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Punicalagin and Catechins Contain Polyphenolic Substructures That Influence Cell Viability and Can Be Monitored by Radical Chemosensors Sensitive to Electron Transfer

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Supporting Information

ABSTRACT: Plant polyphenols may be free radical scavengers or generators, depending on their nature and concentration. This dual effect, mediated by electron transfer reactions, may contribute to their influence on cell viability. This study used two stable radicals (tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) and tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM)) sensitive only to electron transfer reduction reactions to monitor the redox properties of polyphenols (punicalagin and catechins) that contain phenolic hydroxyls with different reducing capacities. The use of the two radicals reveals that punicalagin's substructures consisting of gallate esters linked together by carbon−carbon (C−C) bonds are more reactive than simple gallates and less reactive than the pyrogallol moiety of green tea catechins. The most reactive hydroxyls, detected by TNPTM, are present in the compounds that affect HT-29 cell viability the most. TNPTM reacts with C−C-linked gallates and pyrogallol and provides a convenient way to detect potentially beneficial polyphenols from natural sources.

KEYWORDS: *punicalagin, catechins, pyrogallol, TNPTM chemosensor, cell viability*

INTRODUCTION

The question of whether natural polyphenols provide benefits in terms of human health is a controversial one among scientists. Ever since Harman published his paper on free radicals and aging,[1] it has been assumed that polyphenols prevent disease and delay aging because they scavenge toxic free radicals, which progressively damage biomolecules in live tissues mainly by oxidation.[2] Because they scavenge potentially oxidizing free radicals, polyphenols are referred to as antioxidants. Nevertheless, although it is true that polyphenols scavenge radicals in solution, their intracellular effectiveness is less obvious, and many authors consider them to be virtually inactive in vivo after oral intake.[3] The reason is that the live organism prevents polyphenols from greatly altering the redox homeostasis by rapidly metabolizing and excreting them, as well as by activating regulatory enzymatic systems. Polyphenols are conjugated into glucuronides, methyl esters, and sulfates mainly in the intestine and liver.[4,5] Most of these conjugates are no longer free radical scavengers, and the very small amounts of remaining intact polyphenolic moieties are very unlikely to modify the redox homeostasis significantly.[3] The skin and intestinal tract may be exceptions to this because local concentrations of intact phenolics may be present in significant amounts in these tissues.[6] Moreover, not only may polyphenols be effective free radical scavengers, they may actually generate free radicals depending on the nature and concentration of the specific polyphenols.[3] This so-called pro-oxidant activity may be behind the moderate toxicity of green tea extracts at very high concentrations[7] and the reason why polyphenols are rapidly transformed and excreted after ingestion. Interestingly, at concentrations that are not so high, this mild pro-oxidant activity may result in an overall antioxidant effect via a mechanism known as hormesis, which can be defined as a low-dose stimulation of defense systems with a subsequent beneficial effect.[8] In the case of foodstuffs in which the redox regulation systems progressively lose their efficiency during the shelf life of the product (e.g., fish rich in PUFA), polyphenols have proven to effectively prevent lipid oxidation.[9] Whatever the case, if polyphenols exert an influence over the redox status of any system, whether it is antioxidant, toxic pro-oxidant, or hormetic pro-oxidant, it is somehow related to the reactivity of the constitutive hydroxyl groups in the polyphenols, the functional groups that first react with oxidants.

Different chemical mechanisms may be involved in the free radical-scavenging and/or free radical-generating effects of polyphenols. To better characterize the scavenging activity of polyphenols, several assays focused on different possible mechanisms of their overall action should be considered.[10] The mechanisms that have been proposed are hydrogen atom transfer (HAT), proton-coupled electron transfer (PCET), and sequential proton loss electron transfer (SPLET), with the generation of a

