** and **13**, which bear a hydroxy group at C-3′ and C-4′, respectively. In addition, acetylation of coumarin **19** catechol groups (yielding **20**) also reduced its inhibitory effect.

2.2. Radical scavenging potential

The radical scavenging abilities of all 3-phenylcoumarins synthesized based on the determination of drop in the absorption of the stable free radical DPPH were evaluated. Four compounds (**5**, **15**, **17**, **19**) showed a significant radical scavenging activity and the results were expressed as IC_{50} values (Table 2). The other compounds did not exhibit appreciable activity at a concentration of 50 μM (data not shown).



Scheme 1. Reagents and conditions: (i) 3,4-(methylenedioxy)phenylacetic acid, CH_3CO_2K, Ac_2O, reflux; (ii) HCl 2 N, MeOH; (iii) phenylacetic acid derivatives, CH_3CO_2K, Ac_2O, reflux.

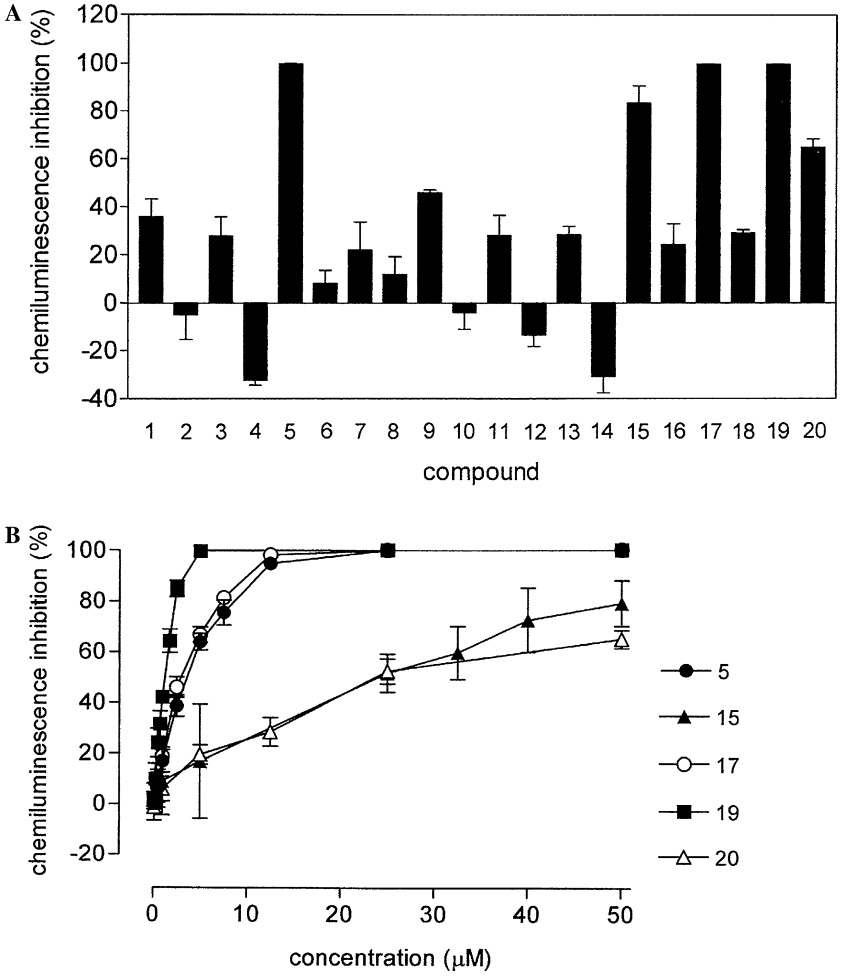


Figure 2. Modulatory effect of the 3-phenylcoumarin derivatives in the HRP catalytic activity, assessed by luminol-enhanced chemiluminescence assay. (A) All compounds were tested at 50 μM. (B) Concentration-dependent plots of the 3-phenylcoumarins showing the highest inhibitory effects in HRP activity. Data are expressed as means ± standard deviation of three independent experiments, with duplicate measurements.

Table 1. Inhibitory effect of 3-phenylcoumarins and flavonoids on HRP catalytic activity

Compound[a]	IC_{50}[c] (μM)	
19	1.2 ± 0.1	X
17	2.8 ± 0.2	X
5	3.4 ± 0.3	X
15	24.6 ± 6.9	Y
20	28.2 ± 7.9	Y
Quercetin[b]	3.0 ± 0.5	X
Myricetin[b]	3.1 ± 0.4	X
Kaempferol[b]	10.2 ± 2.9	Z
Galangin[b]	16.3 ± 2.2	W

[a] Chemical structures are shown in Scheme 1 and Figure 1.
[b] Taken from the literature.[26]
[c] Data expressed as means ± standard deviation of three independent experiments, with duplicate measurements. IC_{50} is the concentration that inhibits HRP catalytic activity by 50%. *Statistics:* X, Y, Z, W: values not sharing the same letter are significantly different from each other (ANOVA and Tukey's post hoc test).

