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Synthesis and evaluation of the platelet antiaggregant properties of phenolic antioxidants structurally related to rosmarinic acid

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ABSTRACT

Polyphenols, such as rosmarinic acid, are widely distributed natural products with relevant antioxidant activity. Oxidative stress plays an important role in the pathogenesis of a number of disorders. Here, we report on the synthesis and biological effects of the polyphenolic esters hydroxytyrosyl gallate (**1**), hydroxytyrosyl protocatechuate (**2**) and hydroxytyrosyl caffeate (**3**), structurally related to rosmarinic acid. The three compounds showed a greater free radical scavenging activity than their precursors and also than rosmarinic acid. Esters **1** and **3** significantly reduced thrombin-evoked platelet aggregation, which is likely mediated to the attenuation of thrombin-stimulated Ca^{2+} release and entry. The three compounds reduced the ability of platelets to accumulate Ca^{2+} in the intracellular stores, probably by enhancing the Ca^{2+} leakage rate and reduced store-operated Ca^{2+} entry in these cells. These observations suggest that the structurally-simplified analogs to rosmarinic acid, compounds **1** and **3**, might be the base of therapeutic strategies to prevent thrombotic complications associated to platelet hyperaggregability due to oxidative stress.

1. Introduction

It is known that intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) controls a number of platelet functions, including aggregation, and platelet agonists, such as thrombin, increase $[Ca^{2+}]_i$, which consists of two components: release of Ca^{2+} from intracellular stores and Ca^{2+} entry through plasma membrane channels [1]. It has been shown that agonist-induced Ca^{2+} mobilization is altered in platelets from patients suffering from oxidative stress, including diseases like type 2 diabetes mellitus (DM), where both Ca^{2+} release and entry are enhanced [2] while Ca^{2+} extrusion by plasma membrane Ca^{2+}-ATPase (PMCA) is reduced [3]. Abnormal Ca^{2+} release, entry and extrusion in platelets from diabetic donors are reversed by treatment with antioxidants, which further support the role of oxidative stress in this phenomenon [4,5]. Consistent to this, we have recently demonstrated that *in vitro* platelet treatment with phenolic compounds showing antioxidant properties reverses the enhanced oxidant production, altered Ca^{2+} mobilization and hyperaggregability described in platelets from diabetic patients, supporting that natural antioxidants may be used to develop therapeutic strategies to reduce cardiovascular complications in type 2 DM [6–8].

Rosmarinic acid is a naturally occurring polyphenol widely distributed in Labiatae plants, among others. It is one of the main components responsible for the high antioxidant activity of commercial rosemary extracts (the only spice commercially available for use as an antioxidant in food processing in Europe and United States) [9], and it can also be found in sage, basil, peppermint, perilla or lavender [10]. The biological activities and potential applications of rosmarinic acid have been studied widely. It exhibits antioxidant [11], anti-inflammatory [12] or anxiolytic-like [13] activities. It also has hepatoprotective [14] and neuroprotective [15] effects, inhibits the enzymatic thrombin activity [16], and induces apoptosis of activated T cells from rheumatoids arthritis patients [17], among other activities. Some rosmarinic acid derivatives have been claimed for the prevention and improvement of insulin resistance in DM [18] and as new anti-HIV-1 agents [19]. Although the chemical synthesis of rosmarinic acid has already been described [20], the required amounts of this compound are still isolated from natural sources or *in vitro* [21] and biotechnologically [22] produced, since the

Abbreviations: ARP, antiradical power; DIAD, diisopropyl azodicarboxylate; DM, diabetes mellitus; DPPH, 1,1-diphenyl-2-picrylhydrazyl; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} entry; TG, thapsigargin; TPP, triphenylphosphine.

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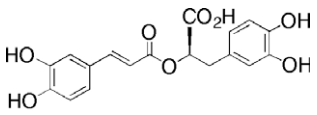
stereogenic carbon at the 3,4-dihydroxyphenyllactic acid moiety adds disadvantages to a commercial synthetic approach. For this reason, the investigation of low-cost ways to produce rosmarinic acid or structurally-simplified analogs with interesting biological activities may be an attractive and profitable aim.



Rosmarinic acid

In this line, here we report on the synthesis of three antioxidants structurally related to rosmarinic acid, such as esters hydroxytyrosyl gallate (**1**) [23], hydroxytyrosyl protocatechuate (**2**) and hydroxytyrosyl caffeate (**3**) [24], from abundant polyphenolic starting materials like gallic acid (**4**) [25], protocatechuic acid (**5**) [26], caffeic acid (**6**) [26] and hydroxytyrosol (**7**) [27] (Scheme 1), and their ability to attenuate thrombin-stimulated platelet aggregation. In addition, we have explored the possible mechanisms involved in the attenuation of platelet function such as their role in Ca^{2+} mobilization. These phenolic compounds might be the base of therapeutic strategies for disorders involving oxidative stress.