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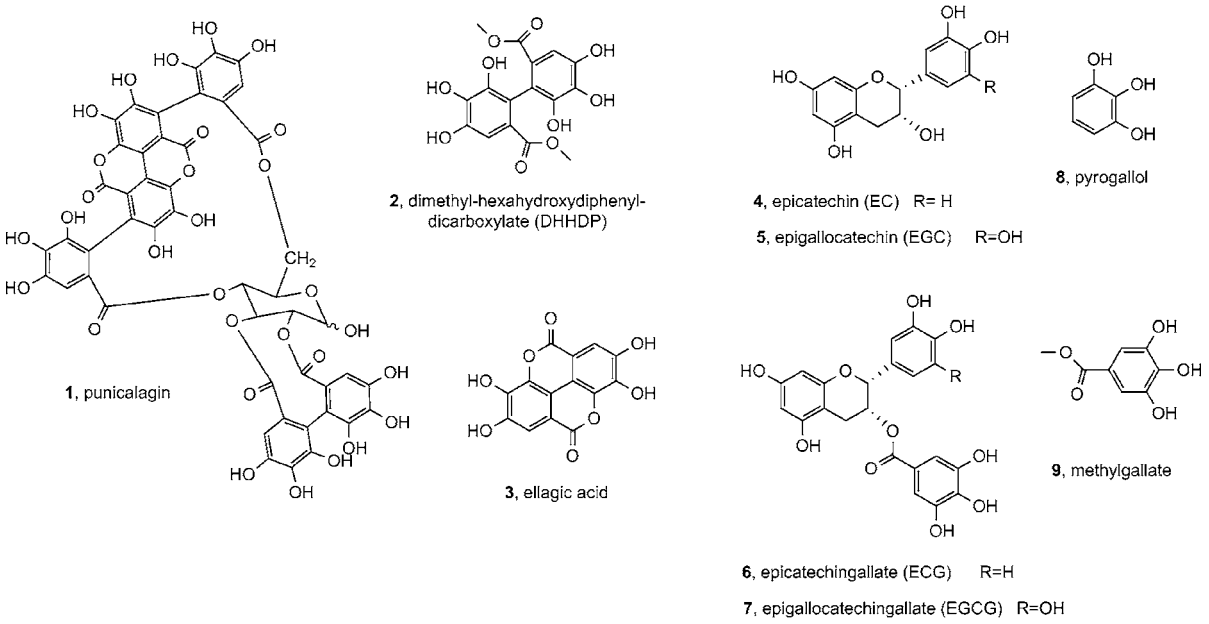

Figure 1. Structures of punicalagin (**1**), related compounds (**2** and **3**), green tea catechins (**4**–**7**), and simple phenols (**8** and **9**).

more stable phenoxyl radical.[11−13] Electron transfer to oxygen generates the superoxide radical $O_2^{\bullet-}$, which is enzymatically converted into hydrogen peroxide[14,15] and ultimately into the deleterious hydroxyl radical in the presence of transition metal cations (e.g., Fe^{2+}).[16] Moreover, the superoxide radical seems to mediate apoptosis.[17,18] Electron transfer appears to be most relevant in the redox cascades involving polyphenols, whether they scavenge or generate reactive radicals. To evaluate the electron transfer capacity of polyphenols, we developed stable radicals of the (2,4,6-trichlorophenyl)methyl (TTM) and perchlorotriphenylmethyl (PTM) series, which react exclusively by electron transfer.[13,19,20] We and others have used these radicals to evaluate the electron transfer capacity of natural and synthetic phenolic scavengers.[21,22] As the activity of the stable radicals of the TTM and PTM series essentially depends on the electron-withdrawing or electron-donating character of the meta- and/or para- substituents introduced into the phenyl rings, radicals with different redox potentials can be designed. The advantage of devising assays using this combination of radicals is that they can discriminate between oxidizing agents by their oxidizing ability, in contrast to the ferric ion reduction method that also operates exclusively by electron transfer processes but measures only the reducing ability based upon the redox potential of the ferric ion. Moreover, the outcome of the ferric ion method is also influenced by binding of the polyphenol to the ion.

