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Simultaneous Detection of the Antioxidant and Pro-oxidant Activity of Dietary Polyphenolics in a Peroxidase System

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The ability to reduce the peroxidase (myeloglobin/H_2O_2)-generated $ABTS^{\bullet+}$ [2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) radical cation] has been used to rank the antioxidant activity of various agents including dietary flavonoids and chalcones. Surprisingly, we found that in the presence of catalytic concentrations of the phenol B-ring containing flavonoids, apigenin, naringenin and the chalcone phloretin, the formation of the $ABTS^{\bullet+}$ was initially increased. The enhanced formation of the $ABTS^{\bullet+}$ was attributed to the peroxidase/H_2O_2 mediated generation of polyphenolic phenoxyl radicals that were able to co-oxidize ABTS. The relative $ABTS^{\bullet+}$ generating ability of these dietary polyphenolics correlated with their ability to co-oxidize NADH to the NAD* radical with the resultant generation of superoxide. This pro-oxidant activity was not observed for either luteolin or eriodyctiol, which are B-ring catecholic analogues of apigenin and naringenin, respectively, suggesting that these antioxidants are incapable of the transition metal-independent generation of reactive oxygen species. This pro-oxidant activity of the polyphenolics therefore needs to be taken into account when quantifying antioxidant activity.

Keywords: Oxidative stress; Flavonoid; Phenoxyl radical; ABTS; Peroxidase; Stopped-flow

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); $ABTS^{\bullet+}$, 2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) radical cation; DETAPAC, diethylenetriaminepenta-acetic acid; GSH, glutathione; GS*, glutathione thiyl radical; GSSG, glutathione disulfide; HRP, horseradish peroxidase; NADH, nicotinamide adenine dinucleotide (reduced form); NAD*, nicotinamide adenine dinucleotide radical; NAD^+, nicotinamide adenine dinucleotide (oxidized form); TEAC, trolox equivalent antioxidant capacity

INTRODUCTION

Flavonoids are a class of structurally diverse polyphenolic compounds found ubiquitously in the plant kingdom (Fig. 1) which have been suggested to have potent pharmacological potential such as anti-inflammatory, cancer chemopreventative,[1,2] anti-viral[3] and antioxidant activity.[4] However, it has also been demonstrated that some flavonoids containing a catechol or pyrogallol B ring (e.g. myricetin or quercetin) have the potential to cause oxidative DNA, protein and membrane damage as they generate reactive oxygen species as a result of the transition metal catalyzed autoxidation[5] although it is unlikely that this occurs *in vivo* as transition metals are bound to proteins.[6] Instead, oxidative damage from phenoxyl radicals formed by peroxidases could occur *in vivo*.[7]

Previously, we have shown that in the absence of transition metals, flavonoids containing a phenol B ring (e.g. apigenin and naringenin, abundant in celery and grapefruit, respectively) can also undergo a peroxidase-catalyzed bioactivation to form reactive phenoxyl radicals which co-oxidized glutathione (GSH) and nicotinamide adenine dinucleotide

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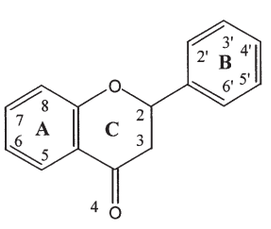


Flavonoid Phloretin

Flavonoid or chalcone	Position of OH group 4′	3′	7	5	2-3 double bond
Chalcone					
Phloretin	OH	H	OH	OH	No
Phenolic Flavonoids					
Apigenin	OH	H	OH	OH	Yes
Naringenin	OH	H	OH	OH	No
Catecholic Flavonoids					
Luteolin	OH	OH	OH	OH	Yes
Eriodictyol	OH	OH	OH	OH	No
Others					
Chrysin	H	H	OH	OH	Yes
7-hydroxy flavone	H	H	OH	H	Yes
5-hydroxy flavone	H	H	H	OH	Yes

FIGURE 1 Flavonoid and phloretin structure.

