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Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements

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A cellular antioxidant activity (CAA) assay for quantifying the antioxidant activity of phytochemicals, food extracts, and dietary supplements has been developed. Dichlorofluorescin is a probe that is trapped within cells and is easily oxidized to fluorescent dichlorofluorescein (DCF). The method measures the ability of compounds to prevent the formation of DCF by 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP)-generated peroxyl radicals in human hepatocarcinoma HepG2 cells. The decrease in cellular fluorescence when compared to the control cells indicates the antioxidant capacity of the compounds. The antioxidant activities of selected phytochemicals and fruit extracts were evaluated using the CAA assay, and the results were expressed in micromoles of quercetin equivalents per 100 μmol of phytochemical or micromoles of quercetin equivalents per 100 g of fresh fruit. Quercetin had the highest CAA value, followed by kaempferol, epigallocatechin gallate (EGCG), myricetin, and luteolin among the pure compounds tested. Among the selected fruits tested, blueberry had the highest CAA value, followed by cranberry > apple = red grape > green grape. The CAA assay is a more biologically relevant method than the popular chemistry antioxidant activity assays because it accounts for some aspects of uptake, metabolism, and location of antioxidant compounds within cells.

KEYWORDS: Antioxidant; antioxidant activity; antioxidant activity assay; dietary supplements; free radicals; fruits; phenolics; phytochemicals; vegetables

INTRODUCTION

Heart disease and cancer are the two leading causes of death in the United States (*1*), and oxidative stress is thought to be an important contributing factor in their development. Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense and may lead to oxidative damage (*2, 3*). It can result from a deficiency in antioxidant defense mechanisms, or from an increase in ROS, due to exposure to elevated ROS levels, the presence of toxins metabolized to ROS, or excessive activation of ROS systems, such as those mediated by chronic infection and inflammation (*4*). In addition to endogenously produced antioxidants and enzymes that catalyze the metabolism of ROS, ROS can be scavenged by exogenously obtained antioxidants, such as phenolics, carotenoids, and vitamins found in fruits and vegetables. Fruits and vegetables are excellent sources of phenolic compounds (*5, 6*). Consumption of these compounds from dietary plant sources may increase protective antioxidants in the body and help combat cardiovascular diseases and cancer, as supported by epidemiological studies (*7–13*). The 2005 Dietary Guidelines for Americans recommends consumption of at least four servings of fruits and five servings of vegetables per day based on a dietary requirement of 2000 kcal (*14*).

The measurement of antioxidant activity is an important screening method to compare the oxidation/reduction potentials of fruits and vegetables and their phytochemicals in various systems. Many chemistry methods are currently in wide use, including the oxygen radical absorbance capacity (ORAC) (*15*), total radical-trapping antioxidant parameter (TRAP) (*16, 17*), Trolox equivalent antioxidant capacity (TEAC) (*18*), total oxyradical scavenging capacity (TOSC) (*19*), and the peroxyl radical scavenging capacity (PSC) assays, the latter of which was recently developed by our laboratory (*20*). The ferric reducing/antioxidant power (FRAP) assay (*21*) and the DPPH free radical method (*22*) measure the ability of antioxidants to reduce ferric iron and 2,2-diphenylpicrylhydrazyl, respectively.

Despite wide usage of these chemical antioxidant activity assays, their ability to predict in vivo activity is questioned for a number of reasons. Some are performed at nonphysiological pH and temperature, and none of them take into account the

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bioavailability, uptake, and metabolism of the antioxidant compounds (*23*). The protocols often do not include the appropriate biological substrates to be protected, relevant types of oxidants encountered, or the partitioning of compounds between the water and lipid phases and the influence of interfacial behavior (*24*). Biological systems are much more complex than the simple chemical mixtures employed, and antioxidant compounds may operate via multiple mechanisms (*25*). The different efficacies of compounds in the various assays attest to the functional variation. The best measures are from animal models and human studies; however, these are expensive and time-consuming and not suitable for initial antioxidant screening of foods and dietary supplements (*23*). Cell culture models provide an approach that is cost-effective, relatively fast, and address some issues of uptake, distribution, and metabolism. Therefore, there is an urgent need to develop a cell-based antioxidant activity assay to screen foods, phytochemicals, and dietary supplements for potential biological activity.

The objective of this research was to develop a quantifiable cellular antioxidant activity (CAA) assay, which would serve as more suitable method to measure antioxidant activity than the currently used "test tube" chemistry methods. We believe that this model better represents the complexity of biological systems than the popular chemistry antioxidant activity assays and is an important tool for screening foods, phytochemicals, and dietary supplements for potential biological activity.

MATERIALS AND METHODS

Chemicals. Folin–Ciocalteu reagent, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), ethanol, glutaraldehyde, methylene blue, ascorbic acid, caffeic acid, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin gallate (EGCG), ferulic acid, kaempferol, luteolin, myricetin, phloretin, quercetin dihydrate, resveratrol, and taxifolin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Gallic acid was obtained from ICN Biomedicals, Inc. (Aurora, OH). Dimethyl sulfoxide and acetic acid were obtained from Fisher Scientific (Pittsburgh, PA), and 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Sodium carbonate, acetone, and methanol were obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). The HepG2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Fruit Samples. Wild blueberries were obtained from the Wild Blueberry Association of North America (Orono, ME). Red Delicious apples were obtained from Cornell Orchards (Ithaca, NY). Green and red seedless table grapes and frozen cranberries were purchased at a local supermarket (Ithaca, NY).