Polyphenols may contain more than one reactive polyphenolic substructure. Punicalagin (**1**) (Figure 1), the most abundant polyphenol in pomegranate (*Punica granatum* L.),[23] is a hydrolyzable tannin of the ellagitannin kind because it contains an ellagic acid substructure (**3**). Punicalagin (**1**) releases ellagic acid (**3**) in the small intestine via spontaneous lactonization with later conversion into urolithin A by the gut microbiota.[24] Punicalagin (**1**) also contains in its structure gallate (three geminal phenolic hydroxyls and a carboxylate function) esters linked by carbon−carbon (C−C) bonds either to themselves (hexahydroxy-2,2′-diphenyl, HHDP moiety) or to the ellagic acid substructure. This ensemble of substructures and their metabolites contributes to the bioactivity of the whole molecule. The C−C bond structures constitutive of ellagitannins appear to be important for their activity. Pedunculagin, another hydrolyzable tannin that contains the HHDP moiety, shows higher cytotoxic activity than pentagalloylglucose, a hydrolyzable tannin that contains only simple gallate esters in its structure.[25] Catechins (flavanols of the flavan-3-ol type) are another family of polyphenols that display different polyphenolic substructures and are relevant to dietary considerations. Green tea is a common source of catechins, mainly, in order of abundance, (−)-epigallocatechin gallate (EGCG) (**7**), (−)-epigallocatechin (EGC) (**5**), (−)-epicatechin (EC) (**4**), and (−)-epicatechin gallate (ECG) (**6**) (Figure 1).[26] Tea flavanols scavenge reactive oxygen and nitrogen species, interfere with pro-oxidant processes, or inhibit pro-oxidant enzymes.[27] Polyphenols appear to exert their biological activity through different mechanisms involving redox reactions and protein−ligand interactions. Because the present paper focuses on the redox reactivity of different phenolic moieties and its possible relationship to cell viability in vitro, we selected pomegranate punicalagin (**1**) and green tea flavanols **4**–**7** for our study; together they contain a broad range of polyphenolic substructures. Here, we examine the electron transfer capacity (reducing power) of punicalagin (**1**), and its metabolite ellagic acid (**3**), its related substructure **2**, and green tea flavanols **4**–**7** bearing the catechol, pyrogallol, and gallate moieties, and we evaluate the effect of all these molecules on the viability of colon carcinoma HT-29 cells.

MATERIALS AND METHODS

Tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl (TNPTM) and tris-(2,4,6-trichloro-3,5-dinitrophenyl)methyl (HNTTM) were synthesized in our laboratory as described previously.[19,20] 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO). Punicalagin (**1**) (≥98% (HPLC)) was obtained from Biopurify (Sichuan, China), ellagic acid (**3**) and the catechins **4**–**7** were from from Sigma-Aldrich, and dimethyl-hexahydroxydiphenyl dicarboxylate (DHHDP, **2**) was synthesized in our laboratory following procedures described elsewhere[28] (see the Supporting Information).

Radical-Scavenging Capacity. The scavenging capacity was determined from mixtures (1:1, v/v) of fresh solutions of stable radicals (TNPTM, HNTTM, DPPH; 120 μM) and fresh solutions of polyphenols **1**–**9** in $CHCl_3$/MeOH (2:1) at different concentrations (1−120 μM) at room temperature. All of the solutions were prepared

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and deoxygenated in the darkness. The reactions were monitored by electron paramagnetic resonance (EPR) on an EMX-Plus 10/12 (Bruker BioSpin, Rheinstetten, Germany) after 48 h (TNPTM), 7 h (HNTTM), and 30 min (DPPH). Operating conditions were as follows: center field, 3615 G; scan range, 250 G; microwave power, 5.2 mW; microwave frequency, 9.86 GHz; modulation frequency, 100 kHz; receiver gain, 6×10^3; and time constant, 4.1 s. The scavenging capacity of polyphenols is given as EC_{50}, which corresponds to the amount (micrograms or micromoles) of polyphenol able to consume half the amount of free radical divided by micromoles of initial radical. The results in micrograms per micromole convey the idea of the scavenging capacity of a given amount of polyphenol, and the results in micromoles per micromole provide information about the number of equivalents per molecule. To facilitate the comparison between structures, the results were also expressed as antiradical capacity (ARC), which is the inverse of EC_{50} in micrograms per micromole and hydrogen atoms donated or electrons transferred per molecule of polyphenol (H/e), which is the inverse of $2 \times EC_{50}$ in micromoles per micromole.

Kinetic Measurements. The rate constants of the reactions between TNPTM and polyphenols **2** and **8** were estimated by EPR. Freshly prepared solutions of TNPTM in CH_3Cl/MeOH (2:1) (240 μM) and the polyphenol (48 μM in the same solvent) were mixed (1:1, v/v, molar ratio 5:1), and the decay of the TNTPM band was followed at room temperature. Operating conditions were as follows: center field, 3450 G; scan range, 250 G; microwave power, 1.0 mW; microwave frequency, 9.86 GHz; modulation frequency, 100 kHz; receiver gain, 8.9×10^3; and time constant, 40.96 s. The rate constants and the total number of electrons transferred per polyphenol (n_e) were estimated with a simple and general kinetic model reported by Dangles et al.[29] defined by eq 1. The values for the rate constant, k were calculated from the integrated eq 2.