2. Material and methods

2.1. Materials

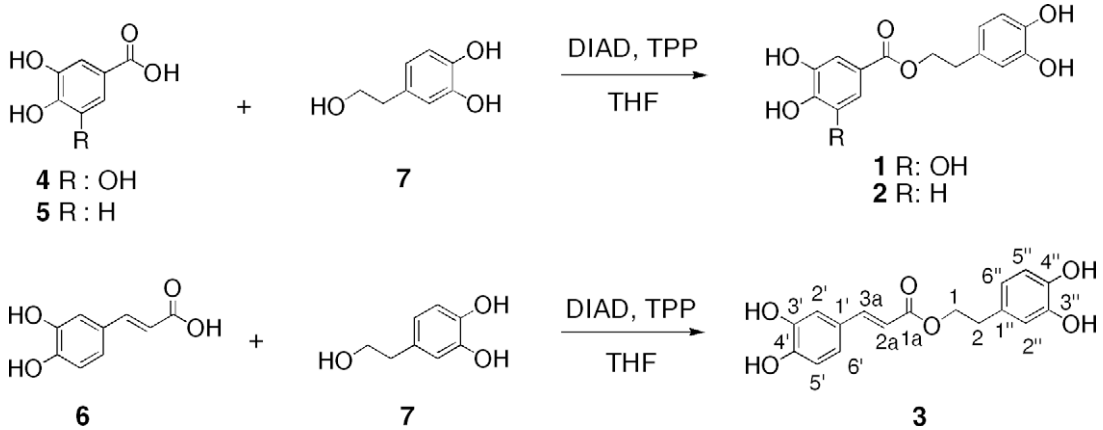
Hydroxytyrosol (**7**) was prepared by reducing 3,4-dihydroxyphenylacetic acid according to Capasso method [28]. Gallic acid (**4**), protocatechuic acid (**5**), caffeic acid (**6**) and diisopropyl azodicarboxylate (DIAD) [29] were purchased from Fluka (Madrid, Spain). Rosmarinic acid, triphenylphosphine (TPP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3,4-dihydroxyphenylacetic acid, apyrase (grade VII), aspirin, thapsigargin (TG), thrombin, ionomycin (Iono) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Madrid, Spain). Fura-2 acetoxymethyl ester (fura-2/AM) and calcein-AM were from Molecular Probes (Leiden, The Netherlands). All reagents were used without further purification. The tetrahydrofuran (THF) used in the syntheses of **1**–**3** was purchased from Panreac (Barcelona, Spain) and distilled under argon immediately before use from sodium/benzophenone.

2.2. Chromatography and structure determination techniques

Chromatographic separations were performed on SCHARLAU silica gel (230–400 mesh) and MERCK silica gel (70–230 mesh) using the solvent systems indicated. Solvent systems are reported as v/v percent ratios. All reactions were monitored by high-performance liquid chromatography (HPLC) (see below) and thin-layer chromatography (TLC) using MERCK silica gel 60 F_{254} precoated aluminum sheets (0.25 mm). Yields refer to chromatographically and spectroscopically pure compounds except when otherwise indicated. HPLC analyzes were performed by analytical RP-HPLC (Waters Spherisorb® C18 S5 ODS2, 250 mm × 4.6 mm i.d., 5 μm) and HPLC purifications by semipreparative RP-HPLC (Waters Spherisorb® C18 S5 ODS2, 250 mm × 22 mm i.d., 5 μm) on a Waters 600E instrument equipped with a photodiode array detector (DAD), scan range: 190–800 nm, and operating at 30 °C. The HPLC analyzes were performed by a step gradient with MeOH/AcOH 99.8:0.2 (v/v) (solvent A) and H_2O/AcOH 99.8:0.2 (v/v) (solvent B) at a flow rate of 0.7 mL/min: linear gradient from 10% to 25% A in 15 min; linear gradient from 25% to 50% A in 5 min; isocratic conditions of 50% A for 5 min, and other 5 min to return to the initial conditions. The total run time excluding equilibration was 30 min. The semipreparative HPLC purifications were performed by a step gradient with MeOH/AcOH 99.8:0.2 (v/v) (solvent A) and H_2O/AcOH 99.8:0.2 (v/v) (solvent B) at a flow rate of 5 mL/min; linear gradient from 20% to 50% A in 5 min; isocratic conditions of 50% A for 5 min, and other 5 min to return to the initial conditions. The total run time excluding equilibration was 15 min. UV spectra were recorded in EtOH on a Varian Cary 4000 UV/vis spectrophotometer. Melting points were determined on a Barnstead Electrothermal apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker DPX 400 spectrometer (400 MHz). Chemical shift values are reported in parts per million (ppm, δ scale) and coupling constants (J) are in hertz (Hz). All described coupling constants are referred to a three-bond coupling distance and further (^nJ, $n \geqslant 3$). The splitting patterns are abbreviated as follows: s (singlet), d (doublet), t (triplet), dd (double doublet). ^{13}C NMR spectra were recorded on the same instrument (100 MHz). Chemical shifts are also reported in ppm and carbon substitution degrees were established by DEPT multipulse sequence. Infrared (IR) spectra were recorder on a FT-IR Perkin–Elmer 1760X spectrometer. High resolution mass spectra (HRMS) were obtained on a LC–MS equipment composed of an Alliance 2795 Waters HPLC chromatograph fitted to a Micromass LCT Premier mass spectrometer, with an electrospray ionization (ESI) source and a time-of-flight (TOF) analyzer.