Fruit Extractions. Extracts were obtained from the fruits using 80% acetone, as described previously (*6*).

Determination of Total Phenolic Content. The total phenolic contents of the fruit extracts were determined using the Folin–Ciocalteu colorimetric method (*26*), as modified by our laboratory (*27, 28*). Results were expressed as mean micromoles of gallic acid equivalents (GAE) per 100 g of fresh fruit ± SD for three replicates.

Preparation of Chemical and Fruit Sample Solutions. A 20 mM stock solution of DCFH-DA in methanol was prepared, aliquoted, and stored at −20 °C. A 200 mM ABAP stock solution was prepared, and aliquots were stored at −40 °C. Working phytochemical and fruit extract solutions were prepared just prior to use. Caffeic acid, (+)-catechin, EGCG, (−)-epicatechin, ferulic acid, gallic acid, kaempferol, myricetin, phloretin, resveratrol, and taxifolin were dissolved in ethanol, luteolin was dissolved in methanol, and quercetin was dissolved in dimethyl sulfoxide before further dilution in treatment medium (WME with 2 mM L-glutamine and 10 mM Hepes). Fruit extracts were diluted in treatment medium. Final treatment solutions contained <2% solvent, and there was no cytotoxicity to HepG2 cells at those concentrations.

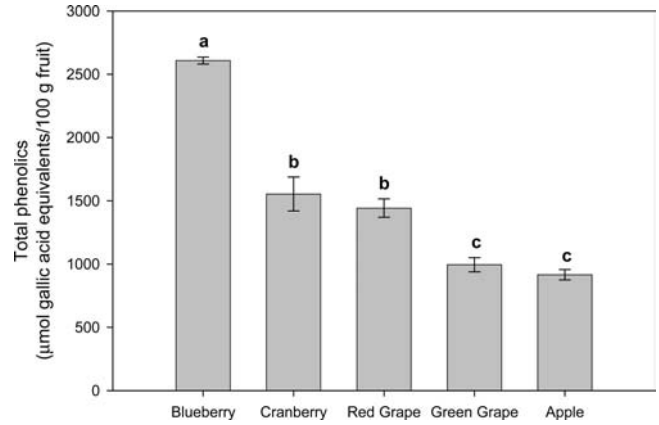

Figure 1. Total phenolic contents of selected fruits (mean ± SD, $n = 3$). Bars with different letters are significantly different ($p < 0.05$).

Cell Culture. HepG2 cells were grown in growth medium (WME supplemented with 5% FBS, 10 mM Hepes, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin) and were maintained at 37 °C and 5% CO_2 as described previously (*29, 30*). Cells used in this study were between passages 12 and 35.

Cytotoxicity. Cytotoxicity was measured using the method of Oliver et al. (*31*) with modifications by our laboratory (*32*). HepG2 cells were seeded at 4×10^4/well on a 96-well plate in 100 μL of growth medium and incubated for 24 h at 37 °C. The medium was removed, and the cells were washed with PBS. Treatments of fruit extracts or antioxidant compounds in 100 μL of treatment medium (WME supplemented with 2 mM L-glutamine and 10 mM Hepes) were applied to the cells, and the plates were incubated at 37 °C for 24 h. The treatment medium was removed, and the cells were washed with PBS. A volume of 50 μL/well methylene blue staining solution (98% HBSS, 0.67% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37 °C for 1 h. The dye was removed, and the plate was immersed in fresh deionized water three times, or until the water was clear. The water was tapped out of the wells, and the plate was allowed to air-dry briefly before 100 μL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a bench-top shaker for 20 min to allow uniform elution. The absorbance was read at 570 nm with blank subtraction using the MRX II DYNEX spectrophotometer (DYNEX Inc., Chantilly, VA). Concentrations of pure compounds or fruit extracts that decreased the absorbance by >10% when compared to the control were considered to be cytotoxic.

CAA of Pure Phytochemicals and Fruit Extracts (Figure 2). Human hepatocellular carcinoma HepG2 cells were seeded at a density of 6×10^4/well on a 96-well microplate in 100 μL of growth medium/well. The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Twenty-four hours after seeding, the growth medium was removed and the wells were washed with PBS. Triplicate wells were treated for 1 h with 100 μL of pure phytochemical compounds or fruit extracts plus 25 μM DCFH-DA dissolved in treatment medium. When a PBS wash was utilized, wells were then washed with 100 μL of PBS. Then 600 μM ABAP was applied to the cells in 100 μL of HBSS, and the 96-well microplate was placed into a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 37 °C. Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 h. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with dye and HBSS without oxidant.

Quantification of CAA. After blank subtraction from the fluorescence readings, the area under the curve of fluorescence versus