$$-\mathrm{d}[\mathrm{TNPTM}]/\mathrm{d}t = k \times n[(\mathrm{poly})\mathrm{phenol}][\mathrm{TNPTM}] = k_1[(\mathrm{poly})\mathrm{phenol}][\mathrm{TNPTM}] \quad (1)$$

$$\ln \frac{1 - I_f/I_x}{1 - I_f/I_0} = -\frac{k_1 c}{I_0/I_f - 1} t \quad (2)$$

In eqs 1 and 2, n represents the number of reduced moles of TNPTM per mole of polyphenol; I_0 is the initial intensity of the TNPTM signal in the EPR spectra; I_f is the final visible intensity; and c is the initial concentration of polyphenol. The n_e values of the stoichiometry of the polyphenol were calculated using eq 3; ϵ is the molar absorptivity characteristic of the stable free radical.

$$n_e = \frac{I_0 - I_f}{\epsilon \times C} \quad (3)$$

Cyclic Voltammetry. Cyclic voltammetries were carried out in a standard thermostated cylindrical, one-compartment, three-electrode cell. A platinum (Pt) disk of 0.093 cm^2 area was used as the working electrode and a Pt wire as the counter electrode. The reference electrode was a saturated calomel electrode (SCE), submerged in a salt bridge of the same electrolyte, which was separated from the test solution by a Vycor membrane. Solutions of polyphenols ($\sim 10^{-3}$ M) in DMF containing tetrabutylammonium perchlorate (0.1 M) as the background electrolyte were studied. The volume of all test solutions was 50 mL. Electrochemical measurements were performed under an argon atmosphere at 25 °C using an Eco Chemie Autolab PGSTA-T100 potentiostat-galvanostat (Autolab, Utrecht, The Netherlands) controlled by a computer with Nova 1.5 software (Autolab). Cyclic voltammograms of all the solutions were recorded at scan rates ranging from 20 to 200 mV s^{-1}.

Cell Culture and Viability Assay. HT-29 human colon adenocarcinoma cells were obtained from the American Type Culture Collection. HT-29 cells were cultured in Dulbeco Modified Eagle's Medium (DMEM with 4500 mg L^{-1} glucose, L-glutamine, and sodium bicarbonate, without sodium pyruvate; Sigma-Aldrich), supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria) and antibiotics, 100 U mL^{-1} penicillin and 100 mg L^{-1} streptomycin (Invitrogen, Paisley, U.K.), at 37 °C in a humidified atmosphere of CO_2 (5%). The effect of treatment with different polyphenols upon proliferation of HT-29 colon cancer cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay, which is based on the ability of live cells to cleave the tetrazolium ring, thus producing formazan, which absorbs at 570 nm. HT-29 cells (3000 cells/well) were grown on a 96-well plate for 24 h and then incubated with the different polyphenols at different concentrations (10−300 μM) in dimethyl sulfoxide (DMSO) (Sigma-Aldrich), except ellagic acid (**3**), which was dissolved in *N*-methylpyrrolidone because of its poor solubility in DMSO. After 72 h, 100 μL of MTT solution (0.5 mg mL^{-1}) was added to each well. After 1 h of incubation, the formazan salt was resuspended in 100 μL of DMSO. Cell viability was measured by absorbance at 550 nm on an ELISA plate reader (Tecan Sunrise MR20-301, TECAN, Austria). The experiments were also run in the presence of catalase (Sigma-Aldrich), 100 U mL^{-1} in DMEM.[30] The results were expressed as IC_{50}.

RESULTS

Radical-Scavenging Capacity of Polyphenols Measured by TNPTM, HNTTM, and DPPH. The scavenging capacity of punicalagin (**1**) and related compounds **2** and **3**, flavanols **4**−**7**, pyrogallol (**8**), and methylgallate (**9**) was measured by making them react with the stable radicals TNPTM, HNTTM, and DPPH in a mixture that includes a polar hydroxylated solvent ($CHCl_3$/MeOH (2:1) (v/v)) and monitoring the decrease of the EPR radical signal. TNPTM and HNTTM are reduced exclusively by accepting electrons, in contrast to DPPH, which reacts by HAT and/or ET depending on the solvent. Table 1 summarizes the results of the scavenging capacity of **1**−**9** against the three radicals.