Table 2. Radical scavenging potential of 3-phenylcoumarins

Compound [a]	IC_{50}[b] (μM)	
19	9.8 ± 0.6	X
5	13.2 ± 0.6	Y
15	24.9 ± 2.5	Z
17	26.8 ± 1.9	Z

[a] Chemical structures are shown in Scheme 1.
[b] Data expressed as means ± standard deviation of three independent experiments, with duplicate measurements. IC_{50} is the concentration that promoted 50% of DPPH reduction. Statistics: X, Y, Z: values not sharing the same letter are significantly different from each other (ANOVA and Tukey's post hoc test).

Compound **19**, bearing two catechol groups, was the most effective free radical scavenger within the set of compounds tested in this study. Substitution of 3′,4′-dihydroxy group from compound **19** by 3′,4′-methylenedioxy group (yielding **5**) significantly reduced its activity. In addition, compounds **15** and **17** were significantly less active than **5** and **19**. The former (**15**) has hydroxy groups attached to C-6 and C-2′, and the latter (**17**) has hydroxy groups at positions C-6, C-3′, and C-4′.

2.3. Molecular modeling studies by docking

In order to propose a binding model for the HRP inhibitors under study, a flexible docking procedure was employed with 20 coumarins and 4 flavonoids, using the unbound HRP structure solved at 1.57 Å resolution (PDB code 1H5G) as the receptor.[28] The structure of HRP in a complex with benzhydroxamic acid, solved at 2.0 Å resolution (PDB code 2ATJ),[29] was not used due to the close similarity between the bound and unbound structures.

In Figure 3A, the top ranked GOLD solution for quercetin with the unbound HRP structure (PDB code 1H5G) is superimposed to the crystal structure of HRP–benzhydroxamic acid complex (PDB code 2ATJ), as well as the HRP crystal structure in complex with the acetate ion (PDB code 1H5A).[28] In general, results suggest that the compounds here investigated could bind to HRP in a way that their specific hydroxy or acetoxy groups would occupy the positions originally occupied by the oxygen atoms of ligands in the