(NADH), resulting in the activation of molecular oxygen to superoxide as described in Eqs. (1)–(5).[8,9]

$$PhOH + 1/2H_2O_2 \xrightarrow{Peroxidase} PhO^* + H_2O \quad (1)$$

$$PhO^* + (GSH \text{ or } NADH) \leftrightarrow PhOH + (GS^* \text{ or } NAD^*) \quad (2)$$

$$GS^* + GS^- \rightarrow GSSG^{*-} \quad (3)$$

$$GSSG^{*-} + O_2 \rightarrow GSSG + O_2^{*-} \quad (4)$$

$$NAD^* + O_2 \rightarrow NAD^+ + O_2^{*-} \quad (5)$$

This mechanism of NADH and GSH oxidation by xenobiotic radicals has also been proposed by Subrahmanyan and O'Brien, for carcinogenic arylamine peroxidase substrates[10] and is currently thought to be at least partly responsible for the drug toxicity and carcinogenicity associated with the administration of diethylstilbestrol, etoposide and acetaminophen.[11–13]

The 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\bullet+}$) is a stable chromophore which absorbs strongly at 734 nm. Because ABTS is a peroxidase substrate, the inhibition of the formation of the $ABTS^{\bullet+}$ by various chemicals in the presence of myoglobin (in a peroxidase like mechanism) and H_2O_2 has been used to rank the antioxidant activity of a variety of chemicals[14] (Fig. 2).

Using catalytic concentrations of the polyphenolic peroxidase substrates, apigenin, naringenin, phloretin or phenol in a peroxidase system we have found that the co-oxidation of ABTS to the $ABTS^{\bullet+}$ by their phenoxyl radicals preceded their $ABTS^{\bullet+}$ quenching (antioxidant) activity. We also found that the relative rate of ABTS co-oxidation between apigenin, naringenin and phloretin in the presence of peroxidase/H_2O_2 correlated with their ability to generate the $NAD^{\bullet}$. These results suggest that caution should be exercised during antioxidant therapy as the potential pro-oxidant activity of phenolic peroxidase substrates should be addressed.

MATERIALS AND METHODS

ABTS (2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)), naringenin, luteolin, phenol, catechol, 5 hydroxyflavone and 7 hydroxyflavone, sodium phosphate, hydrogen peroxide (H_2O_2), horseradish peroxidase type I (HRP) were purchased from Sigma Chemical Co. Eriodictyol and chrysin were purchased from Extrasynthese Z.I. (Genay, France). Apigenin and phloretin were purchased from Toronto Research Chemicals Inc. (North York, Ont.). All Flavonoid and phenolic stocks were prepared in 95% ethanol.

Stopped-flow Spectrophotometric Determination of Co-oxidizing Potential

Stopped-flow experiments were performed using a stopped-flow spectrophotometer by HI-TECH Scientific connected to an HP 9000 (300) computer running proprietary software. This is a two syringe injection

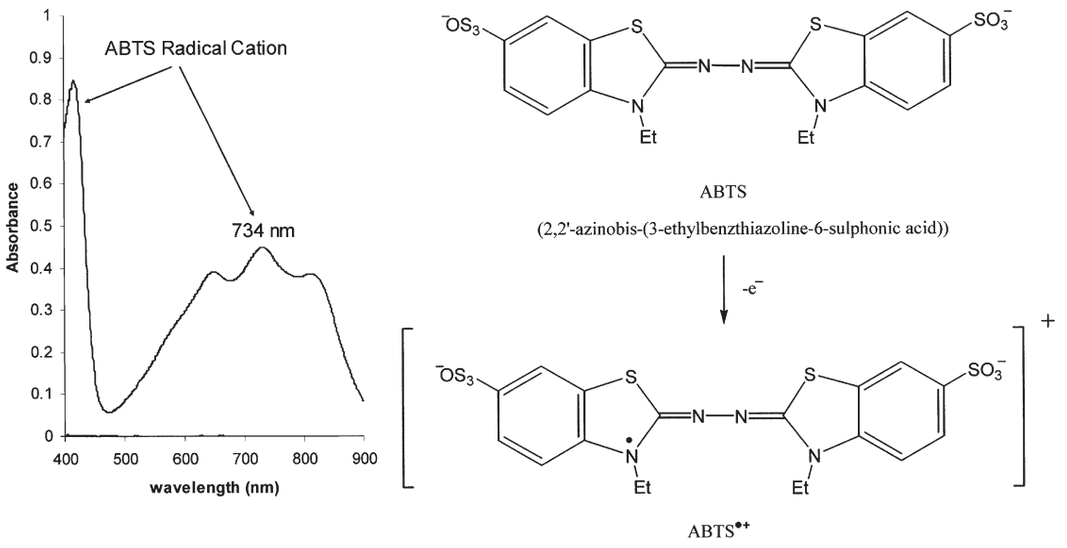


FIGURE 2 Structure of the ABTS and its corresponding radical. The one electron oxidation of ABTS results in the formation of the $ABTS^{\cdot+}$ which absorbs highly at 734 nm.

system which rapidly mixes the contents of both syringes. Peroxidase (0.2 units/ml) in 0.1 M phosphate buffer (pH 7.22) was contained in syringe 1 while ABTS (100 μM), H_2O_2 (100 μM) and analyte (10 μM) in the same buffer were contained in syringe 2. Therefore, the final concentrations of each component during the experiment are peroxidase (0.1 units/ml), ABTS (50 μM), H_2O_2 (50 μM) and analyte 5 μM each. For the measurement of $ABTS^{\bullet+}$ formation over longer periods of time the conditions stated above were repeated by monitoring the absorbance at 734 nm using a Pharmacia Ultrospec 1000 spectrophotometer connected to a PC.