Punicalagin (**1**), ECG (**6**), and EGCG (**7**) were the most active polyphenols against HNTTM and DPPH. The number of electrons transferred to HNTTM roughly corresponded to the number of putative reactive positions (geminal hydroxyls) of the flavanols except for ECG (**6**), which consumed a larger amount of radical. Surprisingly, DHHDP (**2**) transferred 4.3 electrons instead of 6, and punicalagin (**1**) transferred 14.2 electrons instead of 16. The scavenging capacities of the polyphenols against TNPTM radical were lower than those obtained with HNTTM and DPPH because TNPTM reacts only with the most reactive hydroxyls. One molecule each of EGC (**5**), EGCG (**7**), and pyrogallol (**8**) reacted with 3 molecules of TNPTM (roughly 1 electron transferred from each of the three geminal hydroxyls); 1 molecule of punicalagin (**1**) and its substructure DHHDP (**2**) reacted with 3.3 and 2 molecules of TNPTM, respectively (roughly 1 electron transferred from each C−C linked gallate). In contrast, ellagic acid (**3**), EC (**4**), ECG (**6**), and methylgallate (**9**) did not react at all with TNPTM. Figure 2 shows graphically the selective reactivity of characteristic phenolic moieties with TNPTM, monitored by the decrease of the TNPTM radical EPR signal upon reaction with DHHDP (**2**), pyrogallol (**8**), and methylgallate (**9**).

Kinetic Measurements. To further characterize the scavenging activity of the hexahydroxydiphenyl moiety within punicalagin (**1**) and pyrogallol (**8**), which are the only simple structures that react with TNTPM, we made kinetic measurements of the reactions of DHHDP (**2**) and pyrogallol (**8**) with TNPTM. The course of the reaction was monitored using EPR by recording the decay of the TNPTM signal as a result of the addition of the polyphenol in $CHCl_3$/MeOH (2:1) at a molar ratio TNPTM/polyphenol of 5:1. To calculate the stoichiometric factor, the reaction was monitored to completion over a period of 48 h.

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Table 1. Scavenging Capacity of Ellagitannins and Flavanols against Stable Radicals[a]

radical	polyphenol	EC_{50} $\mu g\ \mu mol^{-1}$	EC_{50} $\mu mol\ \mu mol^{-1}$	ARP[b]	e/H[c]
TNPTM	ellagitannins				
	1	50.3 (2.6)	0.15 (0.01)	6.5 (0.3)	3.3 (0.2)
	2	51.2 (0.0)	0.26 (0.00)	3.9 (0.1)	1.9 (0.0)
	3	–[d]	–	–	–
	flavanols				
	4	–	–	–	–
	5	55.2 (6.5)	0.18 (0.02)	5.6 (0.6)	2.8 (0.3)
	6	–	–	–	–
	7	83.3 (5.9)	0.18 (0.01)	5.5 (0.3)	2.7 (0.1)
	simple phenols				
	8	21.7 (1.6)	0.17 (0.01)	5.8 (0.4)	2.9 (0.2)
	9	–	–	–	–
HNTTM	ellagitannins				
	1	38.1 (3.9)	0.04 (0.00)	28.4 (2.7)	14.2 (1.4)
	2	42.2 (5.0)	0.12 (0.02)	8.7 (1.1)	4.3 (0.5)
	3	30.4 (1.1)	0.10 (0.00)	9.9 (0.3)	5.0 (0.1)
	flavanols				
	4	54.0 (4.0)	0.19 (0.02)	5.3 (0.5)	2.7 (0.2)
	5	50.2 (2.2)	0.16 (0.01)	6.2 (0.0)	3.1 (0.1)
	6	24.0 (2.6)	0.05 (0.01)	18.5 (2.0)	9.3 (1.0)
	7	38.3 (3.2)	0.08 (0.01)	11.9 (0.9)	5.9 (0.4)
	simple phenols				
	8	19.7 (1.2)	0.16 (0.01)	6.4 (0.4)	3.2 (0.2)
	9	30.2 (2.5)	0.15 (0.01)	6.5 (0.5)	3.2 (0.3)
DPPH	ellagitannins				
	1	20.0 (1.6)	0.02 (0.00)	55.0 (3.3)	27.5 (1.7)
	2	31.2 (1.6)	0.08 (0.00)	12.2 (0.4)	6.1 (0.2)
	3	22.1 (0.2)	0.07 (0.00)	13.7 (0.3)	6.8 (0.1)
	flavanols				
	4	36.8 (1.6)	0.13 (0.01)	7.9 (0.3)	3.9 (0.2)
	5	31.5 (1.8)	0.11 (0.00)	9.1 (0.3)	4.6 (0.1)
	6	28.9 (3.1)	0.07 (0.01)	15.4 (1.6)	7.8 (0.8)
	7	31.4 (6.1)	0.06 (0.02)	17.3 (3.4)	8.7 (1.7)
	simple phenols				
	8	12.6 (1.2)	0.10 (0.01)	10.0 (0.8)	5.0 (0.4)
	9	31.7 (3.2)	0.17 (0.02)	5.8 (0.6)	2.9 (0.3)