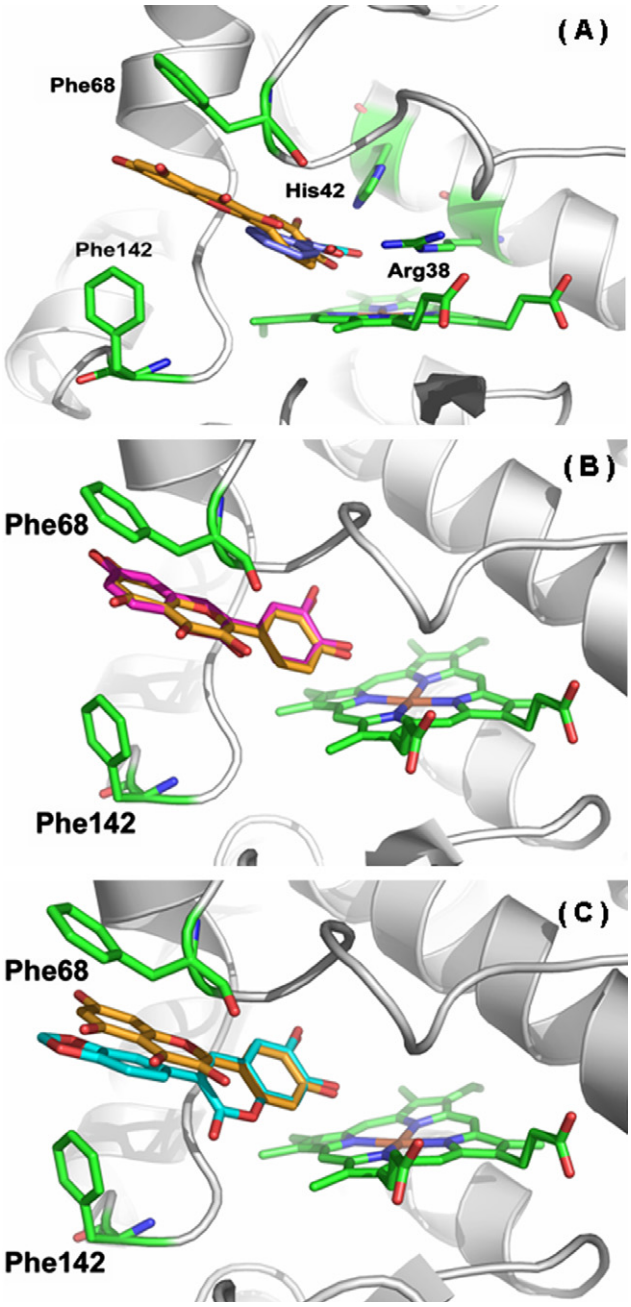


Figure 3. In (A), the top ranked solution of GOLD for quercetin (carbon atoms in orange) with the HRP unbound structure (PDB code 1H5G; represented by a ribbon diagram) is superimposed to the HRP crystal structure in a complex with benzhydroxamic acid (PDB code 2ATJ; ligand with carbon atoms in violet), as well as to the HRP crystal structure in complex with acetate ion (PDB code 1H5A; ligand with carbon atoms in cyan). In (B), the superposition of the top ranked solutions for quercetin (carbon atoms in orange) and coumarin **17** (carbon atoms in magenta) with the HRP unbound structure (PDB code 1H5G) is shown. In (C), the superposition of the top ranked solutions for quercetin (carbon atoms in orange) and **5** (carbon atoms in cyan) with the HRP unbound structure (PDB code 1H5G) is shown. In all structures, the heme group and selected HRP residues are represented with carbon atoms in green, and the hydrogen atoms are omitted for clarity.

crystallographic complexes, close to the heme group. Catechol group of quercetin firstly interacts with the conserved and catalytic His42 and Arg38 residues (Fig. 3A).[30] After the simulations, the positions of the hydroxyls coincide with the oxygen atoms of benzhydroxamic acid and acetate ion in their complexes.

Additionally, quercetin B-ring has occupied the binding site of the benzhydroxamic acid ring, after the simulation. This flavonoid was found to be one of the most active compounds assayed so far, suggesting that both *meta*- and *para*-hydroxy groups of the B-ring (catechol system) play an important role. Moreover, myricetin, which has three hydroxy groups in the B-ring, was as active as quercetin, despite containing one additional hydroxy group. Interestingly, the highly active compound **17** contains a catechol group in the benzene ring and a hydroxy in the coumarinic system. After the docking simulation, GOLD orients this molecule positioning the benzene ring close to the heme group, with the hydroxy at the same binding site observed for the quercetin catechol group, thus reinforcing the importance of the hydroxy oriented in *meta*- and *para*-positions in the benzene ring (Fig. 3B).

On the other hand, results have also indicated that, theoretically, the oxygen atoms of the benzodioxole ring in some of the investigated compounds do not necessarily mimic the hydroxy groups of the flavonoid B-ring. Top-ranked solution for coumarin **5**, one of the most active compounds assayed, indicates an inverted orientation for this molecule inside the HRP active site, after the simulation, with both hydroxy groups positioned close to the heme group, as shown in Figure 3C.

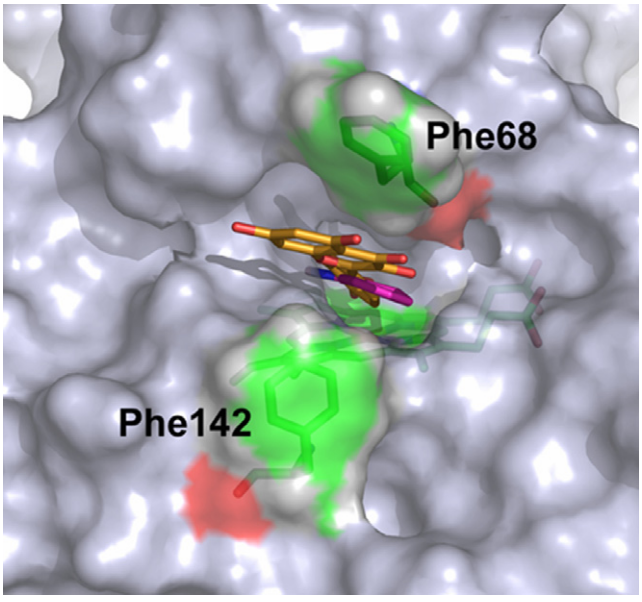


Figure 4. Top ranked solution of GOLD for quercetin (carbon atoms in orange) with the HRP non-bound structure (PDB code 1H5G; represented by a surface) is superimposed to the HRP crystal structure in a complex with benzhydroxamic acid (PDB code 2ATJ; ligand with carbon atoms in magenta). Flavonoid fused rings are flanked by Phe68 and Phe142 residues in HRP.

In addition, the fused rings of quercetin were observed to be flanked by Phe68 and Phe142 of HRP, suggesting π-stacking interactions (face-to-face and edge-to-face). These residues are conserved across other peroxidases, and Phe68 plays a role in the HRP–benzhydroxamic acid complex, shielding the inhibitor from the solvent such as a cap, as represented in Figure 4.[29] Furthermore, the hydrophobic interactions due to the Phe68 and Phe142 residues complement the main interaction observed with the catalytic residues.

