High-Throughput Evaluation of Antioxidant and Pro-oxidant Activities of Polyphenols with Thymidine Protection Assays

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A recently reported high-throughput screening strategy has been applied to the rapid selection of new water-soluble antioxidants that display strong protective activities. Based on a competitive immunoassay, a triple-screening procedure was used to evaluate the ability of different compounds to protect thymidine under different oxidative stresses. The pro-oxidant effect of norbadione A in the presence of iron was observed, while some pulvinic acid derivatives proved strongly protective during γ radiolysis, UV irradiation, and Fenton-like oxidation.

Introduction

Flavonoids are an important class of polyphenolic metabolites in plants whose abundance in various vegetables, fruits, spices, and mushrooms, as well as in beverages such as wine and tea, make them important components of the human diet.[1] Many flavonoids are considered to be efficient antioxidants, that is, compounds able to protect biomolecules from oxidative degradation, in particular through the quenching of reactive oxygen species (ROS).[2] Several epidemiological studies provide support for the protective effect of the consumption of flavonoid-rich products against cancer, heart disease, and stroke.[3] However, several in vitro studies have demonstrated that, in the presence of transition metals, many flavonoids (quercetin, catechin, mirycetin, epicatechin) can also display pro-oxidant effects on biological targets.[4] These deleterious effects might arise from the formation of reactive aryloxyl radicals that are able to i) reduce dioxygen to a superoxide radical, ii) reduce transition metals,[5] iii) initiate peroxidative chain reactions,[6] and iv) lead to electrophilic species that may covalently modify biomolecules.[7] The biological and pharmaceutical implications of these properties are important, as illustrated by the in vitro characterization of the mutagenic effect of some flavonoids resulting from these pro-oxidative effects.[8] As a consequence, the inactivation of transition metal-induced pro-oxidant activity could afford essential benefits when using flavonoids or any other ROS-scavenging agent.[9]

The observation of such dual activities for one compound underlines the importance of considering different sources of oxidative stress for the selection of an antioxidant candidate. The best strategy of rapidly identifying a promising compound would be to assess the potential for broad protection by screening libraries for putative antioxidants.

We have recently described a new high-throughput-screening method for selecting powerful antiradiation compounds that allowed the exceptional in vitro radioprotective properties of norbadione A, a mushroom pigment, to be characterized.[10] In this work, the same strategy was applied to screen a library for protective properties under three types of oxidative conditions. From the results of these screenings, we shall discuss the pro-oxidant effects of flavonols, flavones, norbadione A, and pulvinic acid derivatives on biological targets, such as DNA.

Results

Validation of the analytical tool

The screening procedure is based on the degradation of thymidine (dThd) by oxidative stress under aerobic conditions. The unmodified thymidine remaining after this degradation step is quantified by a competitive enzyme immunoassay (EIA) by using a specific antithymidine antibody. In addition to the previously described protocol, that is, γ irradiation with a ^{137}Cs

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source (135 min at 2.5 Gy min^{-1}),[10] two other oxidative conditions (UV irradiation or the presence of Fe^{2+}) were studied in a 96-well microtiter plate format in the presence of hydrogen peroxide. We first determined the optimal conditions required for the degradation of thymidine under the different oxidative stresses. 98% of the thymidine (70 μM) was destroyed by the UV irradiation at 254 nm (1.75 J cm^{-2}) of a hydrogen peroxide solution (5 mM) buffered at physiological pH. On using a Fenton-like system, thymidine (70 μM) was oxidized in the presence of Fe^{2+}/EDTA (1:1, 700 μM) and hydrogen peroxide (70 mM) for 20 minutes and reached a similar level of degradation (≈92%).

As previously observed for γ irradiation, Fenton-like oxidation or UV irradiation of thymidine led to the production of various oxidized compounds (Figure 1). The three chromatograms exhibited quite different patterns; this reflects the different degradation pathways of the target. The γ and UV irradiations mainly led to many UV-active products of low polarity and probably deriving from the oxidation of the thymine base. On the other hand, for Fenton-like oxidation, we observed a majority of very polar compounds, which might be smaller fragments resulting from a higher degradation of thymidine. The implication of different oxidative species within the three assays will be discussed below. For each experimental condition, the resulting mixture of compounds was resolved by HPLC, and the fractions were analyzed by EIA. This experiment demonstrated the specificity of the antithymidine antibody, since none of the fractions, except those corresponding to the intact target, was detected by immunoassay (Figure 1).

As a model, the effect of the well-known antioxidant Trolox was first evaluated under the three selected degradation conditions (Figure 2). The results, expressed as a percentage of thymidine protection, presented a good precision and reproducibility (for protection >30%, the coefficients of variation were <15%).

