JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Novel Protein-Based Solid-Biosensor for Determining Pro-oxidant Activity of Phenolic Compounds

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ABSTRACT: To develop a protein-based biosensor measuring the pro-oxidant activities of phenolic compounds, egg white proteins were precipitated with calcium chloride to obtain an insoluble calcium proteinate complex. This biosensor was used for the determination of Cu(II)-induced pro-oxidant activity of antioxidants such as gallic acid, catechin, epicatechin, quercetin, chlorogenic acid and myricetin, and ascorbic acid. This assay involved the reduction of Cu(II) ions to Cu(I) by antioxidant compounds (simultaneously giving rise to reactive oxygen species) and binding of the formed Cu(I) to the solid biosensor. The protein-bound Cu(I), an indicator of pro-oxidant activity of antioxidants on proteins, was colorimetrically determined at 450 nm with neocuproine (Nc). The method was applied to synthetic mixtures and herbal (sage, green tea, mint, and marjoram) infusions, and its findings were compared to those of a modified carbonyl detection assay. This low-cost biosensor can be prepared in large quantities and used for a long time.

KEYWORDS: *pro-oxidant biosensor, phenolics, protein precipitation, copper detection, cupric neocuproine, carbonyl assay*

INTRODUCTION

A pro-oxidant effect is a general term used when biochemical or biophysical events initiate or intensify, by different mechanisms, a redox imbalance in favor of oxidant species. One alternative definition of pro-oxidant activity is the ability of antioxidant compounds to reduce transition metal ions to their lower oxidation states, stimulating the production of reactive species via Fenton-type reactions.[1]

Phenolic compounds are secondary metabolites derived from the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These compounds exhibit a wide range of physiological properties such as antiallergenic, anti-inflammatory, antimicrobial, and antioxidant effects. Although most of the health-beneficial effects of these compounds are assumed to originate from their antioxidant activity, there is growing evidence that natural or synthetic antioxidants such as phenolic compounds (curcumin, catechin, epicatechin, gallic acid, quercetin, and anthocyanidins), ascorbic acid, α-tocopherol, and carotenoids can act as pro-oxidants.[1−7] These pro-oxidant effects usually depend on the concentration of compounds,[2,4,8−11] free radical source,[4] and other molecules in the medium.[3,6,9] In the presence of O_2, transition metal ions such as copper(II) and iron(III) catalyze the redox cycling of these compounds leading to the formation of reactive oxygen species (ROS) and phenoxyl (Ar—O$^{\bullet}$) radicals that can damage biomacromolecules such as DNA, lipids, and proteins.[12,13]

The possible pro-oxidant effects of flavonoids may be important in vivo where free transition metal ions are involved in the oxidation process. Flavonoids are capable of reducing Cu(II) to Cu(I) and simultaneously enabling the formation of free radicals. The copper-initiated pro-oxidant activity of a flavonoid depends on the number and position of hydroxyl substituents in its molecular structure. Structure−activity relationships may lead flavonols to show much higher copper-initiated pro-oxidant activity than flavonones with the same number of hydroxyl substitutions.[4]

In recent years, postulates have emerged that pro-oxidant effects can be beneficial under certain conditions. In some reviews, the pro-oxidant activity of individual dietary polyphenols and their ability to induce mitocondrial dysfunction and consequently apoptosis have been suggested as a possible anticancer mechanism.[3,6,13] It is also recognized that the pro-oxidant action of natural poyphenols, unlike their antioxidant properties, has a more specific preference against certain cellular targets, since it appears to play an important role in prevention of certain types of cancer.[14] Halliwell stated that pro-oxidant effects can be beneficial, as the burdening a mild degree of oxidative stress might raise the levels of antioxidant defenses and xenobiotic-metabolizing enzymes, thereby supporting general cytoprotection.[15] Procházková et al. hypothesized that pro-oxidants can exhibit cell signaling properties which are essential to life via contributing to the coordination of cell functions.[7]

In biological systems, iron and copper ions are essential for electron-transfer reactions. These ions, in their unbound form, may lead to the generation of ROS via Haber Weiss and/or Fenton reactions. Because of these reactions, ROS are generated which can lead to oxidative damage to biomolecules.[16−18] It is widely accepted that the damage observed in some pathologies (e.g., Alzheimer's, Parkinson's, and Wilson's diseases) is the consequence of the pro-oxidant properties of these ions. Iron is the most likely candidate for promoting oxidative reactions, whereas the occurrence of copper-catalyzed reactions in vivo is controversial.[19] In fact, organisms take great care in sequestering transition metal ions. Indeed, this

Received: April 10, 2017
Revised: June 15, 2017
Accepted: June 20, 2017
Published: June 21, 2017

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

sequestration can be regarded as a part of antioxidant defense. However, the release of "free" metal ions (i.e., in unbound forms) from sequestered sites can occur as a result of tissue injury by disease, trauma, toxins, and other causes.[19,20]

As a result of oxidation, proteins are covalently modified by the direct action of ROS or indirectly by reaction with secondary byproducts of oxidative stress. The oxidation of amino acid residues in proteins causes several modifications such as deamination, carbonyl group formation and peptide bond cleavage.[21] Protein carbonyl formation in vitro and in vivo is induced by a diverse range of agents, including metal-catalyzed oxidation, ozone, HOCl, singlet oxygen, and ionizing radiation,[22,23] and is used as a biomarker of oxidative stress.[24] Colorimetric detection of carbonyl groups, emerging as the most widely used measurement of protein oxidation, involve their derivatization with 2,4-dinitrophenylhydrazine (DNPH), leading to the formation of a stable dinitrophenyl (DNP) hydrazone product.[25,26] The disadvantages of the carbonyl assay may be listed as low sensitivity, narrow linear range, and high intercept values in the calibration equations. In addition, it can be adversely affected by biological molecules like nucleic acids, hemoglobin, and myoglobin.[23] In spite of these drawbacks, the carbonyl assay may still be regarded as a comparison method for pro-oxidative assay developments on proteins.