2.3. Mitsunobu esterification of polyphenolic acids (**4**–**6**) with hydroxytyrosol (**7**). General protocol

To a cooled (0 °C) solution of recently prepared **7** (see above) [28] (1 mmol) and a given polyphenolic acid (**4**–**6**) (1.5 mmol,



Scheme 1. Synthesis of hydroxytyrosyl gallate (**1**), hydroxytyrosyl protocatechuate (**2**) and hydroxytyrosyl caffeate (**3**).

1.5 M equiv) in dry THF (1 mL/75 mg of **7**) were added TPP (2.53 mmol, 1.5 M equiv) and DIAD (2.53 mmol, 1.5 M equiv). The reaction mixture was stirred at room temperature for 2 days. Then, the reaction was worked up by removal of the solvent, re-dissolved on EtOAc and extracted with 1 N $NaHCO_3$ (*ca.* 50 mL/250 mg of **7**). The organic phase was washed with brine, dried (Na_2SO_4) and evaporated. The resultant ester (crude reaction) was subjected to two successive silica gel column chromatographies, eluting with $CHCl_3$–MeOH mixtures, and eventually purified by semipreparative RP-HPLC.

2.3.1. 2-(3,4-Dihydroxyphenyl)ethyl 3,4,5-trihydroxybenzoate (*1*)

A crude reaction (1.60 g) containing compound **1** was obtained following the general protocol (hydroxytyrosol (**7**): 260 mg; gallic acid (**4**): 430 mg; THF: 3.5 mL; TPP: 663 mg; DIAD: 527 μL), which was first chromatographed on silica gel (70–230 mesh) using $CHCl_3$–MeOH mixtures. It was obtained crude **1** (410 mg) that was submitted to a second column chromatography on silica gel (230–400 mesh) using an 85:15 $CHCl_3$–MeOH mixture. Thus, compound **1** (211 mg, 41%) was obtained as a yellowish powder, and an aliquot amount was re-purified by semipreparative HPLC as described above (NMR purity: 95%). Mp 189–190 °C (lit. [23] 189–191 °C). Spectral data of this compound were identical to those previously reported [23].

2.3.2. 2-(3,4-Dihydroxyphenyl)ethyl 3,4-dihydroxybenzoate (*2*)

A crude reaction (3.50 g) containing compound **2** was obtained following the general protocol (hydroxytyrosol (**7**): 845 mg; protocatechuic acid (**5**): 1.27 g; THF: 12 mL; TPP: 2.13 g; DIAD: 1.59 mL), which was first chromatographed on silica gel (70–230 mesh) using $CHCl_3$–MeOH mixtures. It was obtained crude **2** (1.63 g) that was submitted to a second column chromatography on silica gel (230–400 mesh) using a 95:5 $CHCl_3$–MeOH mixture. Thus, compound **2** (500 mg, 31%) was obtained as a brown-pale powder, and an aliquot amount was re-purified by semipreparative HPLC as described above (NMR purity: *ca.* 96%). Mp 153–156 °C; UV (EtOH) λ_{max} (log ε): 264 (4.33), 290 (4.17) nm; IR (KBr) 3436 and 3308 (phenols), 1677 and 1613 ($ArCO_2R$) cm^{-1}; HRMS (ESI) ($C_{15}H_{14}O_6$) *m/z* calculated $M-H^+$ 289.0712, measured $M-H^+$ 289.0710; ^1H NMR (CD_3OD) δ 2.86 (2H, t, *J* = 7.0, H-2), 4.35 (2H, t, *J* = 7.0, H-1), 6.58 (1H, dd, *J* = 1.9 and 8.1, H-6″), 6.69 (1H, d, *J* = 8.1, H-5″), 6.71 (1H, d, *J* = 1.9, H-2″), 6.78 (1H, dd, *J* = 1.5 and 7.5, H-6′), 7.38 (1H, d, *J* = 1.5, H-2′), 7.39 (1H, d, *J* = 7.5, H-5′); ^{13}C NMR (CD_3OD) δ signals for the hydroxytyrosyl moiety: 35.61 (C-2), 66.75 (C-1), 116.40 (C-2″), 117.04 (C-5″), 121.29 (C-6″), 130.93 (C-1″), 144.87 (C-4″), 146.10 (C-3″), δ signals for the 3,4-dihydroxybenzoate moiety: 115.82 (C-6′), 117.40 (C-2′), 122.70 (C-1′), 123.65 (C-5′), 146.10 (C-3′), 151.65 (C-4′), 168.35 (C-1a).

2.3.3. (E)-2-(3,4-Dihydroxyphenyl)ethyl 3-(3,4-dihydroxyphenyl)acrilate (*3*)