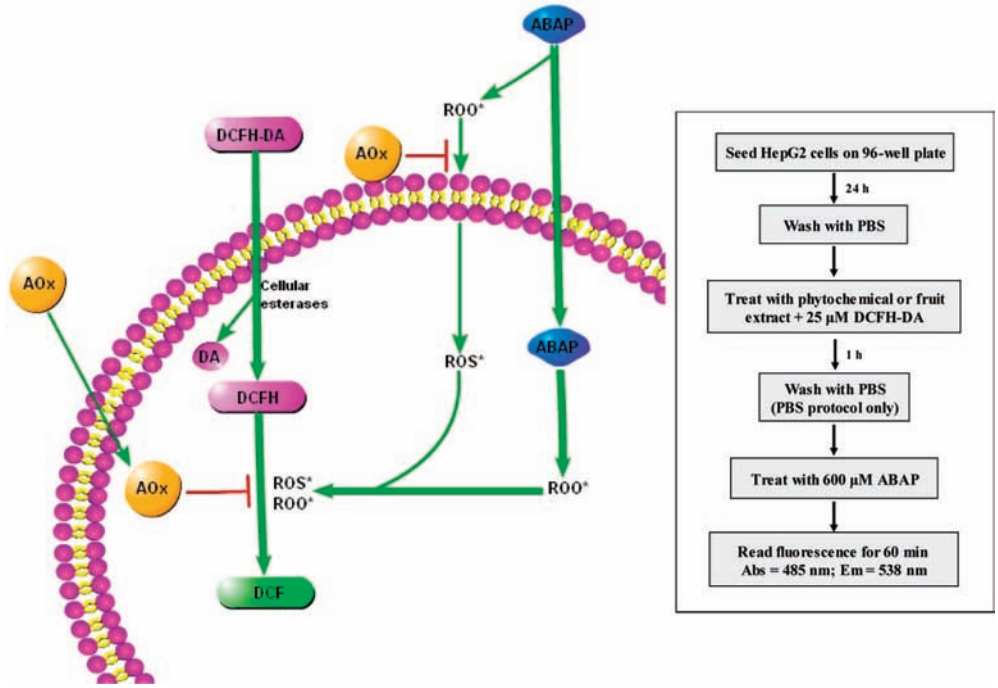

Figure 2. Method and proposed principle of the cellular antioxidant activity (CAA) assay. Cells were pretreated with antioxidant compounds or fruit extracts and DCFH-DA. The antioxidants bound to the cell membrane and/or passed through the membrane to enter the cell. DCFH-DA diffused into the cell where cellular esterases cleaved the diacetate moiety to form the more polar DCFH, which was trapped within the cell. Cells were treated with ABAP, which was able to diffuse into cells. ABAP spontaneously decomposed to form peroxyl radicals. These peroxyl radicals attacked the cell membrane to produce more radicals and oxidized the intracellular DCFH to the fluorescent DCF. Antioxidants prevented oxidation of DCFH and membrane lipids and reduced the formation of DCF.

time was integrated to calculate the CAA value at each concentration of pure phytochemical compound or fruit extract as follows:

$$\text{CAA unit} = 100 - \left(\int \text{SA} / \int \text{CA}\right) \times 100$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve and $\int$CA is the integrated area from the control curve. The median effective dose (EC_{50}) was determined for the pure phytochemical compounds and fruit extracts from the median effect plot of log (f_a/f_u) versus log (dose), where f_a is the fraction affected and f_u is the fraction unaffected by the treatment. To quantify intraexperimental variation, the EC_{50} values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. Interexperimental variation was obtained for some representative pure phytochemical compounds and fruit extracts by averaging the fluorescence values from triplicate wells in each trial to obtain one EC_{50} value per experiment and calculating the mean ± SD for at least four trials. In each experiment, quercetin was used as a standard, and cellular antioxidant activities for pure phytochemical compounds were expressed as micromoles of quercetin equivalents (QE) per 100 μmol of compound, whereas for fruit extracts they were expressed as micromoles of QE per 100 g of fruit. To compare the antioxidant quality of different fruits, CAA was also calculated as micromoles of QE per 100 μmol of total phenolics.

Statistical Analyses. All results were presented as mean ± SD. Comparisons between two means were performed using unpaired Student's *t* tests. When there were more than two means, differences were detected by ANOVA followed by multiple comparisons using Fisher's least significant difference test. Differences were considered to be significant when $p < 0.05$.

RESULTS

Total Phenolic Contents of Fruit Extracts. To characterize the fruit extracts used in the cellular antioxidant activity assay, the total phenolic contents of the fruits were quantified (**Figure 1**). Blueberry contained the most phenolics with 2609 ± 28 μmol of GAE/100 g of fresh fruit, followed by cranberry (1554 ± 134 μmol of GAE/100 g), red grape (1443 ± 72 μmol of GAE/100 g), green grape (994 ± 56 μmol of GAE/100 g), and apple (916 ± 41 μmol of GAE/100 g).

CAA. The proposed principle of the CAA assay is shown in **Figure 2**. On the basis of the optimization trials (data not shown), a concentration of 25 μM DCFH-DA was used because lower levels did not yield consistent fluorescence measurements and higher concentrations decreased the sensitivity of the assay. ABAP caused oxidation of DCFH-DA in a dose–response manner up to a dose of 2 mM (data not shown). The treatment level of 600 μM was chosen because it yielded adequate fluorescence readings while inducing a reasonable level of oxidation that could be inhibited by many phytochemicals and fruit extracts. The kinetics of DCFH oxidation in HepG2 cells by peroxyl radicals generated from ABAP is shown in **Figure 3**. The increase in fluorescence from DCF formation was inhibited by pure phytochemical compounds and fruit extracts in a dose-dependent manner, as demonstrated by the curves generated from cells treated with quercetin (**Figure 3A**,**B**), gallic acid (**Figure 3C**,**D**), and blueberry extracts (**Figure 3E**,**F**). Inhibition of oxidation was seen when no PBS wash was done between antioxidant and ABAP treatments (**Figure 3A**,**C**,**E**) and when a PBS wash was performed (**Figure 3B**,**D**,**F**).