3. Discussion

In the present study, a series of twenty 3-phenylcoumarins was synthesized and evaluated as free radical scavengers and inhibitors of the HRP catalytic activity. As shown in Tables 1 and 2, the catechol group is the main structural requirement to achieve a high inhibitory effect in both investigated activities. The experimental results for the inhibitory effect of 3-phenylcoumarins on the HRP catalytic activity were compared to those previously reported by our research group for flavonoids.[26] Taken together, the results of the present investigation highlighted the importance of similar structural features between flavonoids and 3-phenylcoumarins for the HRP inhibition, as the presence of the catechol group and the number and position of hydroxy groups.

Furthermore, molecular modeling studies by docking with both classes of compounds revealed similar binding models for the most active compounds in the HRP catalytic site. In general, we found that the catechol group of flavonoids and 3-phenylcoumarins was positioned close to the heme group and the two hydroxyls coincide with the position of the oxygen atoms in the bezhydroxamic acid and acetate molecules, whose crystallographic orientations inside the HRP active site are known.[28,29] Although we did not have an extended series of compounds with HRP inhibitory activity, our attempt in ascertaining the experimental results obtained was supported by the agreement of the top-ranked GOLD orientations of the inhibitors with the crystallographic ligands reported from PDB, regarding the oxygen atoms that bind to HRP catalytic residues, as well as to an aromatic binding site.

The excellent radical scavenging ability of dihydroxylated derivatives of 3-phenylcoumarins and flavonoids may be explained by the fact that the ortho-dihydroxy system is able to form a resonance-stable radical and yield quinone or semi-quinone products, which are less reactive. In addition, the better interaction of these molecules with the enzyme active site when compared to the monophenolic compounds favors the scavenging of the two electrons involved in the HRP catalytic cycle by one catechol group.[31] Bors et al.[32] suggested that three structural features are important determinants of the free radical-scavenger potential of flavonoids: (a) the *O*-dihydroxy (catechol) system in the B-ring; (b) the 2,3-double bond conjugated with a 4-oxo function; and (c) the presence of 3- and 5-OH groups. Considering these requirements, the present investigation suggests

that the catechol group is the most significant determinant of electron-donating activity and the possible competitive inhibition of HRP catalytic activity. Accordingly, the ability of catechol-containing 3-phenylcoumarins to inhibit enzyme activity was comparable with the catechol-containing flavonoids quercetin and myricetin.

The present work also identified structural features other than the catechol group that contributed to the inhibition of HRP activity, such as the 6,2′-dihydroxylation (**15**) and the 6,7,3′,4′-tetraacetoxylation (**20**). Regarding compound **15**, we suggest that the spatial proximity between the 2′-hydroxy group and the lactone carbonyl group probably mimics the catechol group interactions with the HRP active site. However, the inhibitory activity in the presence of the 2′-hydroxy group was significantly lower. Moreover, the inhibitory effect of compound **15** was similar to that observed for the acetoxylated compound **20**.

Although numerous phenol derivatives may act as substrates and donate hydrogen to HRP,[31] some acetylated compounds such as formylphenylacetic acid ethyl ester and acetylated derivatives of hydroxamic acids[33] have also been reported to be metabolized by the enzyme, but at lower rates when compared to their hydroxylated parent compounds. Similar results were observed for the acetoxylated coumarin **20**, which had the same activity pattern of its hydroxylated counterpart but with lower intensity. The other acetoxylated 3-phenylcoumarin derivatives did not show inhibitory effect on the HRP catalytic activity, which suggests a specific structural requirement of acetoxylated compounds to this biological effect.

The free radical scavenger activity of phenolic compounds has been reported to mediate the inhibition of prostaglandin synthesis by cyclooxygenase, since this enzyme catalyzes the controlled peroxidation of arachidonic acid.[16,17] Therefore, inhibition of cyclooxygenase peroxidative activity by phenolic compounds seems to be an important mechanism underlying their anti-inflammatory effect.

In conclusion, the results reported here may be helpful to understand better the interactions between 3-phenylcoumarins and the HRP catalytic site. The structure–activity relationships allowed identification of new relevant structural features to the HRP catalytic activity modulation. Furthermore, as the formation of free radicals through peroxidase-catalyzed reactions is involved in the development of chronic inflammatory diseases, the present study can help in developing new molecules to control inflammation and free radical-mediated tissue injury.

4. Experimental

4.1. Chemicals

Kaempferol (3,4′,5,7-tretrahydroxyflavone), myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), horseradish peroxidase (HRP) type VI-A (EC.1.11.1.7), and luminol (5-amine-2,3-dihydro-1,4-phthalazinedione) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Galangin (3,5,7-trihydroxyflavone) was purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Dimethylsulfoxide (DMSO; Merck-Schuchardt, Hohenbrunn, Germany), ethanol 95% (F. Maia, São Paulo, Brazil), and hydrogen peroxide (H_2O_2; Labsynth, Diadema, Brazil) were the other chemicals used.

4.2. Synthesis, characterization, and identification of coumarins

Compounds **1**, **2**, **3, 4**, and **7** were synthesized as previously described (Scheme 1).[27] Purity of all synthesized compounds was evaluated by thin-layer chromatography, using hexane–EtOAc in different proportions (adjusted to each compound) as the mobile phase.