As expected, protection efficiencies were related to the concentration of Trolox, reaching a recovery of thymidine >90% for each oxidative condition. It should be underlined that the efficiency of Trolox at a single dose cannot be compared on the three different assays due to the differences between the experimental conditions (concentration of thymidine, nature, concentration, and formation kinetics of ROS). Nevertheless, interassay comparisons are possible considering a given set of compounds showing different protective hierarchies on using the three assays. In such a case, the screening results would provide, within the family, a rapid readout of the protective potency of each compound as a function of the oxidative stress.

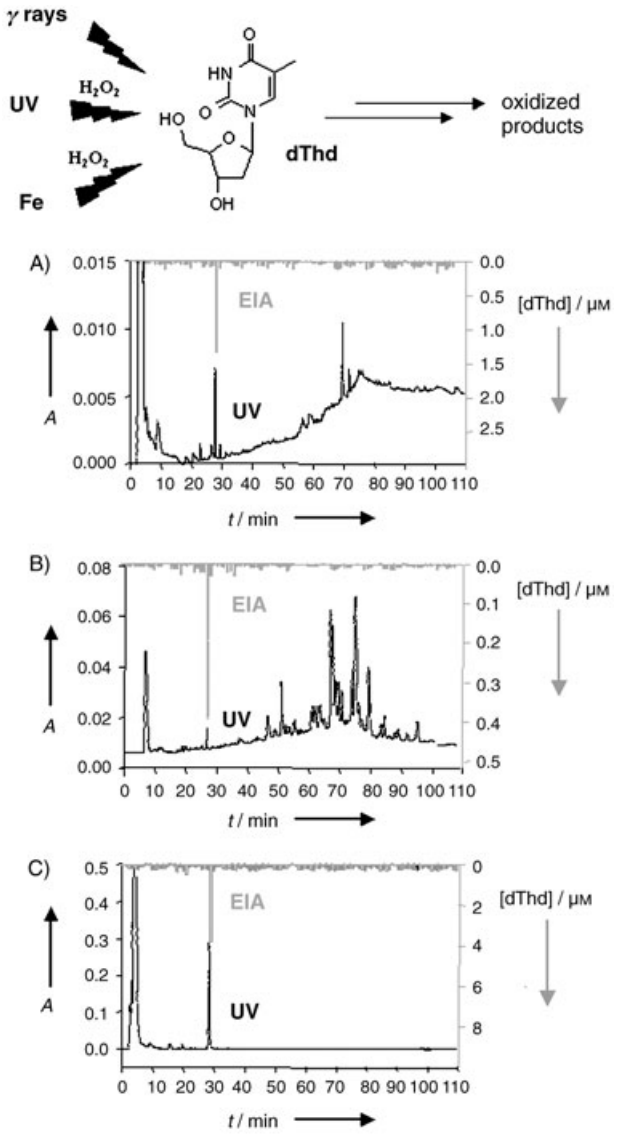


Figure 1. Validation of the screening assay. HPLC chromatography of thymidine submitted to oxidative stress. A) [dThd] = 15 μM under γ rays: 135 min at 2.5 Gy min^{-1}; B) [dThd] = 70 μM under UV/H_2O_2: 254 nm, 1.75 J cm^{-2}, [H_2O_2] = 5 mM; C) [dThd] = 70 μM under Fenton stress: Fe^{2+}/EDTA/H_2O_2 (1:1:100) 700 μM. HPLC was conducted by using a C18 analytical column (250×4 mm, particle 5 μm, 30 °C, gradient elution from water to water/methanol (3:7), flow rate = 1 mL min^{-1}). The concentrations of the remaining thymidine were: A) UV detection (267 nm): 1.16 ± 0.02 μM (92.3% degradation), EIA detection: 1.20 ± 0.01 μM (92.0% degradation); B) UV detection (267 nm): 0.31 ± 0.02 μM (99.5% degradation), EIA detection: 0.44 ± 0.01 μM (99.4% degradation); C) UV detection (267 nm): 6.93 ± 0.02 μM (90% degradation), EIA detection: 3.97 ± 0.01 μM (94.5% degradation).

Stress-dependent protective effect of polyphenols

The screening strategy was applied to the study of a small library of particular interest containing 16 flavonols, five flavones, and five norbadione A and pulvinic acid derivatives (Tables 1, 2, 3, and 4, Scheme 1).[11]

Each compound was assayed three times at a single concentration, either 50 μM for γ radiolysis or 100 μM for UV irradiation and Fenton-like conditions. In order to confirm our observations and evaluate the pro/antioxidant properties of some selected compounds, especially norbadione A and the pulvinic acids, the protection of supercoiled plasmid-DNA was also investigated by using gel electrophoresis as the revealing method.

These experiments clearly showed high discrepancies for the protective effects exerted by the tested polyphenols, depending on the nature of the oxidative stress.