In literature, there are few assays to measure the pro-oxidant activity of phenolics depending on protein damage.[21,27−30] Besides, the existing methods are indirect and laborious, so there is a need to develop easily applied and low-cost methods for testing the in vitro pro-oxidant activity of well-known antioxidant compounds. In a previous work of the authors, an assay was reported to indirectly measure the pro-oxidant activity of phenolics based on their Cu(II)−Cu(I) reducing ability, as the formed Cu(I) ions simultaneously gave rise to the generation of reactive species and was itself bound to egg white proteins. In this solution phase assay, the protein-bound cuprous ions were colorimetrically measured after their liberation with neocuproine (as the colored cuprous-neocuproine chelate).[28] As a result, total pro-oxidant activities (TPAs) of binary synthetic mixtures were calculated as mM quercetin (QUE) equivalent, and of sage, green tea, mint, elderberry, linden, and rosemary extraxts as μmol QUE g^{-1} equivalent. However, the properties of egg white were not fully reproducible from one liquid phase assay to another, and a reproducible solid biosensor utilizing this reaction could not be prepared.

In this work, we aimed to propose a protein-based solid pro-oxidant biosensor for determining the Cu(II)-induced pro-oxidant activity of selected antioxidant compounds including phenolics. In this context, a solid material consisting of egg white proteins instead of the aqueous solution used in the previous study[28] was prepared for the development of the biosensor. Dhara's work was taken as reference in the preparation of this solid material,[31] but we preferred to use $CaCl_2$ solution instead of $Al(NO_3)_3$ to precipitate the proteins, because Al(III) can itself show pro-oxidant properties[32] and form complexes with flavonoids[33] possibly affecting their pro-oxidative mechanism. We believe that this is the first low-cost solid biosensor that can sensitively and reproducibly measure Cu(II)-induced pro-oxidant activity of polyphenols and real samples. We compared our results from the proposed biosensor with those of the modified carbonyl assay.

MATERIALS AND METHODS

Reagents, Solutions and Instrumentation. The following chemical substances of analytical reagent grade were supplied from the corresponding sources: (−) epicatechin (ECAT), and chlorogenic acid (CLA): Sigma (Taufkirchen, Germany); neocuproine (2,9-dimethyl-1,10-phenanthroline) (Nc), and quercetin (QUE): Aldrich (Taufkirchen, Germany); sodium dihydrogen phosphate dihydrate, 2,4-dinitrophenylhydrazine (DNPH), (+) catechin (CAT) hydrate, gallic acid (GA), ascorbic acid (AA), methanol (MeOH), and ethanol (EtOH): Sigma-Aldrich (Taufkirchen, Germany); copper(II) sulfate, and myricetin (MYR): Fluka (Buchs, Switzerland); calcium chloride: Merck (Darmstadt, Germany); ammonium acetate, and disodium hydrogen phosphate: Riedel-de Haën (Seelze, Germany).

Ammonium acetate (NH_4Ac) buffer at pH 7.0, 1.0 M, copper(II) sulfate solution, 1.0×10^{-3} M, and the NaH_2PO_4/Na_2HPO_4 phosphate buffer solution at pH 7.4, 0.2 M, were prepared in distilled water. Neocuproine (Nc) solution, 7.5×10^{-3} M, was prepared daily in absolute ethanol (EtOH). The stock solutions of test antioxidants, 1.0×10^{-2} M, were freshly prepared in distilled water, except those of QUE and MYR, 3.0×10^{-3} M, dissolved in 50% EtOH/H_2O (v/v). 2,4-Dinitrophenylhydrazine (DNPH) solution, 1.0×10^{-2} M, was prepared in MeOH.

The absorbance values were measured with a Varian Cary 1E UV−vis spectrophotometer (Sydney, Australia) using a pair of matched quartz cuvettes of 1 cm optical path length. The pH measurements were made with the aid of an Inolab pH7110 pH-meter using a combined glass electrode. A Select vortex apparatus was used to stir the incubation solutions. An Electromag M4812PII (Istanbul, Turkey) centrifuge apparatus was used for separation of the precipitated egg white protein.

Preparation of Synthetic Mixtures. To test possible additive (or synergistic and antagonistic) effects among binary mixtures of antioxidants in exhibiting pro-oxidant effects, binary combinations that were selected to be appropriate concentrations within the linear ranges of the modified Cu(II)−Nc and carbonyl assays were prepared for determining their pro-oxidant activities as mM ECAT equivalent. The final antioxidant concentrations of the tested binary mixtures are given below:

mixture 1: 400 μM ECAT + 200 μM CLA

mixture 2: 4000 μM AA + 100 μM GA

mixture 3: 4000 μM AA + 100 μM CAT

Herbal Plant Infusion. A 2-g amount of the herbal plant (sage, green tea, mint, and marjoram) supplied from Malatya Pazari A.S. (a company marketing herbal products in the City of Istanbul) was transferred to a stoppered flask, 10 mL distilled water was added, and extracted for 15 min in an ultrasonic bath protected from light at room temperature. The upper phase was decanted, and the extraction process was repeated two times with 10 and 5 mL distilled water, respectively. Thus, the overall extraction took 45 min. The combined supernatants were filtered through glass fiber/polyethylene terephthalate (GF/PET) 1.0/0.45 μm microfilters before analysis. The extracts were generally analyzed freshly, but were stored at −20 °C in the dark when necessary.

Preparation of Protein-Based Solid Sensor. Dhara's work was taken as reference for the preparation of the solid material[31] but was highly modified by selecting $CaCl_2$ instead of $Al(NO_3)_3$ as the protein precipitation agent. The egg white was completely separated from the yolk into a beaker. The final volume was made up to 50 mL by adding distilled water. The mixture was stirred with a magnetic stirrer at 500 rpm until it became homogeneous. Onto the mixture, 100 mL distilled water adjusted to pH 9.5 (i.e., slightly alkalinized with dilute 0.1 M NaOH) was added, followed by the dropwise addition of 50 mL of 0.1 M $CaCl_2$. The mixture was allowed to stand for 2 h at room temperature after heating for 1 h at 10.0 °C. At the end of this period, the Ca−proteinate precipitate was filtered through a filter paper, and the residue washed with distilled water until calcium was completely removed, then left to dry under ambient air conditions. The dried