To calculate the EC_{50}, the dose–response curve from the ratio of the area under the curve of the sample to that of the control and the median effect curve were plotted for each sample. The dose–response curves and median effect plots generated from the data presented from quercetin and blueberry extracts in **Figure 3** are shown in **Figures 4** and **5**, respectively. The EC_{50} is the concentration at which $f_a/f_u = 1$ (i.e., CAA unit = 50), as calculated from the linear regression of the median effect curve.

The EC_{50} values of CAA for pure phytochemical compounds and fruit extracts are listed in **Table 1** along with their cytotoxic

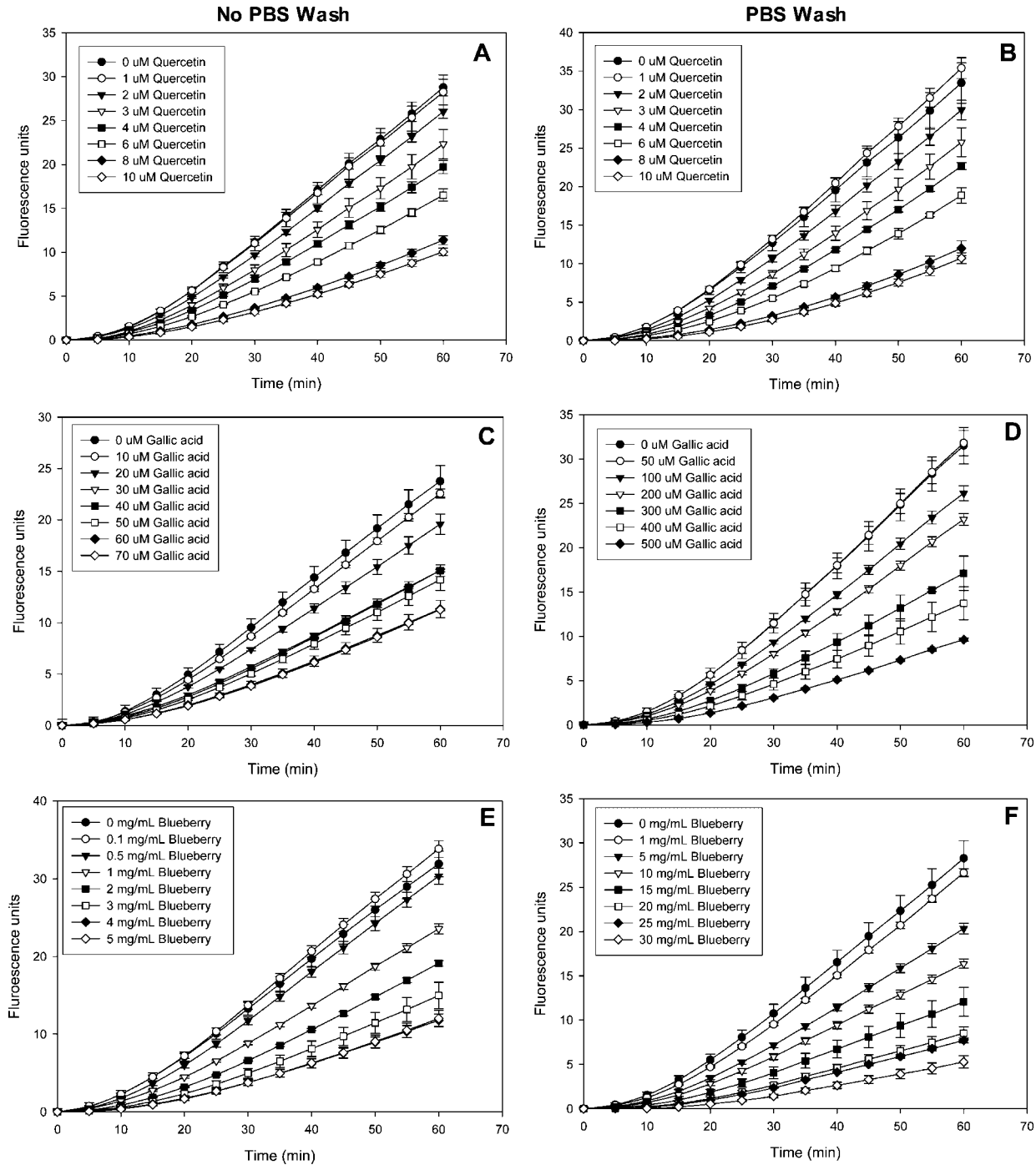

Figure 3. Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by quercetin (**A**, **B**), gallic acid (**C**, **D**), and blueberry extracts (**E**, **F**) over time, using the protocol involving no PBS wash between antioxidant and ABAP treatments (**A**, **C**, **E**) and the protocol with a PBS wash (**B**, **D**, **F**), to remove antioxidants in the medium not associated with cells. The curves shown in each graph are from a single experiment (mean ± SD, $n = 3$).

concentrations. The values presented are from triplicate samples in the same experiment, and the coefficient of variation (CV) represents intraexperimental variation. When more than one experiment was performed for the sample, representative results from one trial were presented. In the protocol involving no PBS wash between antioxidant and ABAP treatments (no PBS wash) for the pure phytochemical compounds, quercetin was the most efficacious antioxidant, followed by kaempferol, EGCG, myricetin, luteolin, gallic acid, ascorbic acid, caffeic acid, and catechin (**Table 1**). Epicatechin and ferulic acid had low activity within the doses tested, and their EC_{50} values could not be calculated. Phloretin, resveratrol, and taxifolin had activity only at doses much higher than their cytotoxic concentrations.