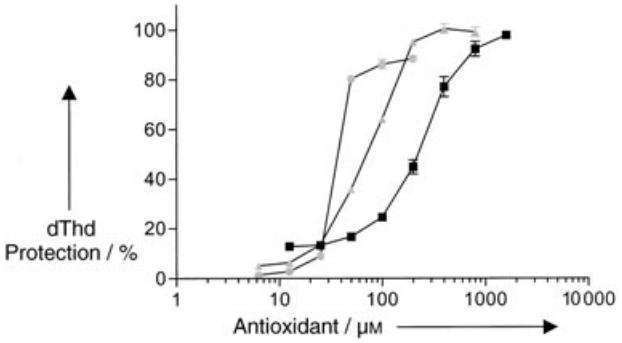


Figure 2. Validation of the screening assay. Protection of thymidine by Trolox. Results are expressed as a percentage of thymidine protection, comparing experiments in the presence or in the absence of the antioxidant. Thymidine in 5 mM phosphate buffer (pH 7.4) was subjected to γ irradiation (▲), [dThd] = 15 μM, 340 Gy; UV/H_2O_2 (■), [dThd] = 70 μM, 254 nm, 1.75 J cm^{-2}, [H_2O_2] = 5 mM; or Fenton system (●), [dThd] = 70 μM, Fe^{2+}/EDTA/H_2O_2 (1:1:100) 700 μM in the presence of varying amounts of Trolox.

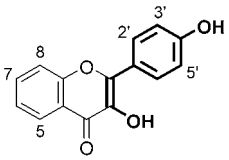
Protective effect of polyphenols during γ and UV exposure

The experiments performed under radiative or UV stress exhibited a good correlation, as revealed by the thymidine protective assays, since the protection hierarchies proved to be almost identical (Figure 3A). This certainly results from the similarity in the nature of the ROS produced. Indeed, HO• radicals, generated through the radiolysis of water or by homolytical cleavage of the oxygen–oxygen bond of H_2O_2 under UV irradiation,[12] probably correspond to the main species responsible for the thymidine degradation. As previously discussed,[10] the protective effects measured by using these two assays are closely related to the HO• radical-scavenging properties of the tested compounds.

To check that the previous results were not limited to the simple thymidine structural features, we evaluated the protection of DNA during γ radiolysis, in the presence of five selected compounds (quercetin (**1**), rutin (**10**), luteolin (**17**), norbadione A (**22**) and the pulvinic acid derivative **26**). The protection efficiencies were characterized by the detection of significant remaining amounts of the supercoiled form of the plasmid pUC18 while, in the control experiments without antioxidant, the oxidized DNA migrated in essentially the same way as linear DNA (Figure 3B compare lane 3 to lanes 5–24). These results clearly demonstrated protection of the DNA structure by the five tested molecules that was associated with their ROS-scavenging properties.

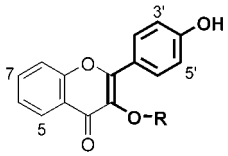
No important variation was observed for the protective effect of the different flavonoid structures when using either the thymidine or the plasmid-DNA assays; this reflects the efficient but close ROS-scavenging properties of these compounds. Nevertheless the data suggest beneficial effects associated with i) the hydroxyl group in position 4′ (compare **1** and **13**, **8** and **15**); ii) the hydroxyl group in position 3 (compare **2** and **18**); iii) the presence of a catechol versus a phenol on ring B (compare **17** and **18**). Interestingly, pulvinic derivatives **24**–**26** presented antioxidant properties similar to those of norbadione A according to the thymidine-protection assay. The

Table 1. Structure of flavonols **1**–**8**.



Compound	Name	Position 5	7	8	2′	3′	5′
1	quercetin	OH	OH	H	H	OH	H
2	kaempferol	OH	OH	H	H	H	H
3	fisetin	H	OH	H	H	OH	H
4	myricetin	OH	OH	H	H	OH	OH
5	morin	OH	OH	H	OH	H	H
6	gossypin	OH	OH	O-glc[a]	H	OH	H

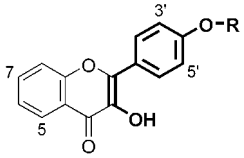
Table 2. Structure of flavonols **9**–**12**.



Compound	Name	R	Position 5	7	3′	5′
9	quercitrin	rham[a]	OH	OH	OH	H
10	(+)rutin	rut[b]	OH	OH	OH	H
11	kaempferol-3-glc	glc	OH	OH	H	H
12	myricitrin	glc	OH	OH	OH	OH

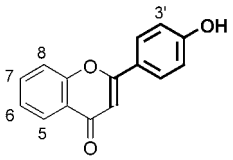
[a] rham = rhamnose. [b] rut = rutinose.

Table 3. Structure of flavonols **13**–**16**.