[a]Values are means (standard deviation), $n = 3$. [b]Antiradical power ($1/EC_{50}$ ($\mu g\ \mu mol^{-1}$)). [c]Moles of reduced radical per mole of polyphenol ($1/(2 \times EC_{50})$) corresponding to the number of electrons or hydrogen atoms transferred per molecule of polyphenol. [d]EC_{50} ($\mu g\ \mu mol^{-1}$) ≥ 132).

The rate constants and stoichiometric factors for these reactions are given in Table 2. The reaction with pyrogallol (**8**) was faster than that with DHHDP (**2**), and the stoichiometric factors were consistent with those estimated from the concentration/activity curve and shown in Table 1, roughly corresponding to 2 and 3 electrons from DHHDP (**2**) and pyrogallol (**8**), respectively. As commented before, methylgallate (**9**) did not reduce the TNPTM.

Anodic Onset Potentials. To explain why most of the phenolic hydroxyls reacted with HNTTM and only some of them with TNPTM, the anodic onset potentials for the oxidation of DHHDP (**2**), ellagic acid (**3**), pyrogallol (**8**), and methylgallate (**9**) were measured by cyclic voltammetry in DMF solutions. The comparative results obtained at 100 mV s^{-1} are summarized in Table 3. The lower the anodic onset potential, the more reactive the phenolic hydroxyl is. Results in Table 3 show that the compounds reactive against TNPTM (**2** and **8**) possess the lowest anodic onset potentials.

Cell Viability of HT-29 Colon Adenocarcinoma Cells. The influence of polyphenols **1**–**9** on the viability of HT-29 colon cells was measured in regular DMEM and also in the presence of catalase[30] to account for artifactual results due to the formation of H_2O_2 from the superoxide radical generated in the medium by electron transfer to oxygen.[3] The results are presented in Table 4.

The active compounds were those that contained pyrogallol, hexahydroxydiphenyl, or gallate moieties (ellagitannins **1** and **2**; flavanols **5** and **7**; and simple pyrogallol **8**). Polyphenols bearing only two geminal hydroxyls (compounds **3** and **4**) were inactive. The effect on cell viability recorded for pyrogallol and structures containing pyrogallol (compounds **5** and **7**) was, at least in part, artifactual because catalase diminished or eliminated the activity. In contrast, catalase did not influence the activity of ellagitannins **1** and **3**, as well as the related compound **2**, which means that this activity was not due to extracellular hydrogen peroxide.[31]