For those experiments including a PBS wash between treatments, the order of efficacies was similar to that obtained from the no PBS wash protocol, except that the CAA values from ascorbic acid and catechin were low and the EC_{50} values of CAA could not be calculated (**Table 1**). Quercetin, kaempferol, and luteolin had slightly higher EC_{50} concentrations when a PBS wash was done between treatments ($p < 0.05$). Myricetin had similar EC_{50} values in each of the two protocols ($p > 0.05$), as did EGCG ($p > 0.05$). Gallic acid

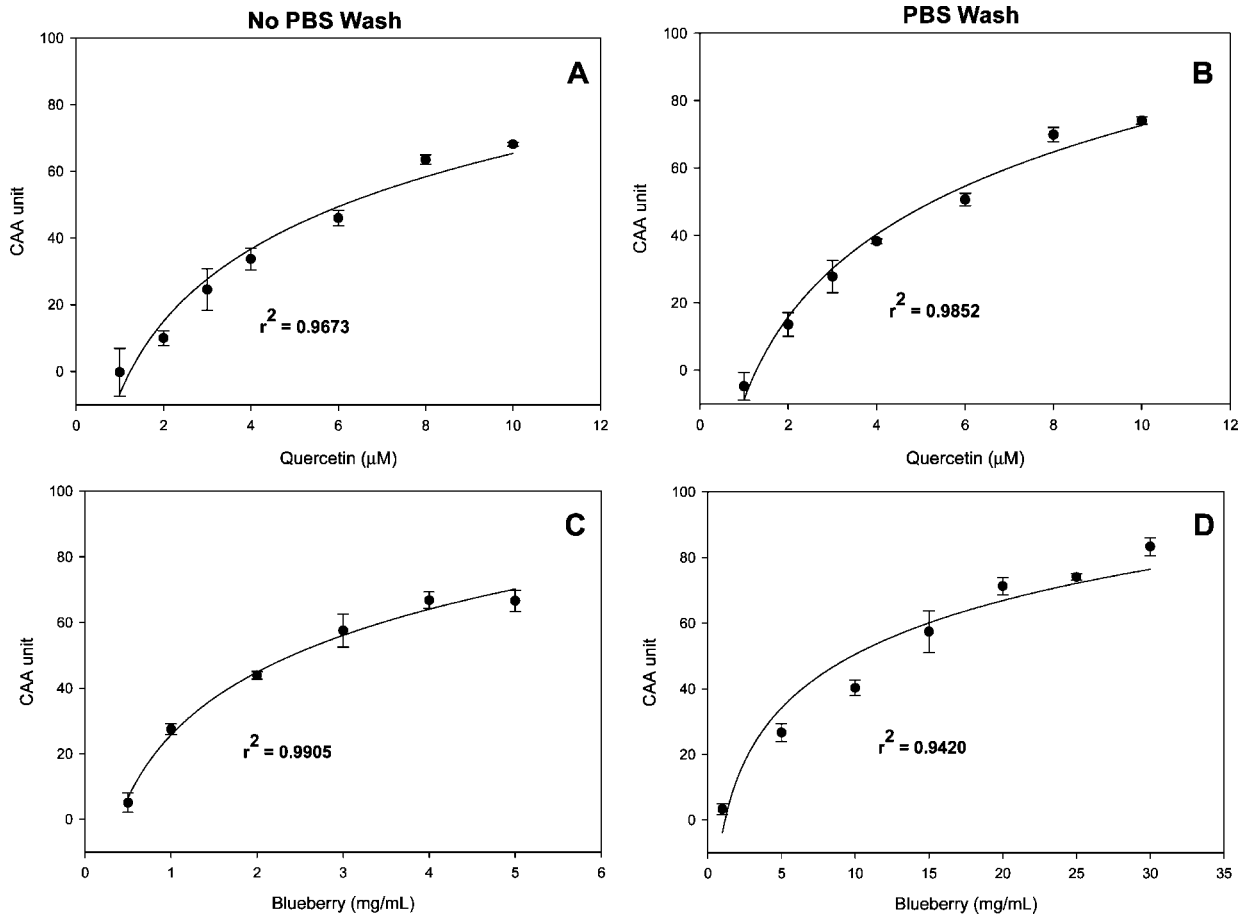

Figure 4. Dose–response curves for inhibition of peroxyl radical-induced DCFH oxidation by quercetin (**A**, **B**) and blueberry extracts (**C**, **D**) without a PBS wash between treatments in the protocol involving no PBS wash between antioxidant and ABAP treatments (**A**, **C**) and the protocol with a PBS wash (**B**, **D**). The curves shown are each from a single experiment (mean ± SD, $n = 3$).

and caffeic acid had much lower activity when a PBS wash was performed compared to that in the protocol without a PBS wash ($p < 0.05$). The intraexperimental coefficient of variation (CV) for the pure compounds was under 10% when a PBS wash was utilized and modestly higher when a PBS wash was not done (**Table 1**).

The EC_{50} values of CAA for the fruit extracts are presented in **Table 1**. Blueberry was the most effective at inhibiting peroxyl radical-induced DCFH oxidation, followed by cranberry, apple, red grape, and green grape. The order of efficacy was the same with or without a PBS wash between fruit extracts and ABAP treatments. The fruit extracts all had lower EC_{50} values in the no PBS protocol than in the PBS wash protocol ($p < 0.05$). The intraexperimental CV ranged from 2.59 to 16.0%, with the majority of trials yielding a CV of <10% (**Table 1**).