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

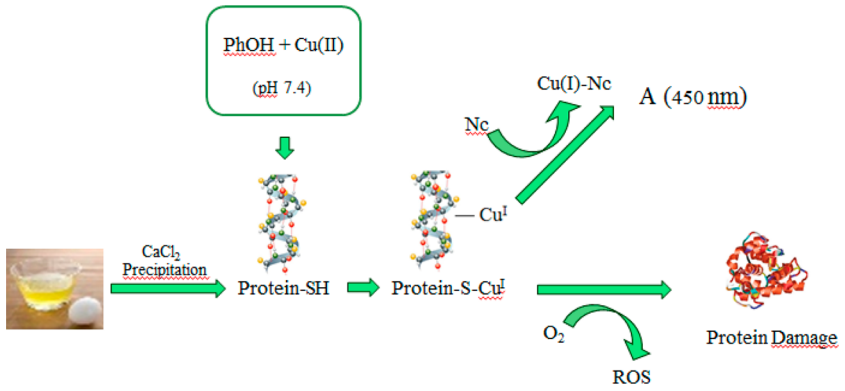

Figure 1. Schematic diagram of the proposed total pro-oxidant activity (TPA) assay using protein-based sensor.

protein residue (protein-based solid pro-oxidant sensor) was ready for use in powder form after grinding in a mortar.

Recommended Procedure for Modified Cu(II)−Nc Assay Using Protein-Based Sensor. To a test tube was weighed 50 mg of protein-based sensor, and added 1.0 mL of phosphate buffer (pH 7.4), 1.0 mL of 1.0 mM copper(II) solution, 1.0 mL standard antioxidant solution at various concentrations (within the range of 0.25−2500 μM, depending on the antioxidant), or 1.0 mL aliquot of the herbal plant infusion (solution appropriately diluted depending on the herb) and 1.0 mL of distilled water. The final mixture at 4.0 mL total volume was vortexed and agitated for 30 min in a rotator at room temperature. At the end of this time, the mixture was centrifuged for 1 min at 5000 rpm, the upper liquid phase decanted, and the protein-based sensor washed three times with 5 mL distilled water each time. To the pro-oxidant sensor were added 1.0 mL of Nc solution and 1.0 mL of NH_4Ac buffer (pH 7.0), and 2 mL distilled water. The mixture was agitated for a second incubation period of 20 min in a rotator at room temperature. Finally, the mixture was filtered through a GF/PET microfilter and the absorbance at 450 nm (A_{450}) was recorded against a reagent blank. The same procedure was followed in preparing the reagent blank, except that the same volume of solvent was added instead of the standard antioxidant or sample solution (Figure 1).

The standard calibration curve for each tested compound was constructed in this manner as absorbance vs molar concentration, and the indirect molar absorptivity of the modified Cu(II)−Nc assay for each antioxidant compound was found from the slope of the calibration line concerned.

Protein Carbonyl Detection Assay. Carbonyl detection assay is based on the measurement of the absorbance of dinitrophenylhydrazones formed from the reaction of 2,4-DNPH with the carbonyl groups emerging from protein oxidation.[21,25−29] Because the solid protein-based sensor was used in our study, the carbonyl assay was modified especially omitting protein precipitation with trichloroacetic acid (TCA). To a test tube were weighed 50 mg of protein-based sensor, and added 1 mL of phosphate buffer (pH 7.4), 1.0 mL of 1.0 mM copper(II) solution, 1.0 mL test compound solution at various concentrations (within the range of 0.25−3000 μM, depending on the antioxidant) or 1.0 mL aliquot of herbal plant infusion (solution appropriately diluted depending on the herb) and 1.0 mL of 10.0 mM DNPH solution. The final mixture at 4.0 mL total volume was vortexed and agitated for 30 min in a rotator at room temperature. At the end of this time, the mixture was filtered through a GF/PET microfilter, and its absorbance at 370 nm (A_{370}) was recorded against a reagent blank. The same procedure was followed in preparing the reagent blank, except that the same volume of solvent was added instead of the sample solution.

The standard calibration curve for each phenolic compound was constructed in this manner as absorbance vs molar concentration, and the indirect molar absorptivity of the carbonyl detection assay for each antioxidant compound was found from the slope of the calibration line concerned.

Statistical Analysis. Spectrophotometric assays were applied in triplicate for each sample and standard. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2016) for calculating the mean and the standard error of the mean. Results were expressed as {mean ± standard deviation (SD)}. The precisions of the two methods were compared with the aid of an *F*-test.[34]

RESULTS AND DISCUSSION

It has been shown that copper ions can irreversibly and nonspecifically bind to thiol groups in proteins. According to the findings of Letelier et al.,[18] copper(II) can display more extensive toxic effects than iron(III), in terms of the oxidative damage and nonspecific binding to biomolecules. The interactions of a number of metal ions (Cr, Mn, Fe, Cu, Zn, Na, Mg, K, and Ca) with antioxidant compounds (e.g., ascorbic acid, caffeic acid, carnosic acid, catechin, chlorogenic acid, eugenol, ferulic acid, gallic acid, quercetin, rutin, γ-tocopherol, and butylhydroxytoluene) were tested by ESR spectroscopy as potential generators of ROS. Only iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{+} and Cu^{2+}) generated ROS by interacting with antioxidants and copper ions had the highest pro-oxidant activity.[35] For these reasons, as in our previous work,[28] we selected Cu(II) and egg white proteins containing ovalbumin that includes six cysteines (Cys) with a single disulfide bond between Cys74 and Cys121 in the amino acid sequence[36] to simulate real cases of pro-oxidant action, where reactive species are generated through transition metal ion−initiated reactions damaging biological macromolecules. However, dissimilar to our previous study, we aimed to develop a solid pro-oxidant sensor due to its repeatability, robustness, and easy use. Calcium chloride precipitation technique was applied to egg white proteins in order to reproducibly prepare the biosensor.