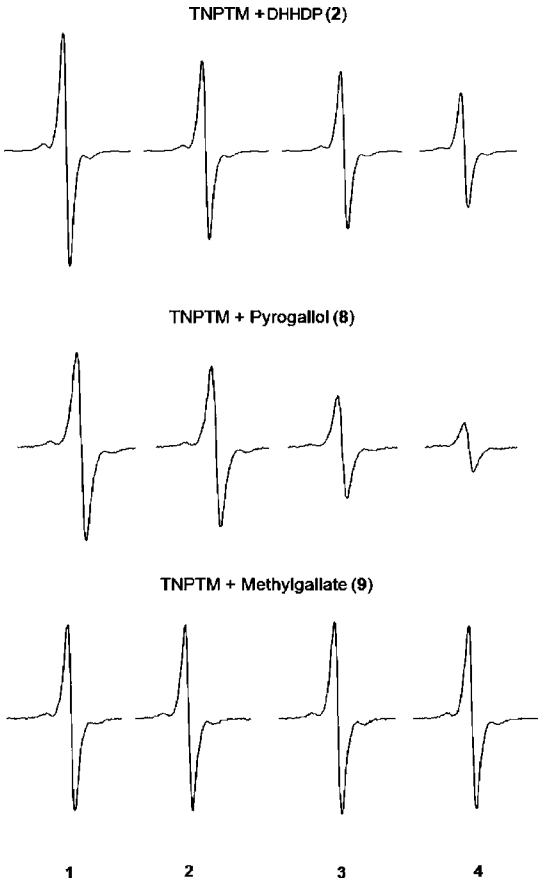

Figure 2. EPR spectra of TNPTM, initial concentration ~120 μM, upon reaction with DHHDP (**2**), pyrogallol (**8**), and methylgallate (**9**) at different initial concentrations: 0 μM (1), 5.7 μM (2), 18.1 μM (3), and 55.1 μM (4) for 48 h. Lande's factor for the TNPTM, g = 2.0026.

Table 2. Rate Constants and Stoichiometric Factors for the Reaction of TNPTM with DHHBD (2), Pyrogallol (8), and Methylgallate (9) in $CHCl_3$/MeOH (2:1)

polyphenol	TNPTM/polyphenol molar ratio[a]	k^b (M^{-1} s^{-1})	n^c
2	4.9–4.9	0.115 (0.010)	1.9
8	4.6–4.6	0.338 (0.070)	3.6
9	4.3–4.0	–	–

[a]Range of ratios for a number of experiments between 2 and 5. Initial concentrations around 120 and 24 μM (molar ratio, 5:1) for TNPTM and polyphenol, respectively. [b]Values are means (standard deviation), n = 2–5. [c]Moles of reduced radical per mole of polyphenol corresponding to the number of electrons transferred per molecule of polyphenol.

DISCUSSION

The biological relevance of polyphenols is still a matter of debate, even after decades of intense research. Particularly, the significant structural features behind polyphenol activities have not been satisfactorily established, probably because they interact with live systems in complex ways at different levels including redox reactions and protein–ligand interactions. Polyphenols may modify redox homeostasis by scavenging reactive radicals, by generating reactive radicals, or by a combination of the two. The electron transfer capacity of different phenolic hydroxyl groups determines the kind of effect elicited, if any. Pyrogallol (**8**) (three geminal hydroxyls) and polyphenols such as EGC (**5**) and EGCG (**7**) (gallocatechins), which contain this substructure, may be both

Table 3. Anodic Onset Potential (AOP) of Polyphenolic Moieties

polyphenol	AOP[a] (V vs SCE)
DHHDP (**2**)	0.50
ellagic acid (**3**)	0.64
pyrogallol (**8**)	0.45
methylgallate (**9**)	0.65

[a]10^{-3} M in DMF solutions with 0.1 M Bu_4NClO_4 on Pt at 100 mV s^{-1} and 25 °C.

Table 4. Viability of HT-29 Cells in the Presence of Polyphenols

	IC_{50}[a]	
polyphenol	μg mL^{-1} in DMEM	μg mL^{-1} in DMEM with catalase
ellagitannins and related compounds		
1	21.5 (3.5)	14.4 (0.4)
2	32.5 (3.9)	34.1 (0.5)
3	≥100	≥100
flavanols		
4	≥100	≥100
5	24.1 (2.7)	≥100
6	53.7 (12.0)	58.9 (8.0)
7	17.5 (3.2)	47.9 (8.0)
simple phenols		
8	5.6 (0.5)	71.4 (7.5)
9	24.6 (8.3)	31.6 (1.8)

[a]Cells were treated with the compounds for 72 h, and viability was monitored with MTT. Values are means (standard deviation), n = 2–3