The relationship between EC_{50} values and total phenolic contents of fruit extracts was examined. When no PBS wash was employed between treatments, the EC_{50} values for CAA were not significantly correlated to total phenolic contents in fruits ($R^2 = 0.450$; $p = 0.215$). EC_{50} values were weakly correlated to total phenolic contents ($R^2 = 0.830$; $p = 0.032$) in fruits when a PBS wash was done.

The reproducibility of EC_{50} values of CAA from similar experiments performed on different days (interexperimental variation) was evaluated for representative compounds tested using the protocols with and without a PBS wash (**Tables 2** and **3**). For pure compounds with no PBS wash performed between treatments, the CV values of interexperimental variation for quercetin and gallic acid were 6 and 17%, respectively (**Table 2**). The CV of the protocol without a PBS wash interexperimental variation for blueberry was 22% (**Table 2**). When a PBS wash was done between antioxidant and oxidant treatments, the CV values for quercetin, gallic acid, and blueberry extracts were 11.3, 11.4, and 7.56%, respectively (**Table 3**).

The EC_{50} values were converted to CAA values, expressed as micromoles of QE per 100 μmol of compound for pure antioxidant compounds (**Figure 6**) and micromoles of QE per 100 g of fresh fruit for fruit extracts (**Figure 7**). When no PBS wash was done between antioxidant and ABAP treatments, quercetin had the highest CAA value ($p < 0.05$), followed by kaempferol (75.3 ± 4.7 μmol of QE/100 μmol), EGCG (42.2 ± 3.1), myricetin (36.8 ± 3.8), and luteolin (22.6 ± 0.2), which were all significantly different ($p < 0.05$). The CAA values for gallic acid, ascorbic acid, and caffeic acid were not significantly different (9.08 ± 0.95, 8.84 ± 1.18, and 5.59 ± 0.70, respectively) ($p > 0.05$), and catechin's CAA value was similar to caffeic acid's at 2.03 ± 0.24 μmol of QE/100 μmol ($p > 0.05$).

When the HepG2 cells were washed with PBS between treatments, the order of activity was nearly the same: quercetin > kaempferol (81.1 ± 2.7 μmol of QE/100 μmol) > myricetin (33.1 ± 1.0) = EGCG (32.3 ± 0.9) > luteolin (22.2 ± 1.0) > gallic acid (1.53 ± 0.12) = caffeic acid (0.997 ± 0.074) at a significance level of $p < 0.05$.

For the fruit extracts in the group without a PBS wash, blueberry had the highest CAA value (171 ± 12 μmol of QE/