The proposed assay of this work involved the reduction of Cu(II) ions to Cu(I) by antioxidant compounds (simultaneously giving rise to reactive oxygen species) and binding of the formed Cu(I) to the solid biosensor. The protein-bound Cu(I), an indicator of pro-oxidant activity of antioxidants on proteins, was colorimetrically determined at 450 nm with neocuproine (Figure 1). The highly colored Cu(I)−neocuproine complex specifically indicated Cu(I)—and not Cu(II). In the widely used protein assays such as Lowry assay with the Folin reagent[37] or cupric−bicinchoninic acid (BCA) assay,[38] the "biuret reaction"—in which cupric ions chelate with protein amides in strongly alkaline solution—is generally coupled to a redox colorimetric reagent to liberate the characteristic chromophore. If only Cu(II) is involved without the use of a complexing agent (such as BCA or phosphotungstomolybdate(VI)), then it may not appreciably

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

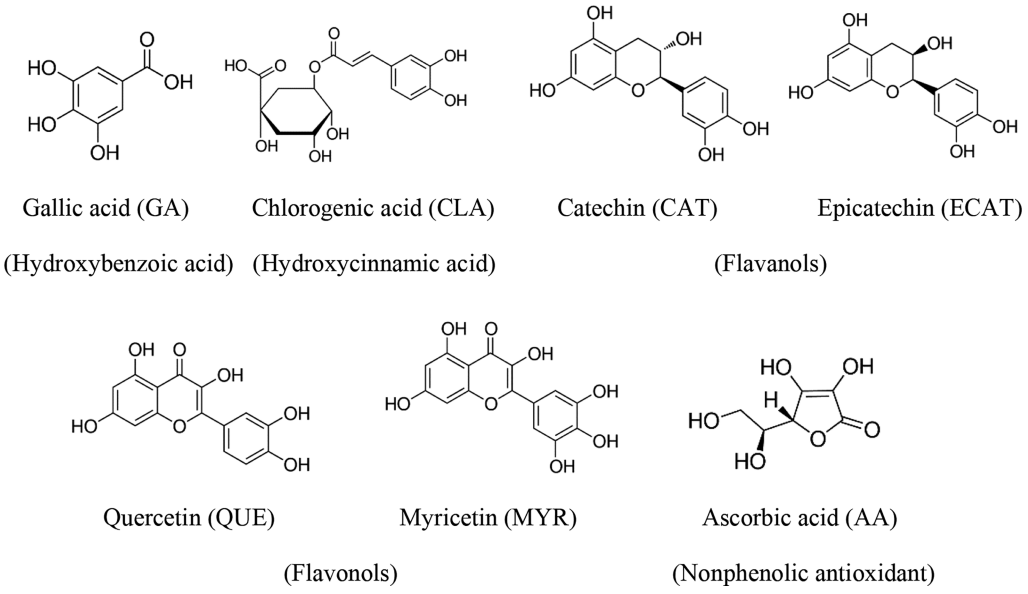

Figure 2. Structures of tested compounds.

oxidize the critical amino acid residues on proteins since Cu(II,I) standard potential is only 0.17 V against SHE. When the biuret reaction is coupled to Cu(II)−BCA or Lowry assay, the Cu(II)−Cu(I) potential is significantly elevated to oxidize the cysteine, tryptophan, and tyrosine residues on peptides/proteins resulting in Cu(I) formation accompanied by molybdenum(V) blue or purple Cu(I)−BCA coloration. In our proposed assay of pro-oxidant activity performed in neutral medium, the high-affinity of egg white cysteines to Cu(I) is coupled to an integrated sequence of Cu(II) complexation followed by Cu(II)−Cu(I) reduction by polyphenols, making this reaction thermodynamically favorable.

The test compounds were chosen to represent different classes of antioxidants and phenols, such as GA (hydroxybenzoic acid), CLA (hydroxycinnamic acid), CAT and ECAT (flavanols), QUE and MYR (flavonols), AA (nonphenolic antioxidant) (Figure 2). We tried to investigate a wide range of concentrations from 0.25 to 2500 μM for specifying the pro-oxidant activity of phenolic compounds. In general, the pro-oxidant activity of polyphenols was most visible above a critical concentration of 2.50 μM according to the modified Cu(II)−Nc assay using the developed solid biosensor. Normal human plasma contains copper close to 1 μg/mL (about 16 μM) which is largely bound to biomacromolecules like ceruloplasmin and albumin.[39] However, a concentration of 1 mM copper was used in the test system to ensure the measurement of considerable pro-oxidant activity within the protocol time of the assay. In other studies related to the measurement of pro-oxidant activity of phenolic compounds using Cu (II), the cupric ion concentration at 1 mM was generally preferred.[35,40]

Optimization of Incubation Periods. There were two incubation steps, explained in detail under the subtitle of "Recommended procedure for modified Cu(II)−Nc assay". To optimize the incubation periods, the time intervals between 5 to 60 min were tested for both investigations with the use of 0.25 mM ECAT, 2.5 mM AA, and 0.025 mM QUE. The obtained results are shown in Figure 3A and B. As can be seen, the combination of 30 min for the first incubation and 20 min for the second incubation was commonly found more convenient for the three antioxidant compounds tested.

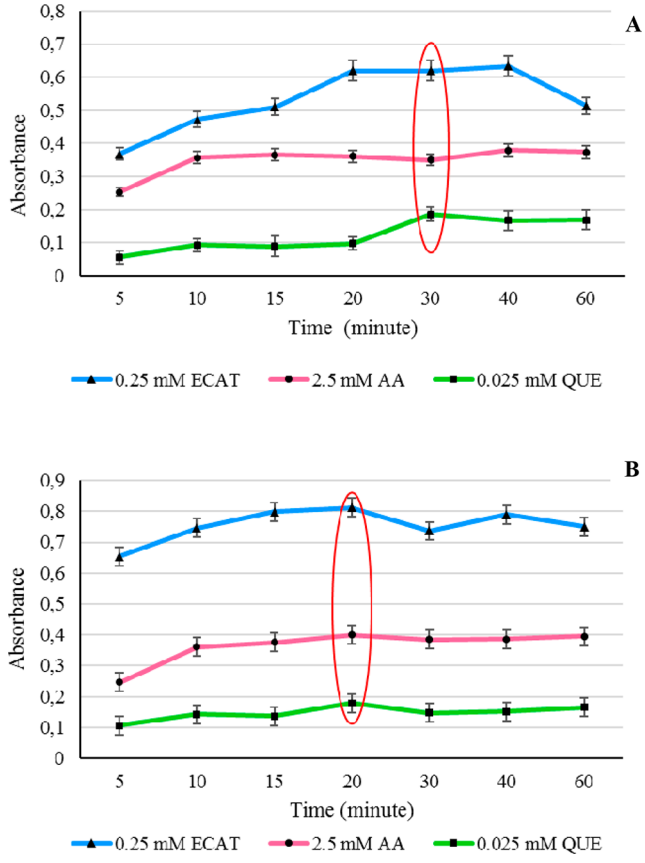

Figure 3. First (A) and second (B) incubation period optimization with 0.25 mM epicatechin (ECAT) (▲), 2.5 mM ascorbic acid (AA) (●), and 0.025 mM quercetin (QUE) (■) by the proposed assay (Second incubation period was 20 min in A; first incubation period was 30 min in B) (n = 3 for each experiment).