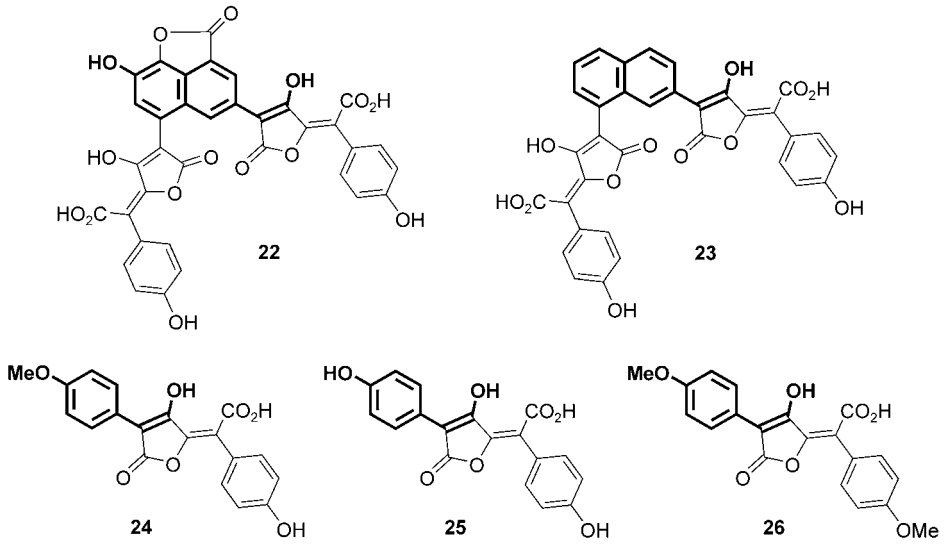


Compound	Name	R	Position 5	7	3′	5′
13	tamarixetin	Me	OH	OH	OH	H
14	myrecitine tri-Me ether	Me	OH	OH	OMe	OMe
15	ombuin	Me	OH	OMe	OH	H
16	robinetin tri-Me ether	Me	H	OH	OMe	OMe

Table 4. Structure of flavones **17**–**21**.



Compound	Name	Position 5	6	7	8	3′
17	luteolin	OH	H	OH	H	OH
18	apigenin	OH	H	OH	H	H
19	apigenin-7-glc	OH	H	O-glc	H	H
20	6,7-dimethoxy-3′,4′,5-trihydroxyflavone	OH	OMe	OMe	H	OH
21	3′,4′,7,8-tetrahydroxyflavone	H	H	OH	OH	OH



Scheme 1. Structures of norbadione A and pulvinic acids.

ROS-scavenging properties of compound **26** were confirmed in DNA-protection experiments and appeared particularly interesting due to the presence of a single hydroxyl group in the structure.

Protective effect of polyphenols under Fenton stress

As illustrated in Figure 4, the results obtained by using the Fenton-like system are quite different from the previous observations related to the two other oxidative stresses. Indeed, large variations in the protective efficiencies were noticed that were closely dependent on flavonoid and pulvinic acids structures.

These important differences for thymidine-protection hierarchies are probably related to the formation of high-valence iron–oxo species under Fenton-like conditions.[13] In this case, efficient trapping agents for such metallic species, or compounds that are able to inhibit their formation, could act as potent protectors. Conversely, some tested molecules could strengthen the Fenton system and therefore display pro-oxidant behavior.