A crude reaction (3.39 g) containing compound **3** was obtained following the general protocol (hydroxytyrosol (**7**): 490 mg; caffeic acid (**6**): 905 mg; THF: 9 mL; TPP: 1.25 g; DIAD: 925 μL), which was first chromatographed on silica gel (70–230 mesh) using $CHCl_3$–MeOH mixtures. It was obtained crude **3** (668 mg) that was submitted to a second column chromatography on silica gel (230–400 mesh) using a 95:5 $CHCl_3$–MeOH mixture. Thus, compound **3** (369 mg, 47%) was obtained as a yellow-dark powder, and an aliquot amount was re-purified by semipreparative HPLC as described above (NMR purity: *ca.* 95%). Mp 118–120 °C; UV (EtOH) λ_{max} (log ε): 296 (3.96), 329 (4.49) nm; IR (KBr) 3485 and 3334 (phenols), 1672 and 1630 ($ArCO_2R$), 976 (asym-trisubstituted benzene and HC = CH–CO) cm^{-1}; HRMS (ESI) ($C_{17}H_{16}O_6$) *m/z* calculated $M-H^+$ 315.0869, measured $M-H^+$ 315.0868; ^1H NMR (CD_3OD) δ 2.82 (2H, t, *J* = 7.1, H-2), 4.28 (2H, t, *J* = 7.1, H-1), 6.22 (1H, d, *J* = 15.9, H-2a), 6.56 (1H, dd, *J* = 8.1 and 2.1, H-6″), 6.69 (1H, d, *J* = 8.1, H-5″), 6.70 (1H, d, *J* = 2.1, H-2″), 6.76 (1H, d, *J* = 8.1, H-5′), 6.92 (1H, dd, *J* = 8.1 and 2.1, H-6′), 7.02 (1H, d, *J* = 2.1, H-2′), 7.51 (1H, d, *J* = 15.9, H-3a); ^{13}C NMR (CD_3OD) δ signals for the hydroxytyrosyl moiety: 35.58 (C-2), 66.49 (C-1), 117.05 (C-2″), 116.49 (C-5″), 121.24 (C-6″), 130.85 (C-1″), 144.91 (C-4″), 146.25 (C-3″), δ signals for the caffeate moiety: 115.14 (C-2′), 115.15 (C-2a), 116.40 (C-5′), 122.78 (C-6′), 127.74 (C-1′), 146.78 (C-3a), 146.87 (C-3′), 149.54 (C-4′), 169.28 (C-1a). NMR data of this compound (recorded in CD_3OD) were in agreement with those previously reported (recorded in DMSO-d_6) [24].

2.4. Free radical scavenging activity

The antioxidant activity of hydroxytyrosol (**7**), the phenolic carboxylic acids **4**–**6**, rosmarinic acid (reference compound) and the esters **1**–**3** was determined by spectrophotometric measurements according to the ability of the tested samples to scavenge the free radical DPPH. This activity was determined using the method employed by Brand-Williams et al. [30] and modified as described below. Methanolic solutions (1.2 mL) of a given compound, at different concentrations between 0.33 and 4.60 μg/mL were mixed in a 1 cm path length disposable plastic cuvette with methanolic solutions (2.4 mL) of DPPH· ($\sim 7 \times 10^{-5}$ M) with an absorbance at 515 nm of 0.80 ± 0.03 AU. The exact DPPH· concentration was calculated from a calibration curve. Triplicate samples were shaken and allowed to stand for 15 min in the dark at room temperature, and the decrease of absorbance at 515 nm was measured using a Perkin–Elmer UV/vis spectrophotometer Lambda 19 (Perkin–Elmer Instruments, Norwalk, CT).

2.5. Platelet preparation

Blood was obtained from healthy volunteers, according to the rules of the Declaration of Helsinki, and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700*g* and aspirin (100 μM) and apyrase (40 μg/mL) added. Cells were then collected by centrifugation at 350*g* for 20 min and re-suspended in HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45, and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase.

2.6. Cell viability

Cell viability was assessed using calcein and trypan blue as described previously [31]. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37 °C, centrifuged and the pellet was re-suspended in fresh HBS. Cells were treated with the different inhibitors, centrifuged and re-suspended in HBS. Fluorescence was recorded from 2 mL aliquots using a spectrophotometer (Varian Ltd., Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique.

2.7. Platelet aggregation

Aggregation of washed platelets was monitored in a Chronolog (Havertown, PA, USA) aggregometer at 37 °C under stirring at 1200 rpm. The percentage of aggregation or amplitude is estimated as the percentage of the difference in light transmission between the platelet suspension in HBS and HBS alone, and indicates the percentage of platelets that aggregate in response to an agonist. Resting platelets in suspension are arbitrarily considered by the

aggregometer as 0% aggregation and HBS is considered to be 100% aggregation. The rate, or slope, of the aggregation is the % change of aggregation per minute.

2.8. Measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Human platelets were loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37 °C. Fluorescence was recorded from 2 mL aliquots of magnetically stirred cellular suspension (2 × 108 cells/mL) at 37 °C using a Cary Eclipse spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm, and emission at 505 nm, as previously described [32]. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated as described previously.

2.9. Statistical analysis

Analysis of statistical significance was performed using one-way analysis of variance (ANOVA) combined with the Dunnett or Tukey test. For comparison between two groups Student's *t* test was used. $p < 0.05$ was considered to be significant for a difference.

3. Results and discussion

3.1. Chemical synthesis

The esterification of phenolic alcohols and phenolic acids via acyl nucleophilic substitution under nucleophilic catalysis requires protection of phenolic hydroxyls, since acids activated *in situ* (isoureas and mixed phosphoric anhydrides) or *ex situ* (chlorides, anhydrides, and mixed anhydrides) show poor discrimination between hydroxyls bound to aliphatic and aromatic carbons [33]. Other methods, such as Fischer esterification using strong protic acids or the Lewis acid-catalyzed acylations have been described with acceptable level of chemoselectivity, but reaction conditions in many cases make these strategies of limited applicability [34]. In order to find a suitable chemoselectivity in the esterification reaction [35] without protecting phenolic hydroxyls, we chose the Mitsunobu couple, which has the advantage of consider to phenolic hydroxyls present on either substrate as inert spectators [33,36]. Thus, compounds **1**–**3** were prepared according to the Mitsunobu protocol [33] with moderate yield. Compound **1** had previously been prepared in five steps and 5% overall yield [23]. However, under Mitsunobu conditions, it could be prepared in one step only (>40% yield) and without resorting to any protecting group. The removal of Mitsunobu by-products and the purification of polar compounds **1**–**3** were achieved by two successive carefully-performed silica gel column chromatographies. This procedure was preferred to the previously described chromatography on Sephadex [33], on the basis of a lower laboratory cost and similar efficiency.