Equipments employed: Melting point: Fisher-Johns 12144 melting-point apparatus. ^{1}H NMR: Bruker DRX400 spectrometer at 400 and 100 MHz or Bruker BPX300 spectrometer at 300 and 75 MHz. Chemical shifts δ in parts per million with $SiMe_4$ as an internal standard, coupling constants J = in hertz. High-resolution mass spectra: HR-ESI-MS was performed in the positive mode (UltrOTOF-Q system, version 1.10, Bruker Daltonics, MA, USA). Samples (0.1 μg/mL) dissolved in dichloromethane were introduced in the mass spectrometer at 5 μL/min. MS experiments were performed using standard isolation and excitation procedures.[34] Nitrogen was the nebulizing and collision gas with the collision energy set at 4 eV. An accurate-mass calibration was obtained through a postacquisition application of a calibration created by the ratio MS/MS of a monensin A $[M-18+Na]^+$ obtained under the same CID and cell conditions or by an internal calibration using a known ion in the spectra.

4.2.1. General procedure for the preparation of coumarin 8 and acetoxylated derivatives. Non-substituted coumarin **8** and acetoxylated derivatives (**6**, **10**, **12**, **14**, **16**, **18**, **20**) were synthesized under anhydrous conditions, by using equipment previously dried at 60 °C for at least 12 h and also at 300 °C during few minutes immediately before use.

The solution containing anhydrous CH_3CO_2K (289 mg, 2.94 mmol), the appropriate phenylacetic acid derivative (1.67 mmol), and hydroxylated benzaldehyde (1.67 mmol) in Ac_2O (1.2 mL) was refluxed (138 °C) with stirring during different periods of time. Except for compound **16** (see item Section 4.2.1.6), the reaction mixture of the other compounds was cooled, neutralized with 10% aqueous $NaHCO_3$, and extracted (3× 30 mL) with EtOAc (**8**, **6**, **18**, **20**) or $CHCl_3$ (**10**, **12**, **14**). The organic layers were pooled, washed with distilled water, dried (anhydrous Na_2SO_4), filtered, and evaporated under reduced pressure. The products were purified by recrystallization in EtOH (**8**, **10**, **12**, **16**, **14**, **20**) or EtOH/EtOAc 2:1 (**6**) and dried.

4.2.1.1. 3-Phenyl-coumarin (8). Prepared as described in Section 4.2.1, using phenylacetic acid (228 mg) and 2-hydroxy-benzaldehyde (0.18 mL), refluxed for 3 h. Yield: 63 mg (17%). Mp: 130–133 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 7.75 (s, H-4), 7.20–7.66 (m, H-5, H-6, H-7, H-8, H-2′, H-3′, H-4′, H-5′ and H-6′). HR-ESI-MS: m/z = 223.0782 $[M + H]^+$ (calcd 223.0759).

4.2.1.2. 6,7-Diacetoxy-3-[3′,4′-methylenedioxyphenyl]-coumarin (6). Prepared as described in Section 4.2.1, using 3,4-(methylenedioxy)phenylacetic acid (301 mg) and 2,4,5-trihydroxy-benzaldehyde (257 mg), refluxed for 2 h. Yield: 118 mg (19%). Mp: 186–189 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 7.67 (s, H-4), 7.37 (s, H-5)[a], 7.24 (s, H-8)[a], 7.20 (d, J = 1.5 Hz, H-2′), 6.89 (d, J = 8.4 Hz, H-5′), 7.17 (dd, J = 8.4, 1.5 Hz, H-6′), 6.04 (s, H-7′), 2.36 (s, OAc), 2.35 (s, OAc). ([a] May be interchangeable.) HR-ESI-MS: m/z = 405.0684 $[M+Na]^+$ (calcd 405.0650).

4.2.1.3. 6-Acetoxy-3-phenyl-coumarin (10). Prepared as described in Section 4.2.1, using phenylacetic acid (228 mg), and 2,5-dihydroxy-benzaldehyde (231 mg), refluxed for 3.5 h. Yield: 140 mg (30%). Mp: 150–153 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 7.68 (s, H-4), 7.23 (d, J = 2.5 Hz, H-5), 7.17 (dd, J = 8.8, 2.5 Hz, H-7), 7.30 (d, J = 8.8 Hz, H-8), 7.60–7.63 (m, H-2′ and H-6′), 7.32–7.41 (m, H-3′, H-4′ and H-5′), 2.26 (s, OAc). HR-ESI-MS: m/z = 303.0693 $[M+Na]^+$ (calcd 303.0661).