Measurement of Oxygen Consumption

NADH (200 μM), polyphenolic analyte (25 μM), H_2O_2 (25 μM) and HRP (0.1 μM) were incubated in a 2 ml solution of TRIS–HCl (pH 7.4, 0.1 M) with DETAPAC (1 mM). The reaction was started by the addition of HRP. Oxygen consumption was monitored using a Clarke type electrode connected to the YSI model 5300 Biological Oxygen Monitor (YSI Inc., Yellow Springs, OH).

Measurement of NADH Oxidation

HRP (0.1 units/ml) was added to a solution of NADH (100 μM), polyphenolic analyte (10 μM) and H_2O_2 (100 μM) in 1 ml of phosphate buffer. NADH was monitored at 340 nm using a Pharmacia Ultrospec 1000 with proprietary Swift software.

Assessment of Antioxidant Capacity

Antioxidant activity of the test substances was assessed by measuring the trolox equivalent antioxidant capacity (TEAC) as described by Miller *et al.*[14] Spectrophotometric readings were performed at 734 nm on a Pharmacia Ultrospec 1000.

RESULTS

All phenolic chemicals showed an elevated rate of $ABTS^{\bullet+}$ (absorbance at 734 nm) formation over the control (without phenolic) (Fig. 3). The order of effectiveness at increasing the rate of $ABTS^{\bullet+}$ formation was phloretin > apigenin > naringenin > phenol. The increase in the rate of $ABTS^{\bullet+}$ formation was found to be concentration dependent for all the phenolics tested (apigenin dose dependence is shown in Fig. 5—inset A and B). Removal of the 4′OH group from apigenin (i.e. chrysin) eliminated the catalytic effect associated with apigenin. The presence of the 5-OH group (as in 5-OH flavone) decreased the initial rate of $ABTS^{\bullet+}$ formation whereas the presence of the 7-OH group (as in 7-OH flavone) did not affect the initial rate of $ABTS^{\bullet+}$ formation compared to the no flavonoid control (Fig. 3).

Though the initial rate of $ABTS^{\bullet+}$ formation for apigenin, naringenin and phenol was greater than the no flavonoid control (Fig. 3), the formation of $ABTS^{\bullet+}$ reached a peak and began to decrease slowly after approximately 1 min (Fig. 4). This behavior occurred much sooner for phloretin (8 s after—Fig. 3). Interestingly, both the initial