3.2. Antiradical measurements of the semi-synthetic antioxidants

The radical scavenging activity of the tested samples was expressed as the molar amount of antioxidant necessary to decrease the initial DPPH· concentration by 50% (efficient concentration = EC_{50} (moles of antioxidant/moles of DPPH·)) and as the antiradical power (ARP = $1/EC_{50}$), for reasons of clarity. In Table 1, hydroxytyrosol (**7**), the referred phenolic carboxylic acids **4**–**6**, rosmarinic acid (reference compound) and the esters **1**–**3** are presented in increasing order of their ARP values. The results of this comparative study showed that esters **1**–**3** are more potent antioxidants than the starting materials used in their syntheses (**4**–**6**) and even the natural antioxidant used as standard, rosmarinic acid. It is worth noting that hydroxytyrosol (**7**), a powerful antioxidant, appears with the lowest ARP value.

Table 1
Efficient concentration (EC_{50})[a] and antiradical power (ARP) values of the semi-synthetic esters **1**–**3**, their precursors **4**–**7** and rosmarinic acid against radical DPPH.

Compound	EC_{50}	ARP
Hydroxytyrosol (**7**)	0.30 ± 0.02	3.39
Caffeic acid (**6**)	0.17 ± 0.01	5.85
Protocatechuic acid (**5**)	0.17 ± 0.02	6.06
Rosmarinic acid (*reference compound*)	0.12 ± 0.01	8.13
Gallic acid (**4**)	0.10 ± 0.01	9.62
Hydroxytyrosyl caffeate (**3**)	0.09 ± 0.01	11.36
Hydroxytyrosyl gallate (**1**)	0.08 ± 0.01	12.50
Hydroxytyrosyl protocatechuate (**2**)	0.06 ± 0.01	18.18

[a] EC_{50} expressed as moles of compound/moles of DPPH· ± SD. ARP = $1/EC_{50}$.

3.3. Effect of the semi-synthetic antioxidants on platelet aggregation

Platelet treatment for 20 min at 37 °C with increasing concentrations (1–100 μM) of esters **1** and **3** significantly reduced the percentage and rate of thrombin-induced aggregation in a concentration-dependent manner (Table 3; $p < 0.05$; $n = 6$–10). The effect of ester **3** cannot be attributed to a reduction in cell viability, as reported in Table 2, and despite the fact that ester **1** reduced cell viability by 8.5%, at the concentration of 100 μM, it reduces the ability of thrombin to induce aggregation by 27%, which suggest a direct effect of **1** on platelet aggregation, rather by inhibition due to a reduction in cell viability. In contrast, neither ester **2** nor any of the precursors of the semi-synthetic compounds had any significant effect on thrombin-evoked platelet aggregation (Table 3). It is noteworthy that some of the precursors, including caffeic acid (**6**), protocatechuic acid (**5**) and, although slightly, hydroxytyrosol (**7**) reduced cell viability, while the semi-synthetic compounds, except hydroxytyrosyl gallate (**1**), had a negligible effect on cell viability (Table 2).

3.4. Effect of the semi-synthetic antioxidants on intracellular Ca^{2+} mobilization

Cytosolic Ca^{2+} concentration, especially sustained increases due to Ca^{2+} entry, has been reported to be important for agonist-induced platelet aggregation [37]. Hence, we have further explored the effect of platelet incubation with the semi-synthetic compounds on Ca^{2+} mobilization in these cells. In a Ca^{2+}-free medium, treatment of platelets with the physiological agonist thrombin (0.1 U/mL) resulted in a transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from the intracellular stores. The subsequent addition of Ca^{2+} (300 μM) to the external medium induced a sustained elevation in

Table 2
Cell viability upon treatment[a] for 20 min at 37 °C with the semi-synthetic esters **1**–**3** and their precursors **4**–**7**.

Compound	Cell viability (% control)
Caffeic acid (**6**)	85.7 ± 2.2*
Protocatechuic acid (**5**)	90.6 ± 3.4*
Gallic acid (**4**)	92.1 ± 5.1
Hydroxytyrosyl gallate (**1**)	91.5 ± 0.5
Hydroxytyrosyl protocatechuate (**2**)	95.4 ± 1.2
Hydroxytyrosyl caffeate (**3**)	94.3 ± 2.6
Hydroxytyrosol (**7**)	95.1 ± 0.7*
Rosmarinic acid (*reference compound*)	85.7 ± 4.1*

[a] Human platelets (10^8 cells/mL) were incubated with 100 μM of **1**–**3** and **4**–**7**, as well as the well known antioxidant, rosmarinic acid, for 20 min at 37 °C. Cell viability was determined using calcein as described in the Experimental section.
* $p < 0.05$ (ANOVA) compared to control.