scavengers and generators of free radicals and are among the most biologically active polyphenols. The gallate moiety (pyrogallol with an esterified carboxylate function) is another important structural feature. It has been widely reported that polyphenols that contain pyrogallols and/or gallates lower cell viability either by disrupting the cell cycle and triggering apoptosis or by other effects that involve redox reactions and/or protein–ligand interactions.[32−34] We focus our attention here on the redox reactions of polyphenols by using chemosensors that are able to discriminate between different phenolic hydroxyls according to their redox potentials. The results are compared with the influence on cell viability in vitro. Polyphenols **1**–**9** reacted with HNTTM, whereas only some of them (**1**, **2**, **5**, **7**, **8**) were able to reduce the TNPTM radical. This was expected for the structures containing pyrogallol (**5**, **7**, **8**)[20] and not for the ellagitanin punicalagin (**1**) because ellagic acid (**3**) was inactive against TNPTM. As expected, TNPTM did not react with catechols (two geminal hydroxyls) (**4**) or gallates (**6**, **9**). Punicalagin (**1**) contains an ellagic acid conjugated substructure and other substructures composed of gallate moieties linked by C–C bonds to each other (hexahydroxydiphenyl) or to an ellagic acid moiety. The stable radical TNPTM is reactive against these C–C-linked gallates as proven by the redox behavior of synthetic DHHDP (**2**). This dimeric gallate transferred two electrons to TNPTM, whereas methylgallate (**9**) was unreactive (Tables 1 and 2, last columns). The C–C bond appears to have activated two hydroxyl positions. Inspection of the structure of punicalagin (**1**) and the number of electrons (3.3) transferred to TNPTM (Table 1, last column) leads us to hypothesize that the C–C bond between the gallate moiety and the ellagic acid moiety produces the same hydroxyl activation that we detected for the

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hexahydroxydiphenyl substructure. The formation of hydrogen bonds between hydroxyls ortho to the C−C bond may be behind the reactivity of these diphenyl structures.[35] This result was corroborated by measuring the anionic onset potential (AOP) of the gallate conjugates **2**, **3**, and **9** and pyrogallol **8**. The reactivity of polyphenols given by the AOP followed the order **8** > **2** > **3** = **9** (Table 3). These results are also in agreement with the kinetic measurements (Table 2).

The outcome of the cell viability assay cannot be related to the redox behavior of the polyphenolic structures in a straightforward way because polyphenols influence cell functions by more than one mechanism. Whatever the case, our results (Table 3) corroborate that pyrogallols and gallates are active against colon adenocarcinoma cells and suggest that the hydroxydiphenyl substructure of punicalagin may play a role involving a particularly reactive redox position. As some of the effects ascribed to pyrogallols in vitro may be due to the artifactual generation of H_2O_2 in the culture medium,[3,15] we ran the in vitro experiments in the presence of catalase. This resulted in a significant decrease in the activity of the polyphenols that contained pyrogallols in their structure. This does not alter the fact that pyrogallols are the most reactive species, because they must be able to generate the superoxide radical as the first step in the formation of H_2O_2; it just shows that the experimental setup does not adequately mimic the situation in vivo, where the extracellular oxygen concentration is much lower.[3] Punicalagin (**1**) affected cell viability as effectively as gallocatechins. In this case, the effect was not artifactual because it was not affected by the addition of catalase to the medium, which suggests that punicalagin (**1**) did not generate the superoxide radical extracellularly, at least not to a sufficient extent to affect cell viability.

By combining the outcome of HNTTM and TNPTM assays, we may generate a picture of both the total electron transfer capacity of polyphenols and the presence of highly reactive hydroxyls. TNPTM detects the most redox reactive phenolics (e.g., pyrogallols and C−C-linked gallates) and may anticipate their influence on cell viability. Independent of whether these highly reactive positions directly scavenge radicals or trigger antioxidant defense responses, TNPTM is a useful chemical probe that easily detects the presence of some of the most biologically significant phenolic structures. This will be useful when the antioxidant potential of extracts and functional foods as well as new synthetic polyphenolic molecules is examined.

In conclusion, we show here that substructures of punicalagin that contain gallate moieties, linked either to each other (hexahydroxydiphenyl moieties) or to the ellagic acid moiety by C−C bonds, present phenolic hydroxyls that are more redox reactive than those in simple gallates and that these structures can be detected by the stable radical TNPTM. The most reactive polyphenolic structures are also those that have the greatest effect on cell viability in vitro. The chemosensor TNPTM may be a useful tool for detecting other potentially beneficial highly reactive polyphenols from natural sources.

■ ASSOCIATED CONTENT

Supporting Information

IR spectrum of TNPTM; plots of scavenging activity against TNPTM, HNTTM and DPPH; kinetics of the reaction between TNPTM and HDDP/pyrogallol; plots of cell viability on HT-29 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DMEM, Dulbecco Modified Eagle's Medium; EGCG, epigallocatechin gallate; EGC, epigallocatechin; EC, epicatechin; ECG, epicatechin gallate; DHHDP, dimethylhexahydroxydiphenyl dicarboxylate; HHDP, hexahydroxy-2,2′-diphenyl; HNTTM, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl; TNPTM, tris(2,3,5,6-tetrachloro-4-nitrophenyl)methyl; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

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