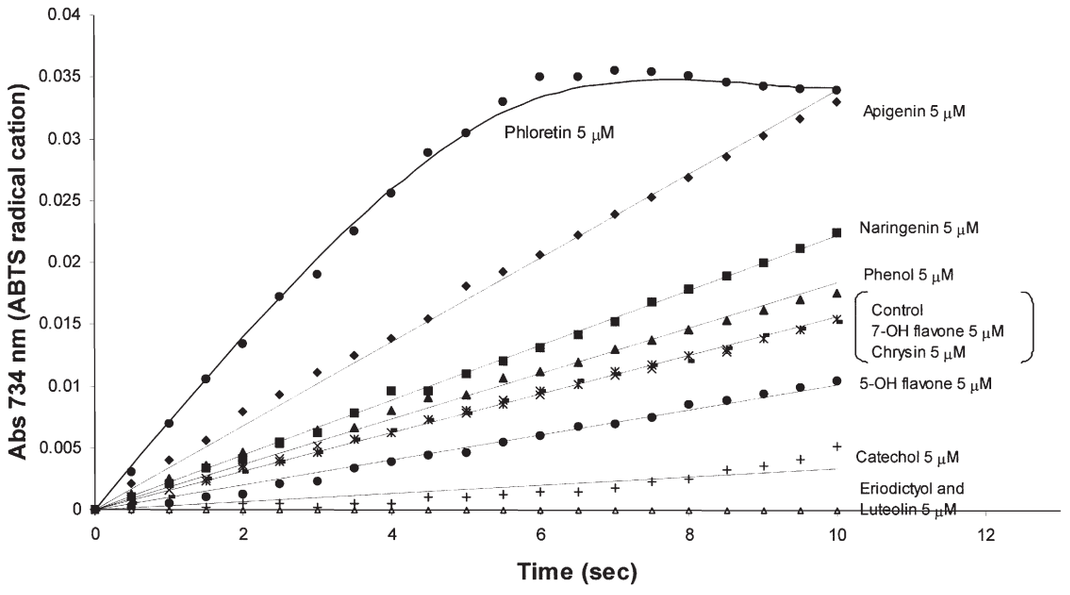


FIGURE 3 Initial increase in the rate of formation of the $ABTS^{\cdot+}$ by various phenoxyl radicals generated by peroxidase and H_2O_2. Reaction was started by mixing a solution of ABTS 100 uM, H_2O_2 100 uM and phenolic flavonoids 10 μM with an equal volume of buffer containing 0.2 units/ml HRP.

increase in the rate of $ABTS^{\bullet+}$ formation and the later reduction of the $ABTS^{\bullet+}$ by apigenin was concentration dependent (Fig. 5 and inset A). The rate of $ABTS^{\bullet+}$ formation in the presence of apigenin could be considered pseudo first order with respect to apigenin concentration (Fig. 5—inset B). Varying the concentration of H_2O_2 from 50 to 200 μM did not affect either the initial rate of increase in

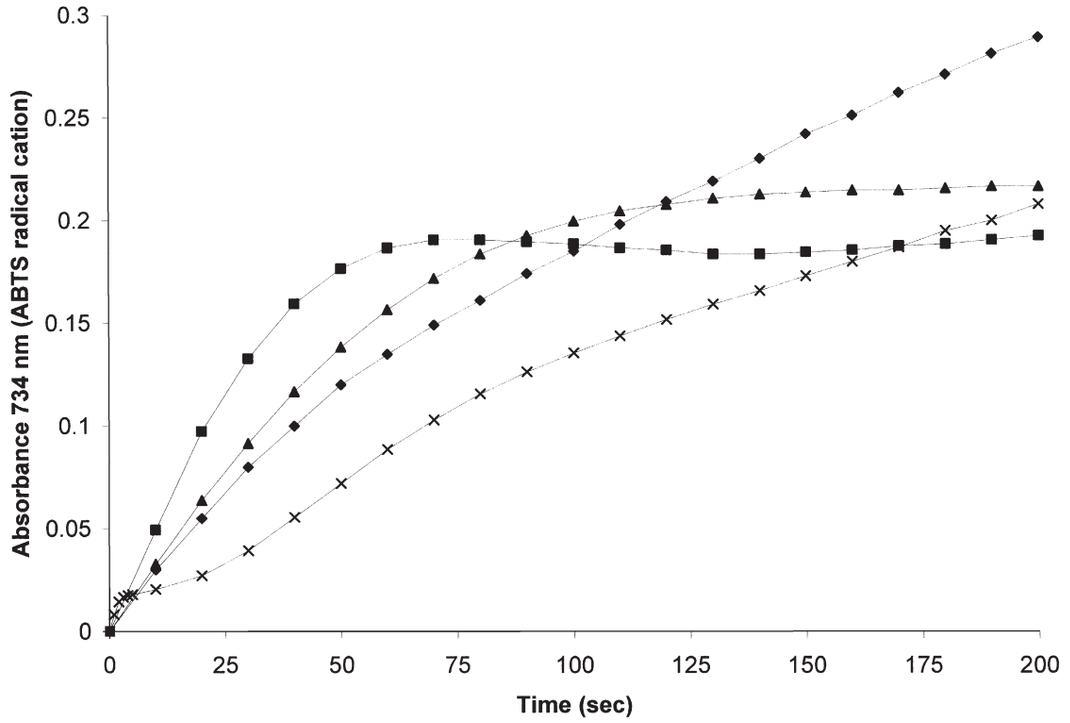


FIGURE 4 Time curve for the initial pro-oxidant and later antioxidant activity of phenol B-ring containing flavonoids (5 μM) and phloretin (5 μM). Apigenin (■), Naringenin (▲), Control (◆) and Phloretin (×) were incubated with ABTS (100 μM), H_2O_2 (100 μM) in phosphate buffer (pH 7.23). The solution was mixed with an equal volume of peroxidase 0.2 units/ml in phosphate buffer pH 7.23. The reaction was monitored on a HI TECH stopped-flow spectrophotometer at 734 nm.