Table 3
Effect of treatment[a] for 20 min at 37 °C with the semi-synthetic esters **1–3** and their precursors **4–7** in thrombin-induced platelet aggregation.

Compound	Lag-time (min)	% Rate	% Aggregation
Control	0.012 ± 0.001	85.8 ± 4.1	85.9 ± 0.8
*Caffeic acid (**6**)*			
1 μM	0.011 ± 0.002	85.6 ± 4.9	84.4 ± 0.9
10 μM	0.012 ± 0.001	87.8 ± 9.7	85.2 ± 1.2
100 μM	0.010 ± 0.001	78.5 ± 2.9	76.7 ± 7.5
*Protocatechuic acid (**5**)*			
1 μM	0.010 ± 0.002	86.0 ± 5.3	86.4 ± 1.3
10 μM	0.010 ± 0.002	81.0 ± 5.9	83.1 ± 2.7
100 μM	0.011 ± 0.001	95.4 ± 14.4	84.8 ± 2.2
*Gallic acid (**4**)*			
1 μM	0.012 ± 0.002	90.6 ± 3.4	84.8 ± 0.9
10 μM	0.018 ± 0.002	91.9 ± 7.2	81.5 ± 5.5
100 μM	0.012 ± 0.001	93.7 ± 5.6	88.5 ± 1.1
*Hydroxytyrosyl gallate (**1**)*			
1 μM	0.011 ± 0.001	83.8 ± 9.5	76.8 ± 7.5
10 μM	0.013 ± 0.003	80.5 ± 4.9	78.2 ± 8.5
100 μM	0.013 ± 0.005	64.1 ± 10.9*	63.0 ± 13.6*
*Hydroxytyrosyl protocatechuate (**2**)*			
1 μM	0.008 ± 0.001*	80.3 ± 2.1	82.2 ± 0.9
10 μM	0.012 ± 0.001	94.5 ± 8.4	83.2 ± 3.4
100 μM	0.014 ± 0.001	94.0 ± 5.0	84.1 ± 3.0
*Hydroxytyrosyl caffeate (**3**)*			
1 μM	0.007 ± 0.001*	75.6 ± 1.3*	82.2 ± 3.5
10 μM	0.010 ± 0.001	76.0 ± 1.3*	81.6 ± 2.4*
100 μM	0.013 ± 0.001	66.1 ± 8.5*	66.5 ± 7.5*
*Hydroxytyrosol (**7**)*			
1 μM	0.012 ± 0.002	84.0 ± 3.1	83.0 ± 2.4
10 μM	0.014 ± 0.002	88.8 ± 7.5	85.5 ± 2.8
100 μM	0.012 ± 0.001	91.8 ± 5.1	85.5 ± 1.1

[a] Human platelets (10^8 cells/mL) were incubated with different concentrations (1–100 μM) of **1–3** and **4–7** for 20 min at 37 °C. Cells were then stimulated with 0.1 U/mL thrombin in the presence of 1 mM extracellular Ca^{2+}. Aggregation of human platelets was induced at a shear rate of 1200 rpm at 37 °C as described in the Experimental section. Values given are presented as mean ± SEM of 6–10 separate determinations.
* $p < 0.05$ (ANOVA) compared to control.