4.2.1.4. 6-Acetoxy-3-[3′-acetoxyphenyl]-coumarin (12). Prepared as described in Section 4.2.1, using 3-hydroxyphenylacetic acid (254 mg) and 2,5-dihydroxy-benzaldehyde (231 mg), refluxed for 2 h. Yield: 193 mg (34%). Mp: 154–156 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 7.72 (s, H-4), 7.24 (d, J = 2.8 Hz, H-5), 7.19 (dd, J = 8.8, 2.5 Hz, H-7), 7.31 (d, J = 9.1 Hz, H-8), 7.41 (dd, J = 2.3, 1.0 Hz, H-2′), 7.09 (ddd, J = 8.1, 2.3, 1.0 Hz, H-4′), 7.38 (t, J = 8.1 Hz, H-5′), 7.50 (ddd, J = 7.8, 1.8, 1.0 Hz, H-6′), 2.26 (s, OAc)[a], 2.27 (s, OAc)[a]. ([a] May be interchangeable.) HR-ESI-MS: m/z = 361.0723 $[M+Na]^+$ (calcd 361.0688).

4.2.1.5. 6-Acetoxy-3-[4′-acetoxyphenyl]-coumarin (14). Prepared as described in Section 4.2.1, using 4-hydroxy-phenylacetic acid (254 mg) and 5-dihydroxy-benzaldehyde (231 mg), refluxed for 2 h. Yield: 162 mg (29%). Mp: 199–200 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 7.75 (s, H-4), 7.31 (d, J = 2.8 Hz, H-5), 7.25 (dd, J = 8.8, 2.8 Hz, H-7), 7.38 (d, J = 9.1 Hz, H-8), 7.73 (d, J = 8.8 Hz, H-2′ and H-6′), 7.19 (d, J = 8.8 Hz, H-3′ and H-5′), 2.33 (s, 6-OAc)[a], 2.34 (s, OAc)[a]. ([a] May be interchangeable.) HR-ESI-MS: m/z = 361.0727 $[M+Na]^+$ (calcd 361.0688).

4.2.1.6. 6-Acetoxy-3-[2′-acetoxyphenyl]-coumarin (16). Prepared as described in Section 4.2.1, using 2-hydroxyphenylacetic acid (254 mg) and 2,5-dihydroxy-benzaldehyde (231 mg), refluxed for 1 h. After the reaction period, distilled water was added to the reaction mixture and the resulting solid filtered and washed with distilled water. Yield: 192 mg (34%). Mp: 161–164 °C. ^{1}H NMR (400 MHz, $CDCl_3$): 7.69 (s, H-2), 7.51 (dl, J = 2.7 Hz, H-5), 7.21 (dd , J = 8.8, 2.7 Hz, H-7), 7.66 (dl, J = 8.8 Hz, H-8), 7.27 (dl, J = 8.5 Hz, H-3′)[a], 7.36 (ddd, J = 8.0, 7.7, 1.3 Hz, H-4′)[b], 7.08 (ddd, J = 7.7, 7.7, 1.0, Hz, H-5′)[b], 7.12 (ddd, J = 8.1, 0.9, 0.5 Hz, H-6′)[a], 2.32 (s, OAc), 2.28 (s, OAc). ([a,b] May be interchangeable.) HR-ESI-MS: m/z = 361.0701 $[M+Na]^+$ (calcd 361.0688).

4.2.1.7. 6-Acetoxy-3-[3′,4′-diacetoxyphenyl]-coumarin (18). Prepared as described in Section 4.2.1, using 3,4-dihydroxyphenylacetic acid (281 mg) and 2,5-dihydroxy-benzaldehyde (231 mg), refluxed for 5 h. After extraction with EtOAc**,** a small amount of solid reaction product remained in the organic layer, which was removed by filtration and washing with EtOAc. The organic layers resulting from these procedures were pooled, washed with distilled water, dried (anhydrous Na_2SO_4), filtered, and evaporated under reduced pressure. Coumarin **18** was obtained as a pure product, and no further purification by recrystallization was carried out. Yield: 181 mg (27%). Mp: 208–212 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 7.79 (s, H-4), 7.62 (m, H-5 and H-7), 7.29 (d, J = 8.9 Hz, H-8), 7.32 (d, J = 2.7 Hz, H-2′), 7.38 (d, J = 8.9 Hz, H-5′), 7.27 (dd, J = 8.9, 2.7 Hz, H-6′), 2.35 (s, OAc), 2.32 (s, OAc). HR-ESI-MS: m/z = 419.0787 $[M+Na]^+$ (calcd 419.0743).

4.2.1.8. 6,7-Diacetoxy-3-[3′,4′-diacetoxyphenyl]-coumarin (20). Prepared as described in Section 4.2.1, using 3,4-dihydroxyphenylacetic acid (281 mg) and 2,4,5-trihydroxy-benzaldehyde (257 mg), refluxed for 1 h. Yield: 100 mg (13%). Mp: 185–188 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 7.77 (s, H-4), 7.40 (s, H-5), 7.26 (s, H-8)[a], 7.60 (m, H-2′ and H-6′), 7.28 (d, J = 9.1 Hz, H-5′), 2.34 (s, OAc), 2.33 (s, OAc), 2.32 (s, OAc). [a] Signal coincident with $CDCl_3$. HR-ESI-MS: m/z = 477.0850 $[M+Na]^+$ (calcd 477.0798).