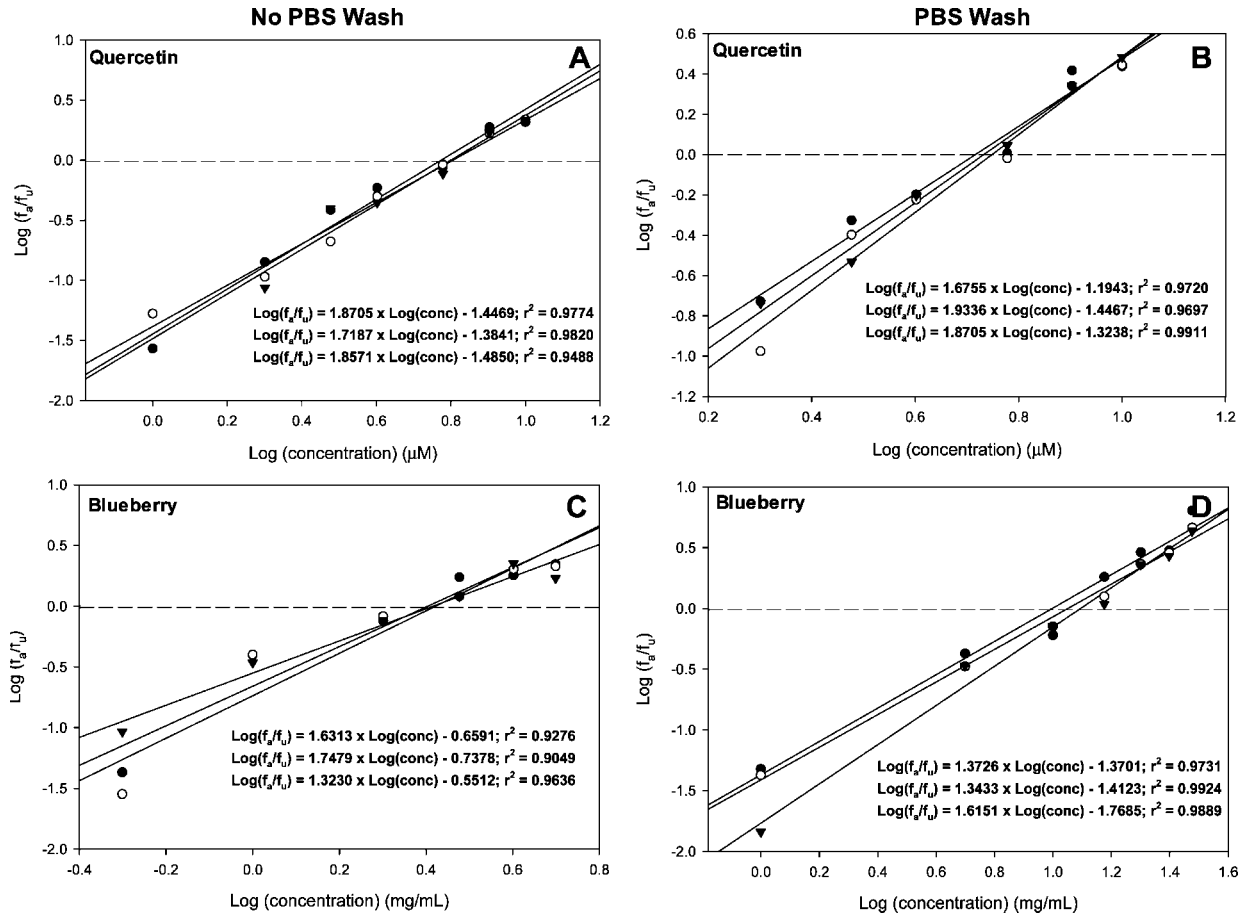

Figure 5. Median effect plots for inhibition of peroxyl radical-induced DCFH oxidation by quercetin (**A**, **B**) and blueberry extracts (**C**, **D**) in the protocol involving no PBS wash between antioxidant and ABAP treatments (**A**, **C**) and the protocol with a PBS wash (**B**, **D**). The curves shown are from a single experiment ($n = 3$).

Table 1. EC_{50} Values for the Inhibition of Peroxyl Radical-Induced DCFH Oxidation by Selected Pure Phytochemical Compounds and Fruits (Mean ± SD, $n = 3$) and Their Cytotoxic Concentrations

	no PBS wash		PBS wash		
compound	EC_{50} (μM)	CV (%)	EC_{50} (μM)	CV (%)	cytotoxicity[a] (μM)
quercetin[b]	5.92 ± 0.07	1.18	5.09 ± 0.19	3.65	>20
kaempferol[b]	7.85 ± 0.51	6.53	6.31 ± 0.21	3.34	30
EGCG	14.0 ± 1.0	7.39	15.8 ± 0.4	2.81	>100
myricetin	16.1 ± 1.70	10.6	15.4 ± 0.5	2.96	200
luteolin[b]	26.1 ± 0.26	1.01	23.1 ± 1.0	4.48	20
gallic acid[b]	65.4 ± 7.3	11.1	335 ± 26	7.81	>500
ascorbic acid	67.5 ± 9.4	14.0	>500		>500
caffeic acid[b]	95.3 ± 15.3	16.1	525 ± 38	7.25	>500
catechin	292 ± 32	11.0	>500		>500
epicatechin	>200		>600		>500
ferulic acid	>250		>500		>500
phloretin	>25		>25		25
resveratrol	>40		>40		40
taxifolin	>150		>150		150

	no PBS wash		PBS wash		
fruit	EC_{50} (mg/mL)	CV (%)	EC_{50} (mg/mL)	CV (%)	cytotoxicity[a] (mg/mL)
blueberry[b]	3.440 ± 0.239	6.94	10.81 ± 0.44	4.09	60
cranberry[b]	11.31 ± 0.29	2.59	36.17 ± 1.20	3.31	60
apple[b]	21.31 ± 3.34	16.0	38.60 ± 3.26	8.45	>100
red grape[b]	24.49 ± 1.73	7.05	42.33 ± 2.22	5.23	>100
green grape[b]	62.89 ± 3.19	5.07	53.01 ± 3.12	5.89	>100

[a] Dose at which the cell number is reduced by >10% after 24 h of treatment. [b] EC_{50} values for no PBS wash and PBS wash are significantly different ($p < 0.05$).

100 g) ($p < 0.05$). The remaining fruits had activity in the order of cranberry (52.1 ± 1.3) > apple (28.1 ± 4.1) = red grape (24.1 ± 1.7) > green grape (9.39 ± 0.49) at a significance level of $p < 0.05$. Again, in the PBS wash protocol, blueberry had

Table 2. Variation among EC_{50} Values for Representative Pure Phytochemical Compounds and Fruit Extracts Obtained When No PBS Wash Was Performed between Antioxidant and ABAP Treatments (Mean ± SD)

		intraexperimental		interexperimental	
compound	trial	EC_{50}[a] (μM)	CV (%)	EC_{50} (μM)	CV (%)
quercetin	1	5.92 ± 0.07	1.18		
	2	6.07 ± 0.25	4.20		
	3	6.21 ± 0.24	3.86		
	4	5.28 ± 0.23	4.40		
	5	5.98 ± 0.18	3.01	5.89 ± 0.36	6.06
gallic acid	1	65.4 ± 7.3	11.13		
	2	51.3 ± 3.0	5.77		
	3	77.3 ± 4.9	6.36		
	4	63.3 ± 2.7	4.19	64.3 ± 10.7	16.6

		intraexperimental		interexperimental	
fruit	trial	EC_{50}[a] (mg/mL)	CV (%)	EC_{50} (mg/mL)	CV (%)
blueberry	1	3.44 ± 0.24	6.94		
	2	3.49 ± 0.39	11.17		
	3	3.83 ± 0.29	7.56		
	4	2.20 ± 0.16	7.39		
	5	2.60 ± 0.06	2.13	3.11 ± 0.68	22.0

[a] $n = 3$.