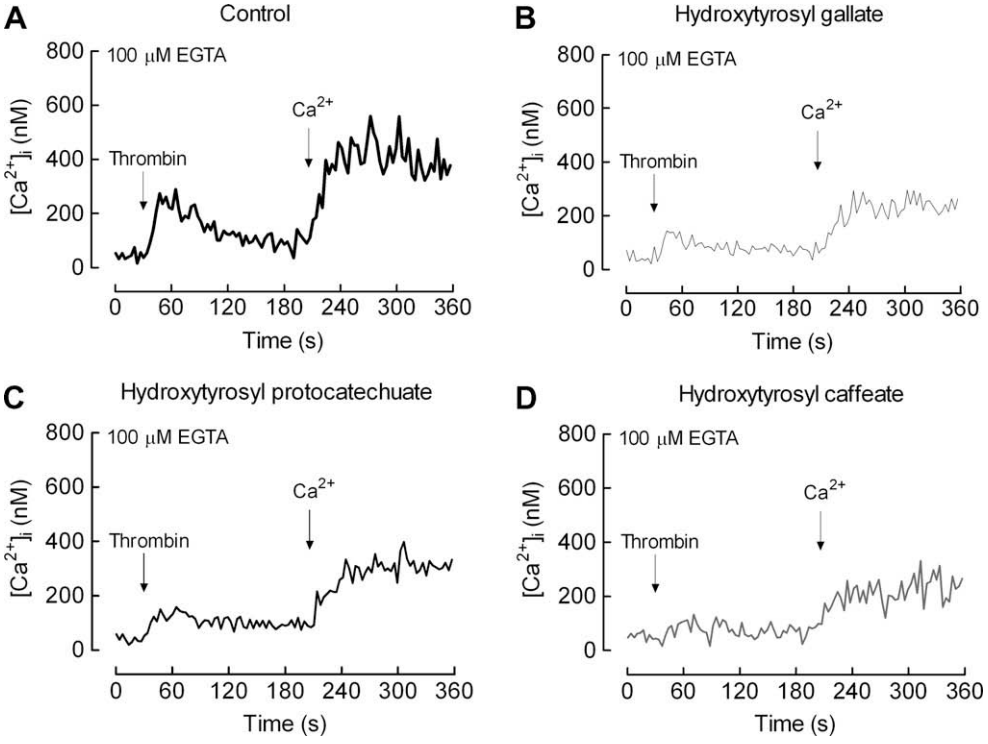


Fig. 1. Effect of treatment with the **1–3** on thrombin-induced Ca^{2+} mobilization in human platelets. Fura-2-loaded human platelets were incubated in the presence of the **1–3** (100 μM), as indicated, or the vehicle (control) for 20 min at 37 °C and then stimulated with thrombin (0.1 U/mL) in a Ca^{2+} free medium (100 μM EGTA was added). $CaCl_2$ (final concentration 300 μM) was added to the medium 3 min later to initiate Ca^{2+} entry. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces shown are representative of six separate experiments.

Table 4
Effect of treatment[a] for 20 min at 37 °C with the semi-synthetic esters **1**–**3** and hydroxytyrosol (**7**) on thrombin-evoked calcium mobilization.

Compound	Calcium release (nM.s)	Calcium entry (nM.s)
Thrombin		
Control	2375 ± 581	10069 ± 742
Hydroxytyrosyl gallate (**1**)	1331 ± 400*	8413 ± 582*
Hydroxytyrosyl protocatechuate (**2**)	1161 ± 182*	9953 ± 1005
Hydroxytyrosyl caffeate (**3**)	1025 ± 119*	8237 ± 846*
Hydroxytyrosol (**7**)	2141 ± 390	10990 ± 768
Thapsigargin + ionomycin		
Control	14253 ± 956	59582 ± 3439
Hydroxytyrosyl gallate (**1**)	12017 ± 718*	50302 ± 3683*
Hydroxytyrosyl protocatechuate (**2**)	10098 ± 1630*	47689 ± 6840*
Hydroxytyrosyl caffeate (**3**)	7081 ± 1076*	31912 ± 6398*
Hydroxytyrosol (**7**)	12435 ± 1280	53717 ± 5270

[a] Fura-2-loaded human platelets (10^8 cells/mL) were incubated with 100 μM of **1**–**3** for 20 min at 37 °C. At the time of experiment 100 μM EGTA was added. Cells were then stimulated either with 0.1 U/mL thrombin or with thapsigargin (1 μM) in combination with 50 nM ionomycin, as indicated, and 3 min later $CaCl_2$ (300 μM) was added to initiate Ca^{2+} entry. Changes in cytosolic Ca^{2+} concentration were determined as described in the Experimental section.
* $p < 0.05$ (ANOVA) compared to control.

$[Ca^{2+}]_i$ indicative of Ca^{2+} entry (Fig. 1A). Treatment of human platelets for 20 min at 37 °C with 100 μM of esters **1**–**3** significantly reduced thrombin-evoked Ca^{2+} release from the intracellular stores and, with the exception of hydroxytyrosyl protocatechuate (**2**), they also significantly reduced thrombin-induced Ca^{2+} entry (Fig. 1 and Table 4; $p < 0.05$; $n = 6$). The fact that ester **2** did not significantly reduced thrombin-stimulated Ca^{2+} entry might provide an explanation to the lack of effect of this compound on platelet aggregation by this agonist. All the semi-synthetic compounds were more effective than their precursor hydroxytyrosol (**7**) inhibiting thrombin-stimulated Ca^{2+} mobilization.

Similarly, we have investigated the effect of compounds **1**–**3** on store-operated Ca^{2+} entry (SOCE), an event activated by depletion of the intracellular Ca^{2+} stores that has been reported as a major mechanism for Ca^{2+} entry in human platelets [38,39]. In the absence of extracellular Ca^{2+}, human platelets were treated with 1 μM thapsigargin (TG), an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) [40], plus a low concentration of ionomycin (50 nM; required for extensive depletion of both internal Ca^{2+} stores in platelets) [41,42]. Platelet treatment with TG + ionomycin in stirred cuvettes at 37 °C evoked a transient elevation in $[Ca^{2+}]_i$ due to release of Ca^{2+} from internal stores. Subsequent addition of Ca^{2+} (300 μM) to the external medium induced a sustained increase in $[Ca^{2+}]_i$ indicative of SOCE (Fig. 2). Treatment of human platelets for 20 min at 37 °C with 100 μM of compounds **1**–**3** significantly attenuated Ca^{2+} release induced by TG + ionomycin (Fig. 2 and Table 4), which indicates that these compounds reduces the ability of platelets to accumulate Ca^{2+} into the stores. We have further investigated whether the reduction in TG + ionomycin-induced Ca^{2+} release by these compounds might be attributed to a reduction in the Ca^{2+} leakage rate from the intracellular stores. As shown in non-stimulated cells (Table 5), the three compounds enhanced significantly the rate of Ca^{2+} leakage from the intracellular compartments, which indicates that the inhibition of TG + ionomycin-evoked Ca^{2+} release is unlikely due to a reduction in the permeability of the Ca^{2+} stores to Ca^{2+} and is more likely attributed to a reduction in Ca^{2+} accumulation due to an increase in the permeability of the membrane of the Ca^{2+} stores for Ca^{2+}. These findings also indicate that these compounds are not Ca^{2+} chelators. In addition, we have investigated the effect of these semi-synthetic compounds on SOCE in human platelets. Treatment with