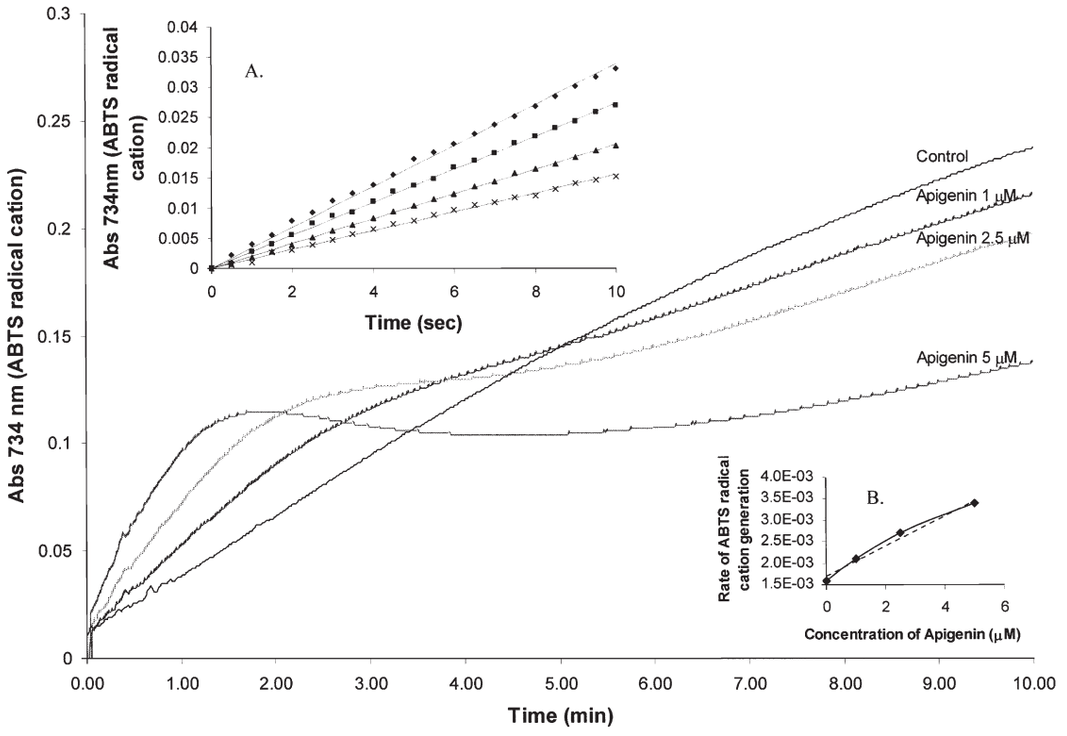


FIGURE 5 Concentration dependence of both the initial pro-oxidant and later antioxidant activities of apigenin. Inset A shows the dose dependent effect of apigenin on the formation of the $ABTS^{+}$ Apigenin 0 (×), 1 (▲), 2.5 (■) or 5 (◆) μM, ABTS 100 uM, H_2O_2 100 μM in phosphate buffer pH 7.23 was mixed with an equal volume of peroxidase 0.2 units/ml in phosphate buffer pH 7.23. The reaction was monitored on a HI TECH stopped-flow spectrophotometer at 734 nm. Inset B shows a plot of apigenin vs. rate of $ABTS^{+\cdot}$ formation.

$ABTS^{\bullet+}$ or the later quenching of $ABTS^{\bullet+}$ for apigenin (result not shown). Luteolin and eriodictyol differ from naringenin and apigenin, respectively, only in that they contain a catechol B-ring instead of a phenol B-ring. These catechol B-ring containing flavonoids as well as catechol itself prevented $ABTS^{\bullet+}$ from forming which was visualized as a decreased rate of formation of the $ABTS^{\bullet+}$ (Fig. 3). All three catecholic chemicals tested were able to suppress the formation of the $ABTS^{\bullet+}$ as shown in Fig. 6 up to 60 s of the reaction.

The formation of superoxide accompanying the oxidation of NADH to the NAD radical by free radicals (as described in Eq. (2)) can be detected using an oxygen electrode. Catechol containing compounds such as the flavonoids eriodictyol and luteolin or catechol, did not co-oxidize either NADH or ABTS to their perspective radicals as indicated by the lack of both $ABTS^{\bullet+}$ formation and NADH associated oxygen consumption. However, the substances were able to oxidize NADH to NAD^{+} more rapidly than the phenolic agents indicating that they were better peroxidase substrates (Table I). NADH oxidation did not occur in the absence of flavonoid as at pH 7.4 NADH is not a peroxidase substrate.