Analytical Performances of Applied Methods. Analytical performance data such as LOD and LOQ values as well as intra- and interday reproducibility (i.e., within- and between-run precision) of the proposed method for the chosen standard compound ECAT are given in Table 1. According to the

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

Table 1. Comparison of Figures of Merit of the Modified Cu(II)−Nc and Carbonyl Detection Assays Tested on ECAT Standard

parameter	modified Cu(II)−Nc	modified carbonyl detection assay
linear range (μM)	12.5−150	50.0−250
limit of detection (μM)[a]	1.2	3.0
limit of quantification (μM)[b]	4.0	10.0
calibration equation[c]	$A = 3472c + 0.142$	$A = 1394c + 0.270$
correlation coefficient (r)	0.9907	0.9944
within-run precision[d], R.SD (%)	2.84	4.26
between-run precision[d], R.SD (%)	3.18	5.05

[a]LOD = $3s_{bl}$ m^{-1} (where m is the slope of the calibration line, and s_{bl} is the standard deviation of a blank) [b]LOD = $10s_{bl}$ m^{-1} [c]Linear equation between absorbance (A) and concentration (c, M) [d]For 0.25 mM ECAT, $n = 3$

Table 2. Linear Regression Equations, Correlation Coefficients (r), and Linear Concentration Ranges of the Tested Compounds with Respect to the Modified Cu(II)−Nc and Carbonyl Detection Assays ($n = 3$)

	modified Cu(II)−Nc assay		modified carbonyl detection assay	
tested compound	linear regression equation and correlation coefficient (r)	linear range (μM)	linear regression equation and correlation coefficient (r)	linear range (μM)
ECAT	$A = 3472\ c + 0.142\ r = 0.9907$	12.5−150	$A = 1394\ c + 0.270\ r = 0.9944$	50.0−250
CAT	$A = 2295\ c + 0.201\ r = 0.9996$	25.0−250	$A = 2043\ c + 0.411\ r = 0.9577$	7.5−100
GA	$A = 1789\ c + 0.225\ r = 0.9996$	25.0−175	$A = 1447\ c + 0.313\ r = 0.9737$	25.0−250
CLA	$A = 1250\ c + 0.209\ r = 0.9996$	25.0−250	$A = 2067\ c + 0.498\ r = 0.9807$	2.5−175
AA	$A = 142\ c + 0.068\ r = 0.9998$	250−2500	$A = 1110\ c + 0.535\ r = 0.9193$	12.5−500
QUE	$A = 5080\ c + 0.035\ r = 0.9853$	2.5−22.5	$A = 29365\ c - 0.091\ r = 0.9515$	2.5−22.5
MYR	$A = 711\ c + 0.0041\ r = 0.9997$	25.0−175	$A = 371\ c + 0.230\ r = 0.9880$	750−3000

modified Cu(II)−Nc and carbonyl detection assays, correlation coefficients (r) and linear concentration ranges were calculated from calibration equations of phenolic compounds and AA solutions in distilled water, and of QUE and MYR in 50% EtOH/H_2O (v/v) mixture (Table 2). The correlation coefficients and linearity ranges of the tested compounds with respect to the modified Cu(II)−Nc and carbonyl detection assays were found to be quite different. This can be explained by the different mechanisms of the methods.

Aerated Cu(II) solutions may generate reactive species (such as superoxide anion and hydroxyl radicals) under certain conditions via the Haber−Weiss cycle.[41] These ROS may form in bulk solution (in an open system) as well as in the vicinity of protein−Cu(I) complexes (in a "cage"-like system) after reaction with antioxidants.[42] As a result, protein carbonyls may form under both types of ROS attack. Thus, antioxidant activity testing by decreased production of protein carbonyls is probably a result of overall ROS scavenging activity of antioxidant compounds, whereas the modified Cu(II)−Nc assay applied to the protein sensor is expected to measure only the "site-specific" damage precursor, i.e., protein-bound Cu(I).

In general, the modified Cu(II)−Nc assay showed higher indirect molar absorptivities for most antioxidants than the carbonyl assay, indicating higher analytical sensitivity. QUE, ECAT, and CAT were the strongest pro-oxidants while MYR and AA were the weakest in both assays (Table 2). As quercetin possesses all the requirements for effective antioxidant action, namely the *ortho*-dihydroxy (catechol) structure in the B-ring, the 2,3-double bond in conjugation with the 4-oxo function (enhancing electron-transfer and radical scavenging actions through electron-delocalization), and finally the presence of both 3- and 5-OH groups, enabling the formation of stable quinonic structures upon flavonoid oxidation, it is expected to show the highest antioxidant capacity in various antioxidant assays. However, in a pro-oxidant activity test based on Cu(II) → Cu(I) reduction followed by the generation of reactive species capable of protein damage, the strongest antioxidants like QUE are probably the most effective pro-oxidants,[4,28] as listed in Table 2. The order of molar absorption coefficients of test compounds with respect to the modified Cu(II)−Nc and the carbonyl assays were as follows: QUE > ECAT > CAT > GA > CLA > MYR > AA and QUE > CLA > CAT > GA > ECAT > AA > MYR, respectively. As in our previous study,[28] AA had the least pro-oxidant activity, while CAT and ECAT had similar effects with the modified Cu(II)−Nc assay using the solid biosensor. Rufián-Henares et al.[43] also measured the pro-oxidant activity based on Cu (II) reduction, with AA scoring the lowest value. However, contrary to our previous finding, the pro-oxidant activity of QUE was found much higher than those of CAT and ECAT in this study. This finding is compatible with the claim that flavones (flavonols in current literature, kaempherol, and QUE) might have much higher copper-initiated pro-oxidant activities than flavonones (eriodictyol and taxifolin) with the same number of hydroxyl substitutions.[4] An investigation involving antioxidant and pro-oxidant activities of phenolic compounds in the presence of copper ions showed that ROS was not detected without Cu^{2+} for the phenolic compounds alone by ESR spectroscopy and also these compounds alone did not increase 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in calf thymus DNA in the absence of Cu^{2+}. ECAT had stronger pro-oxidant effect than CAT and QUE according to the ESR measurements while all three compounds exhibited close changes in 8-OHdG levels in DNA. Most important of all, the report of Iwasaki et al.[40] stating that "*ortho*-dihydroxyl groups of polyphenols that can chelate with Cu(II) induced the greatest pro-oxidant activity" was in accordance with our findings in that the strongest pro-oxidant active phenolics of our work as QUE > ECAT > CAT > GA structurally had *ortho*-dihydroxy catechol groups in common. Comparing the data in Table 2, the slopes were very different especially for QUE and AA for the two assays. It is also noteworthy that in favor of the stronger linear response of the