4.2.2. General procedure for the preparation of the hydroxylated compounds. Hydroxylated coumarins (**5**, **9**, **11**, **13**, **15**, **17**, **19**) were obtained by hydrolysis of their acetoxylated counterparts. In general, the appropriate acetoxylated coumarin mixed with 2 N aqueous HCl and MeOH was refluxed (100 °C) with stirring during different periods of time. The resulting reaction mixtures were cooled in an ice-bath and the reaction products, obtained as solids, were filtered, washed with cold distilled water, and dried under vacuum.

4.2.2.1. 6,7-Dihydroxy-3-[3′,4′-methylenedioxyphenyl]-coumarin (5). A mixture of coumarin **6** (75 mg), HCl (10 mL) and MeOH (4 mL) was allowed to react for 5 h. Yield: 49 mg (84%). Mp: >300 °C. ^{1}H NMR (400 MHz, CD_3OD): 7.86 (s, H-4), 6.99 (s, H-5)[a], 6.77 (s, H-8)[a], 7.21 (d, J 1.8 Hz, H-2′), 6.87 (d, J 8.1 Hz, H-5′), 7.17 (dd, J 8.1, 1.8 Hz, H-6′), 5.98 (s, H-7′). ([a] May be interchangeable.) HR-ESI-MS: m/z = 299.0573 $[M+H]^+$ (calcd 299.0556).

4.2.2.2. 6-Hydroxy-3-phenyl-coumarin (9). A mixture of coumarin **10** (80 mg), HCl (10 mL) and, MeOH (4 mL) was allowed to react for 2 h. Yield: 54 mg (79%). Mp: 202–204 °C. ^{1}H NMR (400 MHz, CD_3OD):

7.99 (s, H-4), 7.06–7.09 (m, H-5 and H-7), 7.26 (ddd, J = 7.6, 1.8, 0.8 Hz, H-8), 7.70–7.73 (m, H-2′ and H-6′), 7.39–7.48 (m, H-3′, H-4′ and H-5′). HR-ESI-MS: m/z = 239.0736 $[M+H]^+$ (calcd 239.0708).

4.2.2.3. 6-Hydroxy-3-[3′-hydroxyphenyl]-coumarin (11). A mixture of coumarin **12** (85 mg), HCl (7 mL), and MeOH (3 mL) was allowed to react for 4 h. Yield: 46 mg (72%). Mp: 267–268 °C. ^{1}H NMR (400 MHz, CD_3OD): 7.95 (s, H-4), 7.03 (d, J = 2.8 Hz, H-5), 7.05 (dd, J = 8.4, 2.8 Hz, H-7), 7.22 (dl, J = 8.4 Hz, H-8), 7.12–7.16 (m, H-2′ and H-4′), 7.25 (t, J = 7.6 Hz, H-5′), 6.82 (ddd, J = 8.1, 2.3, 1.0 Hz, H-6′). HR-ESI-MS: m/z = 255.0679 $[M+H]^+$ (calcd 255.0657).

4.2.2.4. 6-Hydroxy-3-[4′-hydroxyphenyl]-coumarin (13). A mixture of coumarin **14** (90 mg), HCl (10 mL), and MeOH (4 mL) was allowed to react for 1 h. Yield: 55 mg (81%). Mp: 289–292 °C. ^{1}H NMR (400 MHz, $CDCl_3$: CD_3OD 8:2): 7.58 (s, H-4), 6.81 (d, J = 2.8 Hz, H-5), 6.88 (dd, J 8.8, 2.8 Hz, H-7), 7.07 (d, J = 8.8 Hz, H-8), 7.42 (d, J = 8.8 Hz, H-2′ and H-6′), 6.75 (d, J = 8.8 Hz, H-3′ and H-5′). HR-ESI-MS: m/z = 255.0673 $[M+H]^+$ (calcd 255.0657).

4.2.2.5. 6-Hydroxy-3-[2′-hydroxyphenyl]-coumarin (15). A mixture of coumarin **16** (110 mg), HCl (10 mL), and MeOH (3 mL) was allowed to react for 2 h. Yield: 30 mg (36%). Mp: 269–270 °C. ^{1}H NMR (400 MHz, CD_3OD): 7.86 (s, H-4), 6.99 (d, J = 2.8 Hz, H-5), 7.05 (dd, J = 8.8, 2.8 Hz, H-7), 7.24 (dl, J = 8.8 Hz, H-8), 6.88 (dd, J = 7.5, 1.0 Hz, H-3′), 7.21 (ddd, J = 8.3, 7.3, 1.8 Hz, H-4′), 6.89 (dt, J = 7.5, 7.5, 1.0 Hz, H-5′), 7.29 (dd, J = 7.8, 1.8 Hz, H-6′). HR-ESI-MS: m/z = 255.0659 $[M+H]^+$(calcd 255.0657).