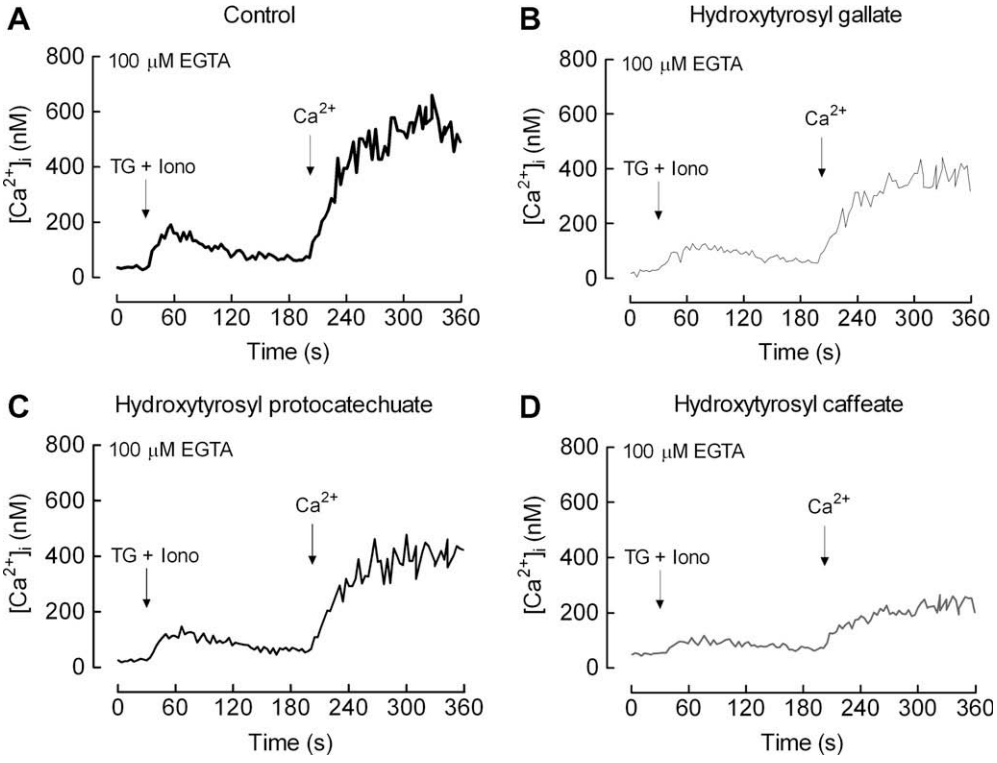


Fig. 2. Effect of treatment with the **1**–**3** on TG + ionomycin-evoked Ca^{2+} mobilization in human platelets. Fura-2-loaded human platelets were incubated in the presence of the **1**–**3** (100 μM), as indicated, or the vehicle (control) for 20 min at 37 °C and then stimulated with 1 μM TG in combination with 50 nM ionomycin in a Ca^{2+} free medium (100 μM EGTA was added). $CaCl_2$ (final concentration 300 μM) was added to the medium 3 min later to initiate Ca^{2+} entry. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces shown are representative of six separate experiments.

Table 5
Effect of treatment [a] for 20 min at 37 °C with the semi-synthetic esters **1**–**3** on Ca^{2+} leakage from the intracellular stores.

Compound	Calcium leakage (nM s)
Control	63 ± 19
Hydroxytyrosyl gallate (**1**)	658 ± 259*
Hydroxytyrosyl protocatechuate (**2**)	588 ± 32*
Hydroxytyrosyl caffeate (**3**)	177 ± 76*

[a] Fura-2-loaded human platelets (10^8 cells/mL) were incubated with 100 μM of **1**–**3** for 20 min at 37 °C. At the time of experiment 100 μM EGTA was added and cells were not stimulated for at least 3 further minutes. Changes in cytosolic Ca^{2+} concentration were determined as described in the Experimental section.
* $p < 0.05$ (ANOVA) compared to control.

compounds **1**–**3** significantly reduced TG + ionomycin-evoked SOCE in these cells (Fig. 2 and Table 4).

4. Conclusion

We have designed and synthesized a series of semi-synthetic antioxidants related to rosmarinic acid by simply joining well known and abundant natural phenolic acids with hydroxytyrosol through an ester linkage. We measured the antiradical properties, and the effect on Ca^{2+} mobilization and platelet aggregation of these compounds in human platelets. Our study shows that the semi-synthetic compounds provide an advantage over their precursors. First of all, the antiradical activity of the semi-synthetic compounds is greater than that of the precursors. Second, the latter show a more marked effect on cell viability than the semi-synthetic compounds. Finally, our results demonstrate that hydroxytyrosyl gallate (**1**), and hydroxytyrosyl caffeate (**3**) exert an inhibitory effect on platelet aggregation stimulated by the physiological agonist thrombin, an effect that was not observed with their precursors. The effect of these compounds on platelet aggregation is likely mediated by attenuation of Ca^{2+} mobilization. This study provides the basis for our ongoing efforts to discover new potent antioxidant and antiaggregant compounds and to elucidate the mechanism of the potentially aggregant activity of compounds **1** and **3**.

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