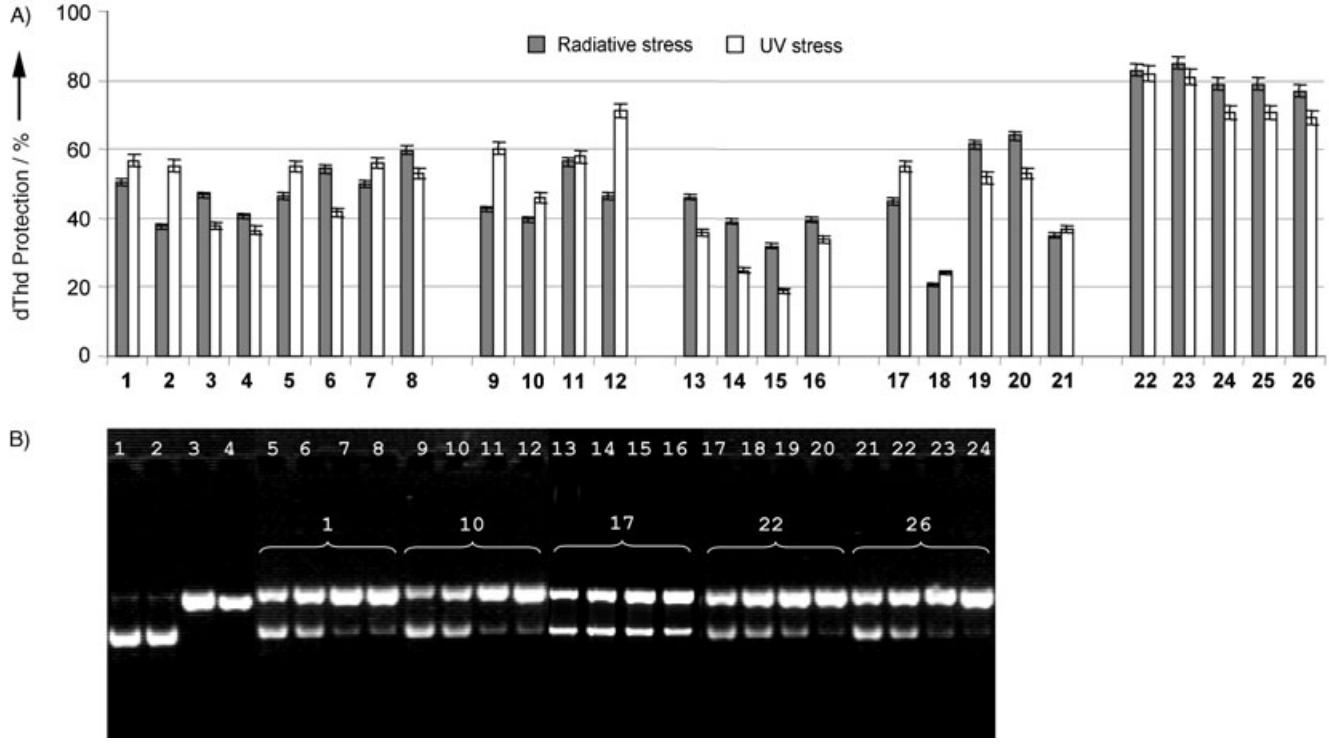


Figure 3. Protective effects of polyphenols under UV and γ exposure. A) Screening results for dThd protection assay. Radiative stress: [dThd] = 15 μM, [antioxidant] = 50 μM, 340 Gy; UV stress: [dThd] = 70 μM, [antioxidant] = 100 μM, 254 nm, 1.75 J cm^{-2}, [H_2O_2] = 5 mM. Experiments were performed in triplicate. B) DNA protection by flavonoids and pulvinic derivatives under γ radiolysis. Supercoiled plasmid pUC18 DNA (lanes 1 and 2) was irradiated at 2.5 Gy min^{-1} for 30 min. Control linear plasmid DNA, digested by *Hind*III (lane 4). Oxidations of DNA in the absence (lane 3) or in the presence of four concentrations (500, 250, 125 and 62.5 μM respectively) of antioxidants **1** (lanes 5–8); **10** (lanes 9–12); **17** (lanes 13–16); **22** (lanes 17–20), and **26** (lanes 21–24). Linear and supercoiled DNA were separated by agarose gel electrophoresis and stained with ethydium bromide.