4.2.2.6. 6-Hydroxy-3-[3′,4′-dihydroxyphenyl]-coumarin (17). A mixture of coumarin **18** (100 mg), HCl (8 mL), and MeOH (3 mL) was allowed to react for 2.5 h. Yield: 64 mg (95%). Mp: 294–297 °C. ^{1}H NMR (400 MHz, CD_3OD): 7.85 (s, H-4), 7.00–7.03 (m, H-5 and H-7), 7.19–7.21 (m, H-8), 7.21 (d, J = 2.3 Hz, H-2′), 6.82 (d, J = 8.3 Hz, H-5′), 7.07 (dd, J = 8.3, 2.3 Hz, H-6′). HR-ESI-MS: m/z = 271.0624 $[M+H]^+$ (calcd 271.0607).

4.2.2.7. 6,7-Dihydroxy-3-(3′,4′-dihydroxyphenyl)-coumarin (19). A mixture of coumarin **20** (75 mg), HCl (7 mL), and MeOH (3 mL) was allowed to react for 3 h. Yield: 35 mg (75%). Mp: >300 °C. ^{1}H NMR (400 MHz, CD_3OD): 7.79 (s, H-4), 6.98 (s, H-5)[a],6.76 (s, H-8)[a], 7.17 (d, J = 2.0 Hz, H-2′), 6.80 (d, J = 8.3 Hz, H-5′), 7.02 (dd, J = 8.3, 2.0, H-6′). ([a] May be interchangeable.) HR-ESI-MS: m/z = 309.0401 $[M+Na]^+$ (calcd 309.0375).

4.3. Evaluation of HRP catalytic activity

HRP activity was evaluated by a modification of the method described by Krol et al.[35] Values in parentheses are final concentrations in a reaction volume of 0.5 mL. Briefly, aliquots of H_2O_2 (50 μM), luminol (140 μM), and each test-compound (0–50 μM) or DMSO (control) were added to 0.1 M sodium phosphate buffer, pH 7.4. After incubation for 3 min at 30 °C, HRP solution (0.2 IU/mL) was added to the reaction medium and chemiluminescence production was measured in a luminometer (AutoLumat LB953, EG&G Berthold, Germany) during 20 min at 30 °C. Luminol and the test-compounds were prepared in DMSO, whereas H_2O_2 and HRP were prepared in 0.1 M phosphate buffer, pH 7.4. DMSO final concentration in the reaction medium was 1%. Values of the integrated area of chemiluminescence were used to calculate percentages of inhibition or increase promoted by each concentration of the tested compounds. IC_{50} values were determined for those compounds with significant inhibitory activity.

4.4. Assay of DPPH radical scavenging

DPPH free radical scavenging ability of the 3-phenyl-coumarin derivatives was evaluated according to a modified method of Blois.[36] DPPH solution (final concentration of 100 μM) in ethanol was diluted in 0.1 M sodium acetate buffer, pH 5.4 (6:4) prior to addition of the test-compounds (0–50 μM) in DMSO. The contents were vigorously mixed and allowed to stand at 25 °C for 5 min. Absorbance was measured at 510 nm, before (100%) and after addition of the test-compounds. Percentage of DPPH reduction by each sample was calculated.

4.5. General docking strategy

Flexible docking simulations were performed using the GOLD 3.0 software,[37] which contains an implemented genetic algorithm. Default parameters are originally optimized for a set of 305 different complexes with solved structures provided in the Protein Data Bank (PDB). A population of 100 conformers, 100000 operations, 95 mutations, and 95 crossovers was used. Docking calculations were performed inside a sphere with a 15 Å radius and centered at the His42 Nε2 oxygen of the horseradish peroxidase structure (PDB code 1H5G),[28] which has been solved at the highest resolution (1.57 Å) amongst all horseradish peroxidases with coordinates deposited in PDB. The residue chosen is situated approximately at the center of the HRP active site. Ten orientations of the highest score obtained for each compound were thus selected through the GoldScore. Based on this function, GOLD classifies the orientations of the molecules by a decreasing affinity order (the scores) with the receptor binding site. Previous to the docking calculations and after the removal of the crystallographic waters inside the active site, residue side-chain hydrogen atoms were added and oriented with constrained minimization procedures, where only these atoms were kept free. Atomic changes and potentials of the AMBER force field were added to the protein structure using the Insight II software.[38]

Conformational search was performed with the 20 coumarins and 4 flavonoids, using the systematic method for molecules with 5 or less rotatable bonds. The MONTE CARLO method was used for the most complex

molecules, with the MMFF molecular mechanics model. Structures were then fully optimized in the gas phase at B3LYP/6-31G* level, using the Spartan 04 1.0.3 software.[39]

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