The initial pro-oxidant activity of the phenolic agents was not affected by their antioxidant activity as indicated by a lack of an inverse correlation between the initial rate of $ABTS^{\bullet+}$ formation and antioxidant value, however, at later time points, the extent of $ABTS^{\bullet+}$ reduction was correlated with antioxidant values (Table I).

DISCUSSION

Phenoxyl radicals generated from peroxidase or redox cycling, heme containing enzymes (e.g. myeloperoxidase, hemoglobin, thyroid peroxidase, cytochrome P450s) have been implicated in drug toxicity[6] and various pathological diseases. For example, the phenoxyl radical of the anticancer agent, etoposide was shown to cause oxidative stress cytotoxicity in HL-60 cells which was attributed to etoposide oxidation to the phenoxyl radical catalyzed by intracellular myeloperoxidase and H_2O_2. Also, the oxidation of the amino acid tyrosine which is present in serum at concentrations as high as 70 uM[15] to the tyrosyl radical by the same enzyme has been implicated in the formation of *o,o′*-dityrosine protein adducts.[16]

Both phenolic and catecholic flavonoids have been shown to be excellent substrates for peroxidase. Flavonoids containing a catechol B-ring undergo

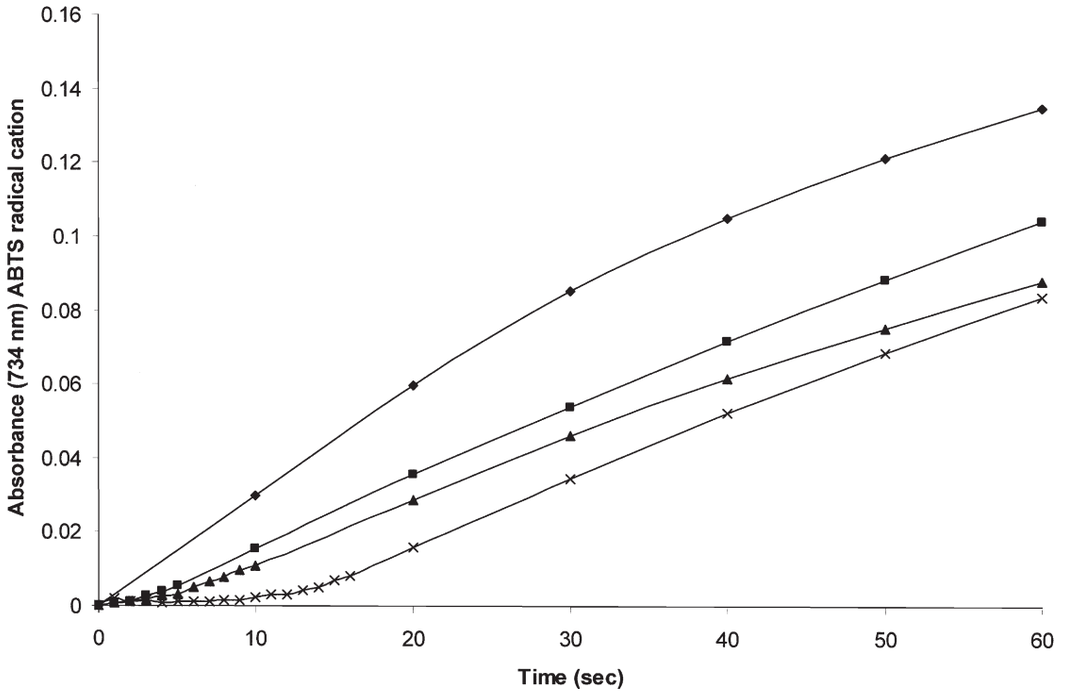


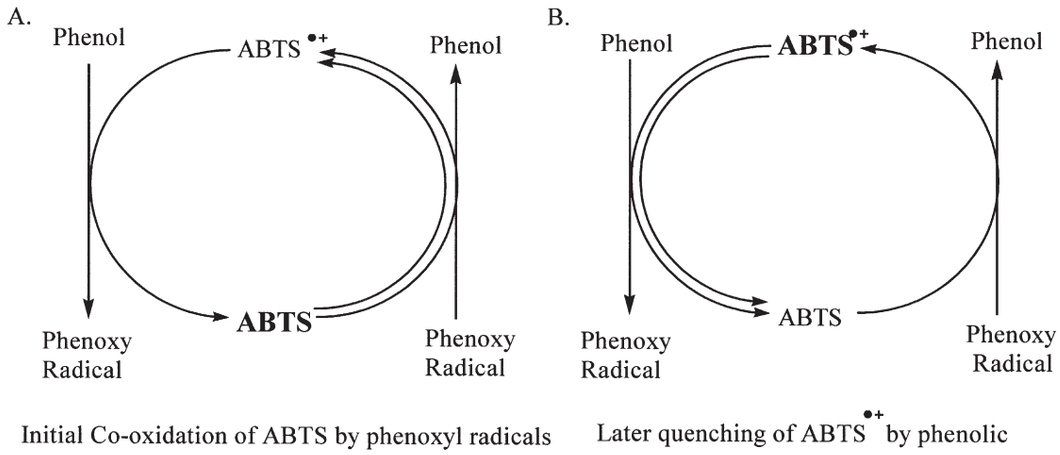
FIGURE 6 Time curve for the antioxidant activity of various catecholic flavonoids (5 μM) and catechol (5 μM). Control (◆), Eriodictyol (▲), Catechol (■) and Luteolin (×) were incubated with ABTS (100 μM), H_2O_2 (100 μM) in phosphate buffer (pH 7.23). The solution was mixed with an equal volume of peroxidase 0.2 units/ml in phosphate buffer pH 7.23. The reaction was monitored on a HI TECH stopped-flow spectrophotometer at 734 nm.