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

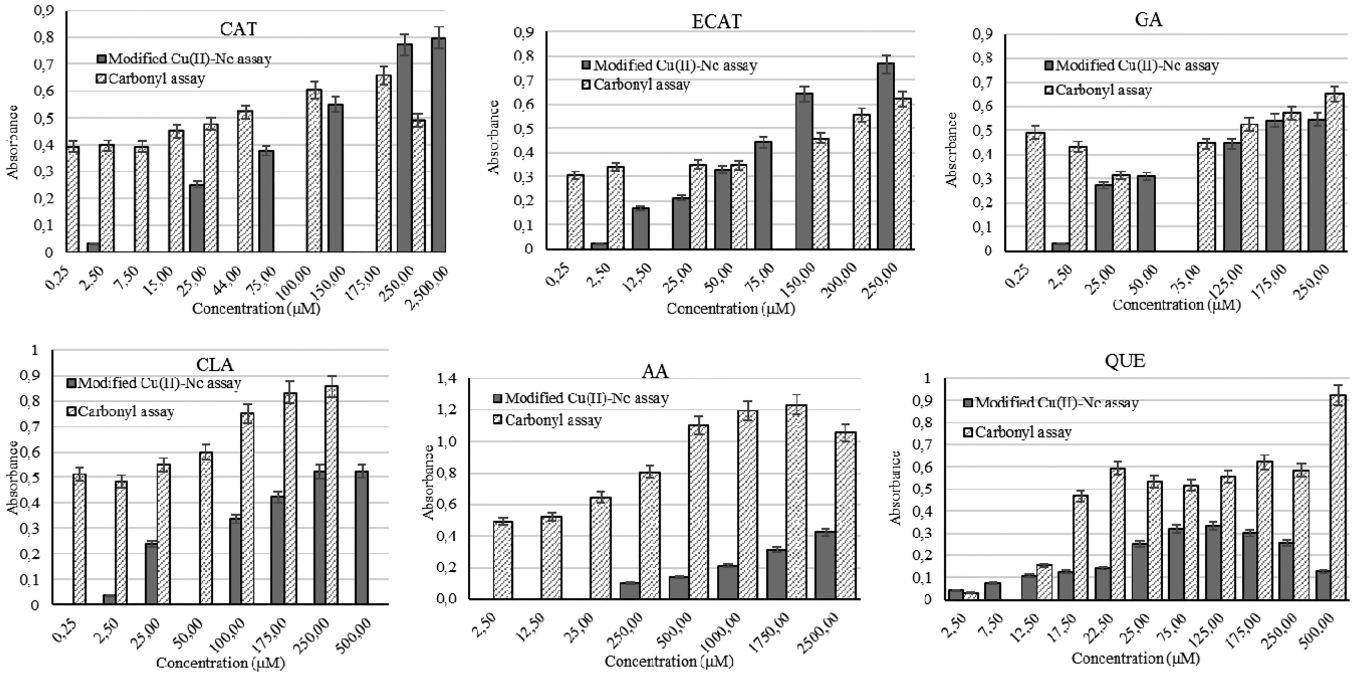

Figure 4. Relationship between absorbance and concentration of tested antioxidant compounds with respect to the modified Cu(II)−Nc and carbonyl assays. CAT, catechin; ECAT, epicatechin; GA, gallic acid; CLA, chlorogenic acid; AA, ascorbic acid; and QUE, quercetin.

Table 3. Statistical Comparison (at 95% Confidence Level) of Total Pro-Oxidant Activity (TPA) of ECAT Standard and Five-Fold Diluted Sage Extract as mg ECAT L^{-1} with Respect to the Modified Cu(II)−Nc and Carbonyl Detection Assays

sample	parameter	modified Cu(II)−Nc assay	modified carbonyl detection assay
ECAT standard	no. of samples	5	5
	average TPA	93.4[a]	104.2[a]
	standard deviation	1.57	2.07
	variance	2.48	4.28
	degrees of freedom	4	
	$F_{calculated}$	1.725	
	$F_{Critical}$	6.390	
sage extract	no. of samples	5	5
	average TPA	125.7[a]	163.0[a]
	standard deviation	0.74	1.08
	variance	0.55	1.17
	degrees of freedom	4	
	$F_{calculated}$	2.127	
	$F_{Critical}$	6.390	

[a](mg ECAT L^{-1} equiv).

proposed biosensor, the modified carbonyl detection assay had higher intercept values and lower correlation coefficients than the modified Cu(II)−Nc assay.

According to modified Cu(II)−Nc assay findings, ECAT, CAT, GA, CLA, QUE, and MYR had pro-oxidant activity above 2.5 μM while AA had pro-oxidant activity above 0.25 mM concentration (Figure 4). AA showed pro-oxidant effect in our proposed assay within the 0.25−2.5 mM concentration range. Considering the results of the carbonyl test, the absorbance values were almost constant within a wide concentration range and a significant increase could only be observed at very high concentrations. Comparative evaluation of data presented in Figure 4 revealed that the absorbances obtained by both methods (reflective of pro-oxidant ability) showed an increasing trend with antioxidant concentration, except for GA and QUE where the two methods displayed significant differences.