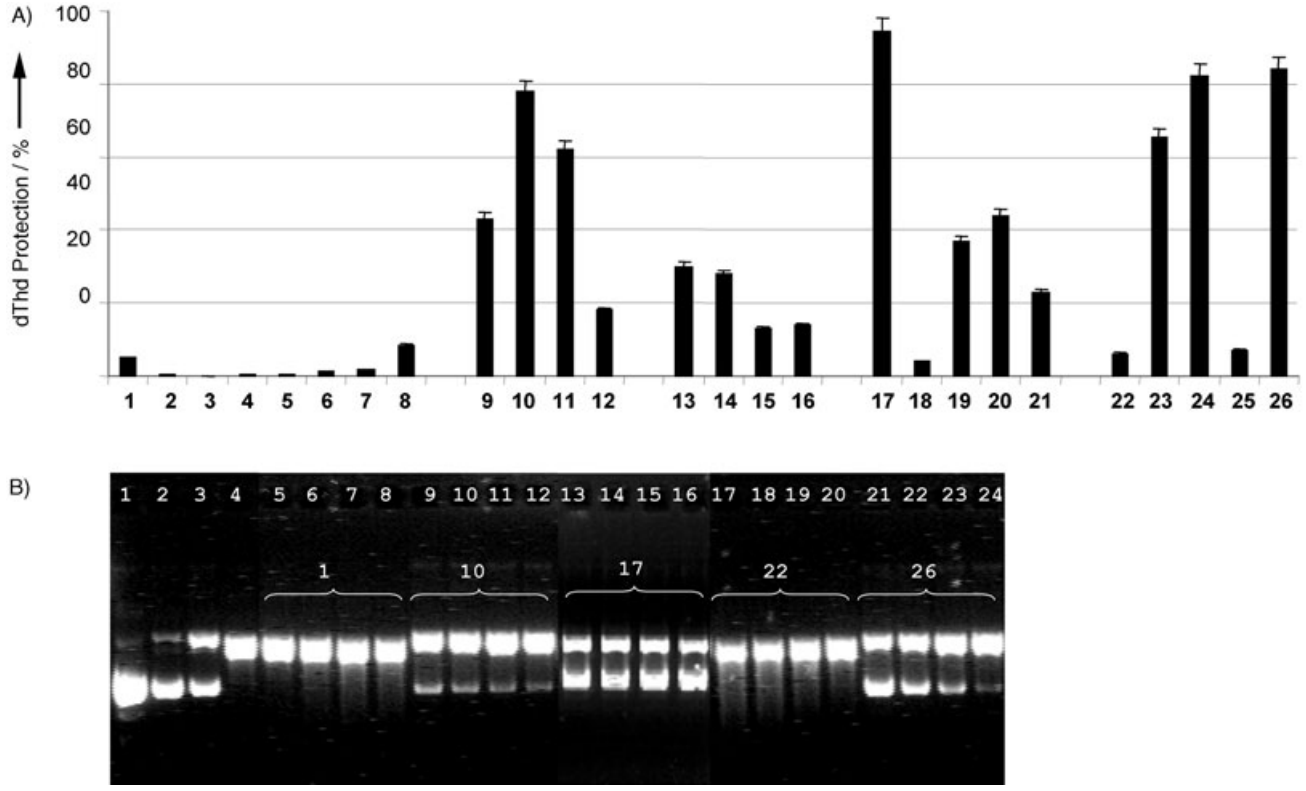


Figure 4. Protective effects of polyphenol under Fenton stress. A) Screening results by using dThd protection assay; [dThd] = 70 μM, [antioxidant] = 100 μM, Fe^{2+}/EDTA/H_2O_2 (1:1:100) 700 μM in phosphate buffer 5 mM (pH 7.4). Experiments were performed in triplicate. B) DNA protection by flavonoids and pulvinic derivatives under Fenton stress. Oxidations of supercoiled plasmid pUC18 DNA (lane 1) were carried out in the presence of 200 μM $Fe^{II}SO_4$/EDTA/H_2O_2 (1:1:100) for 30 min. Control experiments were performed without iron (lane 2) or without H_2O_2 (lane 3). Oxidation of DNA in the absence (lane 4) or in the presence of four concentrations (500, 250, 125 and 62.5 μM respectively) of antioxidants **1** (lanes 5–8); **10** (lanes 9-12); **17** (lanes 13-16); **22** (lanes 17–20); and **26**: (lanes 21–24). Linear and supercoiled DNA were separated by agarose gel electrophoresis and stained with ethydium bromide.

Among the assayed compounds, 11 polyphenols proved to be significantly less efficient for protecting thymidine under Fenton conditions compared to γ- or UV-induced stress (Figure 4A). This might correspond to pro-oxidant properties and/or to weak protective efficiencies towards the oxidative species produced under Fenton conditions. Such behavior is particularly noteworthy for the flavonol family **1**–**8**.

Among these compounds, quercetin **1**, which appears totally inefficient in the present assay, possess well-known metal-initiated pro-oxidant activity.[4c,6,14] No general trend for the protective efficiencies of phenol versus catechol B ring-containing flavonoids was observed. However, the large difference observed between the catechol B ring-containing luteolin **17** and apigenin **18** supports the pro-oxidant activity of the latter, previously reported to result from the formation of a phenoxyl radical that might co-oxidize biological targets.[15]

The DNA-protection experiments further characterized the pro-oxidant effects of two of the five selected compounds. Under Fenton-like oxidative conditions, the presence of quercetin induced a higher degree of plasmid degradation; this reflected pro-oxidant behavior (Figure 4B lanes 5–8 compared to lane 4). Conversely, rutin and mainly luteolin successfully protected DNA structure (Figure 4B lanes 9–12 and 13–16), thus demonstrating the benefit of the glycosylation or the removal of the 3-hydroxyl group on quercetin. Nevertheless, the antioxidant capacity of rutin in the presence of transition metals has already been reported.[4b,6,14]

The pro-oxidant capacity of norbadione A (Figure 4B lanes 17–20) was also clearly demonstrated during these experiments, while the pulvinic acid derivative **26** efficiently protects DNA from degradation (Figure 4B lanes 21–24). Similar protection was observed with the monomethoxylated pulvinic derivative **24** (data not shown).

It is worth noting that the results obtained with plasmid-DNA as a target are in complete agreement with the screening data resulting from the high-throughput thymidine assay (Figures 3A and 4A).