a peroxidase catalyzed oxidation to quinones or quinone methides that can conjugate GSH or protein thiols[17–19] while phenol B-ring containing flavonoids generate phenoxyl radicals (e.g. apigenin and naringenin) which can co-oxidize other molecules (e.g. protein, GSH, NADH and DNA).[6–8,14,16,20] The latter pro-oxidant activity has been implicated in phenoxyl radical mediated hemolysis and thyroid peroxidase inactivation by flavonoids. Contrarily, semiquinone metabolites of catechol B-ring containing flavonoids generally do not readily oxidize NADH or GSH[7] and have

TABLE I The initial rate of ABTS co-oxidation caused by phenoxyl radicals correlates with the peroxidase-catalyzed co-oxidation of NADH to the NAD* by phenolic substrates and is independent of the antioxidant activity of the phenolic substrate

Chemical	Antioxidant activity (TEAC value)	O_2 uptake with NADH (μM)*	NADH oxidation (μM/min)	$ABTS^{\bullet+}$ formation (abs/min) × 10^{-3}
Chalcone				
Phloretin	1.2 ± 0.1	195 ± 20	44.4 ± 0.9	6.5 ± 0.3
Phenolic flavonoids				
Apigenin	1.45 ± 0.08†	88 ± 10‡	15.1 ± 0.7	3.5 ± 0.2
Naringenin	1.53 ± 0.05†	77 ± 7‡	8.8 ± 0.5	2.2 ± 0.3
Catecholic flavonoids				
Luteolin	2.1 ± 0.05†	<1‡	>90	0
Eriodictyol	1.9 ± 0.03†	<1	51 ± 3	0
Flavonoids with no B-ring hydroxyl group				
Chrysin	1.43 ± 0.07†	3 ± 2	<0.1	1.6 ± 0.1
7-OH flavone	0.65 ± 0.05	<1	<0.1	1.6 ± 0.1
5-OH flavone	0.92 ± 0.04	<1	<0.1	1 ± 0.1
Single ring phenols				
Phenol	0.87 ± 0.06	58 ± 3‡	2.2 ± 0.3	1.8 ± 0.1
Catechol	0.46 ± 0.07	2 ± 1	3.3 ± 0.1	0.30 ± 0.02

*NADH dependent oxygen uptake is expressed as the total amount of oxygen reduced. †Ref. [25]. ‡Ref. [7]. TEAC, trolox equivalent antioxidant capacity. TEAC values and NADH and GSH mediated O_2 uptake values were reproduced using the same methods described previously.[7,9] $ABTS^{\bullet+}$ formation in the absence of a phenolic compound was (1.6 ± 0.1) × 10^{-3} abs/min.