In the literature, various concentration values can be found for antioxidant compounds to exhibit anti- and pro-oxidant activities. For QUE, a critical concentration level of 2 μM was established for antioxidant ability, whereas 4.3 μM was set for pro-oxidant activity.[10] AA and GA were shown to have pro-oxidant activities at 0.82 mM and 0.6 mM concentrations, respectively.[2]

For statistical comparison of the modified Cu(II)−Nc and carbonyl detection assays, the total pro-oxidant activities of ECAT standard and 5-times diluted sage extract were calculated as ECAT equivalent using the ECAT calibration curve (Table 3). Although ECAT-equivalent TPA values were reported in this table, this may be easily converted to other units as antioxidant or pro-oxidant activity in terms of a reference

DOI: 10.1021/acs.jafc.7b01649
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Table 4. Experimental and Theoretical TPA Values of Synthetic Mixture Solutions of Tested Compounds (in the Units of mM ECAT Equiv)

	modified Cu(II)−Nc assay		modified carbonyl detection assay	
sample	experimental TPA	theoretical TPA	experimental TPA	theoretical TPA
mixture 1	0.48 ± 0.01	0.55	2.39 ± 0.03	1.38
mixture 2	0.17 ± 0.02	0.18	1.53 ± 0.03	4.17
mixture 3	0.15 ± 0.02	0.21	1.27 ± 0.03	4.49

compound can be given as the ratio of the slope of the calibration curve of the test compound to that of the reference compound drawn under identical conditions. The *F*-test results are also presented in Table 3. It was observed that there was no significant difference between the precisions of the two methods at the 95% confidence level.

Total Pro-oxidant Activity (TPA) of Synthetic Mixtures. Pro-oxidant activities of binary combinations of antioxidant solutions prepared as described under the "Preparation of synthetic mixtures" subtitle were found as mM ECAT equivalents. The results obtained from the modified Cu(II)−Nc and carbonyl assays are shown in Table 4. The theoretical ECAT equivalent pro-oxidant activities of the synthetic mixture solutions were calculated by summing up the individual ECAT equivalent concentrations obtained with the use of the absorbance values found by their linear regression equations.

The modified Cu(II)−Nc assay yielded experimental TPA values (for binary mixtures of tested antioxidant compounds) that were very close to the theoretically expected values, while carbonyl assay gave lower values than expected. In general, antagonistic effects were predominant in mixtures (Table 4). The standard potential of the Cu(II)/Cu(I) redox couple normally does not enable the oxidation of most phenolic antioxidants, but since the to-be-formed Cu(I) eventually binds to protein thiols with a high complexation constant, Cu(II,I) reduction potential is thermodynamically raised to the level required for the oxidation of phenolic compounds (i.e., due to selective stabilization of cuprous state in preference to cupric state). As Cu(II) reduction by antioxidants with a coupled reaction of Cu(I)-protein binding is basically an electron transfer (ET)-reaction, it may be antagonistically affected by hydrogen-bonding interactions among antioxidant compounds, such as hydrogen-bond formation between a partly oxidized semiquinone radical and an unoxidized phenolic (e.g., in the form of Ar—O•... HO—Ar) forming a bulky structure around protein-bound Cu(I) and slowing down the electron transfer process of the tested antioxidant mixture. This may explain the antagonistic behavior of antioxidant mixtures.

Total Pro-oxidant Activity (TPA) of Herbal Plant Extracts. Sage, green tea, mint, and marjoram plants were studied as real samples to test pro-oxidant activity with the developed sensor. Related literature reveals that major phenolic components of mentioned herbal infusions are as follows: in sage, vanilic acid, caffeic acid, rosmarinic acid, glycosides of luteolin, quercetin, and kaempherol;[44,45] in mint, caffeic acid, rosmarinic acid, eriodictyol, luteolin, and apigenin glycosides;[45] in green tea, catechin, epicatechin, epigallocatechin-3-gallate, glycosides of quercetin, kaempherol, and myricetin;[45,46] and in marjoram, protocatechuic acid, syringic acid, gallic acid, *p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, rosmarinic acid, catechin, epicatechin, kaempherol, and quercetin.[44] The infusions prepared as described in the sample preparation section of these plants were diluted at different ratios (between 1:2−1:100 (v/v)), and the modified Cu(II)−Nc and carbonyl assays were applied. The total pro-oxidant activities of herbal plant extracts with respect to these assays were expressed as mM ECAT equivalent (Figures 5A and 5B). As can be seen

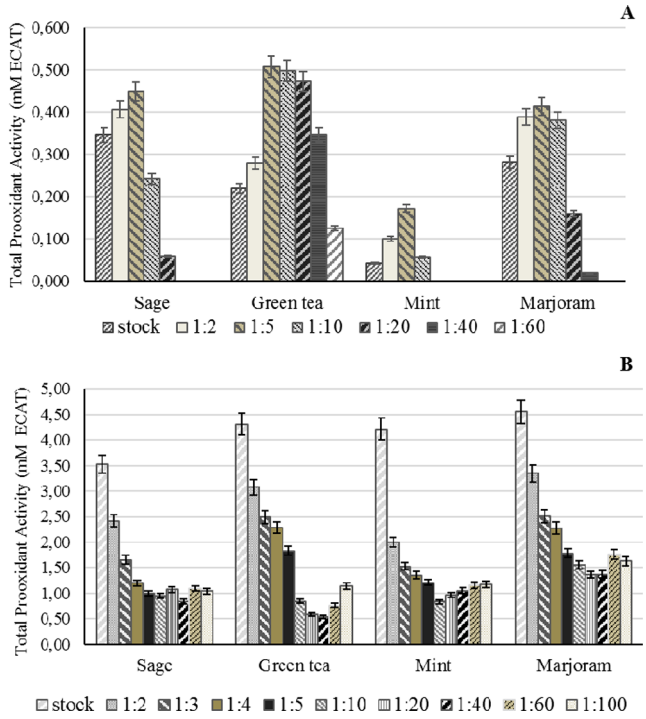

Figure 5. Total pro-oxidant activities (TPA) of herbal plant extracts (diluted at different ratios) with respect to the modified Cu(II)−Nc (A) and carbonyl assays (B).