Discussion

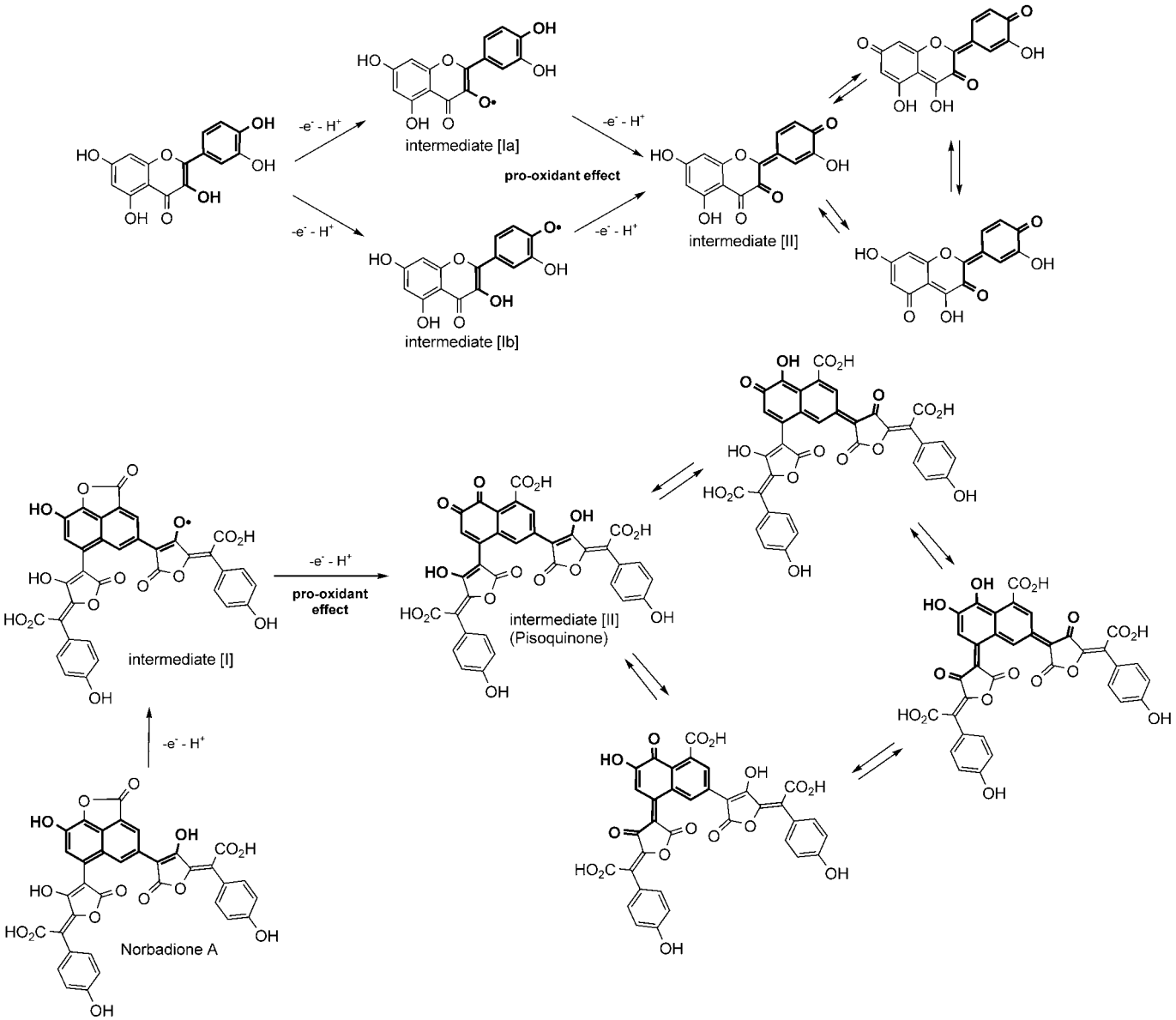
The results from the thymidine- and DNA-protection studies might help to explain the pro-oxidant properties of norbadione A.

Considering quercetin, whose metal-initiated pro-oxidant activity has been extensively demonstrated,[4c,6,14] the results obtained with the three structural analogues **10**, **13**, and **17** clearly indicate the negative impact of the simultaneous presence

of hydroxyls in positions 3 and 4′ (Figure 4A). The best candidates in these series for the selection of a broad-range protective agent would be **10** and **17**, which retain the catechol moiety on ring B to sustain good antioxidant properties, while the hydroxyl group at position 3 is glycosylated or removed to limit the pro-oxidant effect. Interestingly, the structural features highlighted here as being responsible for the pro-oxidant effects of flavonols (i.e., the presence of an enol function conjugated with a 4′-hydroxyl-containing B ring) match the requirements for efficient quinone methide formation. The ability of *o*-quinone-type and quinone methide-type metabolites to form adducts with various tissue macromolecules is a suggested base for the harmful in vivo pro-oxidative effects of some flavonoids.[16]

As previously outlined, pulvinic acids and norbadione A structures include acidic enol functions that are deprotonated at physiological pH and thus act as favorable targets for ROS or other oxidative species.[10,17] Thus, based on a structural analogy between norbadione A and quercetin, that is, the presence of an enol function conjugated with an aromatic hydroxyl, we suggest that the pro-oxidant effect of these molecules can be rationalized by a 2-electron oxidation mechanism that leads to intermediates [II], isomerizable into several *o*-quinone and quinone methide forms (Scheme 2).