SCHEME 1 Co-oxidation of ABTS by phenoxyl radicals. The initial increase in rate of $ABTS^{+\cdot}$ is due to the co-oxidation of ABTS by the phenoxyll radical (A), whereas the loss in $ABTS^{+\cdot}$ levels observed at later time points may result from its quenching due to the accumulation of phenols or oxidation products (B).

lower redox potentials and better antioxidant potential.[6,21,22]

The co-oxidation of ABTS by phenolic flavonoids proceeds as described by Eq. (6).

$$\text{Phenoxyl Radical} + \text{ABTS} \longrightarrow \text{Phenol} + \text{ABTS}^{\bullet+} \quad (6)$$

The relative initial rate of ABTS co-oxidizing capacity of a given phenolic peroxidase substrate was independent of its ability of quench the $ABTS^{\bullet+}$ since initially, no $ABTS^{\bullet+}$ was present at time 0 (Scheme 1, panel A). Also, since the initial concentration of ABTS was in great excess compared to the concentration of the polyphenolic, the initial formation of $ABTS^{\bullet+}$ can be considered a pseudo first order reaction with respect to polyphenolic concentration as was shown for apigenin. This might explain why the rate of $ABTS^{\bullet+}$ scavenging by phenolic flavonoids was initially fast followed by a slower phase, as compared to the catecholic flavonoids which quickly scavenged $ABTS^{\bullet+}$ without exhibiting the biphasic characteristic observed with the phenolic chemicals.[22]

However, at later time points when $ABTS^{\bullet+}$ had accumulated, the antioxidant activity of the reduced chemical significantly prevented the further formation of $ABTS^{\bullet+}$ by subsequently reducing it (Scheme 1, panel B). The chalcone phloretin was also effective at initially increasing the rate of $ABTS^{\bullet+}$ formation. However, the unusually rapid termination of the increase in the rate of ABTS co-oxidation by the phloretin phenoxyl radical could be attributed to its conjugation to ABTS or itself thereby inactivating its catalytic activity. This also explains why at later time points, phloretin was not as effective as naringenin or apigenin at preventing $ABTS^{\bullet+}$ formation.

The major determinant of the rate of ABTS co-oxidation is the rate of substrate turnover by peroxidase as was indicated by the correlation between the rate of NADH co-oxidation with the rate of ABTS co-oxidation within the phenolic group of chemicals. However, unlike ABTS oxidation, NADH oxidation is not specifically carried out by free radical species.

Previously, we and others have shown that the peroxidase-catalyzed oxidation of the catechol containing flavonoids, luteolin and quercetin in the presence of GSH resulted in the formation of GS-flavonoid conjugates[8,17–19] instead of GS radicals suggesting that the formation of quinones generated by the disproportionation of the semiquinone radical intermediate was favored over the co-oxidation of GSH to form a thiyl radical. Therefore, the catecholic substances were co-oxidizing NADH via their *o*-quinone intermediates and not by their semiquinone radical intermediates which explains why they did not initially increase the formation of $ABTS^{\bullet+}$. Although, significant competition for peroxidase could underlie the cause of the decrease in $ABTS^{\bullet+}$ formation in the presence of the catecholic substances, it is more likely that initially, the scavenging of the $ABTS^{\bullet+}$ which is formed is the predominant cause, as ABTS was in great excess. The scavenging activity catecholic compounds as opposed to their phenolic counterparts also strengthens the view that compounds with catecholic moieties have greater radical scavenging capacity than ones with phenolic moieties which is substantiated by the higher TEAC values. Furthermore, the rapid increase in the initial rate of ABTS co-oxidation to the $ABTS^{\bullet+}$ by the phenolic compounds tested here suggests that upon their one-electron oxidation (for instance, by the hydroxy radical) they in fact may serve as radical carriers instead of scavengers (antioxidant) and may themselves participate in causing cellular injury.

We have shown that the rate of formation of $ABTS^{\bullet+}$ is biphasic for both the phenolic and catecholic chemicals tested. However, although a decrease in the rate of formation of the $ABTS^{\bullet+}$ in the later phase is common between the phenolic and catecholic chemicals tested, in the case of the phenolic chemicals the initial rate of formation of the $ABTS^{\bullet+}$ was increased (representing the pro-oxidant activity of these chemicals) compared to ABTS alone, whereas with catecholic chemicals, the rate of formation of the $ABTS^{\bullet+}$ was initially markedly decreased. Since many antioxidants are bioactivated through peroxidase-like pathways,[23] it is necessary to acknowledge their potential pro-oxidant activity. Furthermore, it should also be noted that since the original TEAC method utilized methemoglobin as the peroxidase ($ABTS^{\bullet+}$ generating) activating enzyme, the pro-oxidant activity of the flavonoids described here have the potential to overestimate their antioxidant activity as determined by the TEAC assay.

Furthermore, the applicability of determining the relative phenoxyl radical generating ability of phenolic compounds could complement the already established trolox equivalent antioxidant concentration (TEAC) assay developed by Miller *et al.*[12] which follows the formation of $ABTS^{\bullet+}$ in a ferrylmyoglobin/H_2O_2 system as well as the newly developed decolorization assay developed by Re *et al.*[24]

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