from Figure 5A, TPA values showed an increasing trend at low dilution ratios, whereas a decreasing trend at high dilution ratios. This tendency suggested that the antioxidant properties of plant extracts might be strengthening again at higher concentrations, so the pro-oxidant activities of polyphenolic compounds should be limited to a restricted concentration region depending on the studied herb. On the contrary, modified carbonyl assay results did not support this hypothesis (Figure 5B), because TPA values of herbal extracts exhibited a decreasing trend with increasing dilution ratios, and practically did not change above a given ratio depending on the type of herb. This case, quite similar to the situation observed in Figure 4, suggested that the modified carbonyl assay may have a disadvantage as being unsuitable for wide concentration ranges of polyphenolics and/or herbal extracts containing them. The possible influence of dilution (or more generally antioxidant concentration) on the measured antioxidant activity was previously discussed by Hengst et al.[47] who reached multiple conclusions (rather than a single conclusion) because this effect was greatly dependent on the nature of the assay and

DOI: 10.1021/acs.jafc.7b01649
J. Agric. Food Chem. 2017, 65, 5821−5830

Table 5. ECAT Recoveries from Herbal Infusions Using the Modified Cu(II)−Nc Assay

herbal plant	added (mM)	expected (mM)	found (mM)	recovery %	RSD %
1:10 diluted sage			0.16 ± 0.01		2
	0.1	0.26	0.27 ± 0.01	104	2
	0.2	0.36	0.40 ± 0.01	111	2
1:20 diluted green tea			0.44 ± 0.01		1
	0.1	0.54	0.52 ± 0.02	96	3
	0.2	0.64	0.61 ± 0.01	95	2
1:10 diluted mint			0.24 ± 0.01		2
	0.1	0.34	0.34 ± 0.01	100	2
	0.2	0.44	0.46 ± 0.01	104	2
1:20 diluted marjoram			0.53 ± 0.01		1
	0.1	0.63	0.65 ± 0.02	103	2
	0.2	0.73	0.75 ± 0.02	103	2

antioxidant, as well as on reaction conditions. Antioxidant assays generally have more clear mechanisms than pro-oxidant assays, and this matter has not been fully resolved even for antioxidant assays.

Evaluating the data in Figure 5, TPA values of mentioned herbal extracts were calculated as 13.80 mM ECAT with modified Cu(II)−Nc assay (9.15 mM ECAT with modified carbonyl assays) for green tea, 2.42 (4.84) for sage, 3.20 (6.70) for marjoram, and 0.45 (4.00) for mint, respectively. These ECAT equivalent values were calculated within the linear concentration range of test compounds by taking the dilution ratios into account. Evaluation of recovery values for the modified Cu(II)−Nc assay were performed by spiking the diluted herbal plant extracts with two known amounts (0.1 and 0.2 mM) of ECAT standard solution (Table 5). The tabulated data were expressed as added, expected, and found mM ECAT equivalents. The obtained recovery values (considering the dilution ratios) close to 100% demonstrated that the modified Cu(II)−Nc assay was suitable to determine TPA values of plant extracts containing polyphenolic compounds (Table 5).

In fact, it is quite difficult to make a general assessment of anti- or pro-oxidant behavior of polyphenols, because the ability of polyphenols to act as anti- or pro-oxidants under in vitro and in vivo systems is dependent on a number of factors such as the concentration and structure of the polyphenol, the test system used and the substrate to be protected. Related to this, Maurya and Devasagayam[11] reported that ferulic and caffeic acids might have concentration−dependent antioxidant and pro-oxidant activities; these two compounds displayed hydroxyl radical scavenging effect up to 5 μM concentration, beyond which they started behaving as pro-oxidants. The stronger pro-oxidant ability of caffeic acid compared to that of ferulic acid was explained by the higher iron(III) reducing property of the former. Likewise, potent antioxidants such as gallic acid[48] and epigallocatechin gallate[49] displayed strong pro-oxidant activity in the deoxyribose degradation assay, due to their ability to reduce Fe(III) to Fe(II). Hagerman et al.[50] reported that small-molecule polyphenols such as gallic acid and quercetin, as opposed to high molecular-weight phenolics such as hydrolyzable and condensed tannins showing little or no activity, could exhibit significant pro-oxidant ability by virtue of their easier oxidation. The pro-oxidant activity of plant phenolics was primarily associated with their ability to reduce Fe(III) and Cu(II) to the corresponding lower oxidation states.[51,52] Cirico and Omaye[10] examined the antioxidant and pro-oxidant behavior of catechin, hesperidin, ferulic acid, and quercetin individually and in mixtures, where catechin and hesperidin showed predominantly antioxidant effect on LDL oxidation depending on enrichment concentrations while ferulic acid and quercetin showed pro-oxidant behavior. Furthermore, antioxidant mixtures containing the test compounds did not behave in an additive manner, exhibiting significant antioxidant capacity at all enrichment levels. Strlic et al.[48] reported that small gallic acid-to-iron ratios gave rise to distinctly pro-oxidative effects (probably due to iron chelation and reduction, followed by promotion of $^{\bullet}$OH production), whereas the overall result of higher gallic acid-to-iron ratios (i.e., greater than 2) was predominantly antioxidative behavior. In another study, it was observed that three pure phenols, *p*-coumaric acid, epicatechin, and gallic acid, exhibited different behaviors in Fenton oxidation reactions. Pro-oxidative effects were not observed with *p*-coumaric acid that had the highest redox potential (0.66 V) of the three phenols tested, whereas both epicatechin (0.33 V) and gallic acid (0.56 V) showed pro-oxidative behavior, thought to be associated with iron reduction.[53] All these examples reveal the close relationship between pro-oxidative behavior and transition metal ion reducing ability of phenolic compounds, constituting the major standpoint of this work.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

NH_4Ac, ammonium acetate; AA, ascorbic acid; CAT, catechin; CLA, chlorogenic acid; CYS, cysteine; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazine; ECAT, epicatechin; ESR, electron spin resonance; EtOH, ethanol; GA, gallic acid; GF/PET, glass fiber/polyethylene terephthalate; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; MeOH, methanol; MYR, myricetin; Nc, neocuproine; Ar—O$^{\bullet}$, phenoxyl; QUE, quercetin; ROS, reactive oxygen species; SD, standard deviation; TPA, total pro-oxidant activity; TCA, trichloroacetic acid

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