We hypothesize that the second electron transfer leads to the reduction of Fe^{III} to Fe^{II} or O_2 to $O_2^{\cdot-}$, or allows the formation of a metallic oxidative species, due to the low potential of the intermediate [I]/[II] redox couple and/or the relatively slow disproportionation of intermediate [I]. The inhibition of the pro-oxidant effect results from the glycosylation of the enol function on rutin, the absence of the hydroxyl in position 3 on luteolin, and from the protection of the aromatic hydroxyl as a methoxyl on **26**, preventing the 2-electron transfer mechanism from happening in either case.



Scheme 2. Suggested mechanism for the pro-oxidant properties of quercetin **1** and norbadione A, **21**.

Among the 26 molecules tested, the best overall protective properties were shown by **24** and **26**, which contain only two or one hydroxyl group, respectively. These results underline the atypical and very promising structural features of pulvinic acids for the design of powerful scavenging agents for HO• radicals and oxidative iron species.

Conclusion

The recently reported high-throughput screening strategy allows, for the first time, the rapid and efficient triple screening of water-soluble antioxidant libraries. This method provides a unique view of the scope of their protective scavenging potencies under different biologically relevant stress conditions. These assays are fully automatizable and allow the analysis of more than 1000 samples per day. By using a complementary study on DNA, the capability of the assays to detect pro-oxidant effects under Fenton-like oxidative conditions was also demonstrated.

The results obtained for the protection of thymidine and plasmid DNA by selected flavonols and pulvinic acid derivatives led to the rationalization of the pro-oxidant properties of these molecules. The suggested mechanism for these deleterious effects involves the presence of an enol function conjugated with an aromatic hydroxyl that leads to the possible formation of quinone methide metabolites.

This study constitutes the first report on the iron-induced pro-oxidant activity of norbadione A and other pulvinic acid derivatives. Moreover, the results characterized the best overall protective properties in the three screening assays of compounds **24** and **26**, which appeared to be devoid of pro-oxidant effects. Pulvinic acids, whose synthesis is straightforward and flexible, thus represent very promising compounds for the design of new broad-range protective agents.

Experimental Section

Thymidine protection assay under γ radiolysis: This procedure has been previously described.[10]

Thymidine protection assay under UV radiation: Each well of a microtiter plate (Maxisorb–Nunc) contained thymidine (70 μM, Aldrich), antioxidant (100 μM), and hydrogen peroxide (5 mM) in phosphate buffer (100 μL, 25 mM, pH 7.4). The plates were irradiated with a Bio-Sun 3W irradiator at 254 nm to deliver 1.75 J cm^{-2}. Samples of oxidized solutions (5 μL) were quenched by addition of EIA buffer (50 μL) containing bovine serum albumin (1 mg mL^{-1}, Sigma) in phosphate buffer (100 mM, pH 7.4) before adding acetylcholinesterase (AChE)–thymidine conjugate (50 μL, prepared and stored as previously described)[18] and the specific monoclonal antithymidine antibody (50 μL) in EIA buffer in a second plate (Maxisorb–Nunc) coated with polyclonal goat anti-mouse antibody (Jackson Immuno. Research Laboratories Inc., California). After incubation for 2 h at room temperature, the plates were washed, and Ellman's reagent was added. The absorbance related to the solid-phase-bound AChE activity was measured at 414 nm. Results are expressed as a percentage of thymidine protection. Thymidine quantification was achieved by using a calibration curve (fitted by using a linear log–logit transformation) obtained with pure thymidine. All measurements were made in duplicate.

Thymidine protection assay under the Fenton-like oxidative system: Each well of a microtiter plate (Maxisorb–Nunc) contained thymidine (70 μM, Aldrich), antioxidant (100 μM), freshly prepared $FeSO_4$/EDTA complex (700 μM), and hydrogen peroxide (7 mM) in phosphate buffer (100 μL, 25 mM, pH 7.4). The plates were shaken at room temperature for 30 min. Samples of oxidized solutions (5 μL) were quenched by addition of EIA buffer (50 μL) containing bovine serum albumin (1 mg mL^{-1}, Sigma) in phosphate buffer (100 mM, pH 7.4), and then assayed for the quantification of thymidine by using the protocol described above.

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Keywords: antioxidants · high-throughput screening · immunoassays · natural products · pro-oxidants

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