Table 3. Variation among EC_{50} Values for Representative Pure Phytochemical Compounds and Fruit Extracts Obtained When a PBS Wash Was Performed between Antioxidant and ABAP Treatments (Mean ± SD)

		intraexperimental		interexperimental	
compound	trial	EC_{50}[a] (μM)	CV (%)	EC_{50} (μM)	CV (%)
quercetin	1	5.55 ± 0.09	1.60		
	2	4.48 ± 0.18	3.94		
	3	5.40 ± 0.20	3.73		
	4	5.09 ± 0.19	3.65		
	5	5.06 ± 0.19	3.77	5.12 ± 0.58	11.3
gallic acid	1	289 ± 12	4.05		
	2	270 ± 32	11.78		
	3	350 ± 21	6.14		
	4	347 ± 37	10.60		
	5	335 ± 26	7.81	318 ± 36	11.4

		intraexperimental		interexperimental	
fruit	trial	EC_{50}[a] (mg/mL)	CV (%)	EC_{50} (mg/mL)	CV (%)
blueberry	1	11.2 ± 1.2	11.08		
	2	10.3 ± 0.8	7.95		
	3	11.6 ± 0.4	3.45		
	4	9.52 ± 0.48	5.05		
	5	10.8 ± 0.4	4.09	10.7 ± 0.8	7.56

[a] $n = 3$.

the greatest activity (47 ± 1.9 μmol of QE/100 g) ($p < 0.05$), followed by cranberry (14.2 ± 0.5) ($p < 0.05$). The CAA value of apple (13.3 ± 1.1) was not significantly different from that of cranberry ($p > 0.05$), and the CAA value of red grape (12.1 ± 0.6) was similar to that of apple ($p > 0.05$). Green grape had the lowest CAA value (9.67 ± 0.57) ($p < 0.05$) when a PBS wash was performed between treatments.

To compare the antioxidant quality of different fruits, CAA values can be expressed as micromoles of QE per 100 μmol of total phenolics (**Table 4**). This value makes it possible to compare the antioxidant quality of the total phytochemicals in whole foods compared to pure phytochemical compounds. In the protocol without a PBS wash, blueberry exhibited the highest antioxidant quality (8.70 ± 0.09 μmol of QE/100 μmol of total phenolics) ($p < 0.05$), followed by similar values from cranberry (3.36 ± 0.09) and apple (3.07 ± 0.45) and then red grape (1.67 ± 0.12) and green grape (1.04 ± 0.05). These values are comparable to the activities of 100 μmol of gallic acid, ascorbic acid, caffeic acid, and catechin (**Figure 6**). When a PBS wash was done, the antioxidant quality values were 1.82 ± 0.07 μmol of QE/100 μmol of total phenolics for blueberry, 1.45 ± 0.12 for apple, 0.973 ± 0.057 for green grape, 0.914 ± 0.030 for cranberry, and 0.839 ± 0.044 for red grape, comparable to the efficacies of 100 μmol of gallic acid or caffeic acid (**Figure 6**). There were no significant differences between the antioxidant qualities of cranberry and green grape or cranberry and red grape ($p > 0.05$) in the PBS wash protocol.

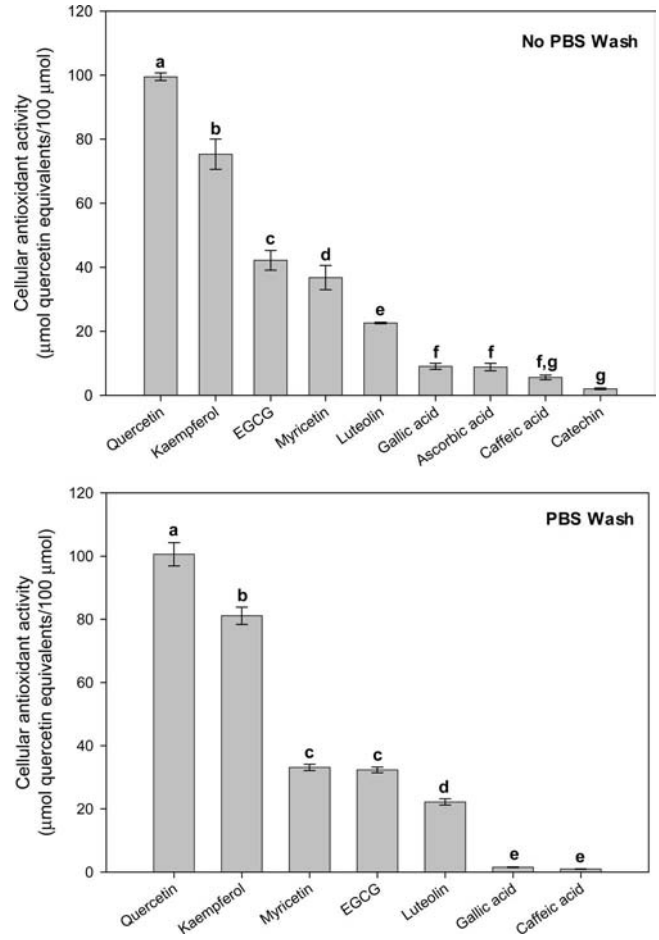

Figure 6. Cellular antioxidant activity (CAA) of selected pure phytochemical compounds (mean ± SD, $n = 3$). Bars with different letters are significantly different ($p < 0.05$).

DISCUSSION

Principle of the CAA Assay. We have developed a much-needed method to measure antioxidant activity in cell culture. As indicated at the First International Congress on Antioxidant Methods, there is an urgent requirement for more appropriate methods to evaluate the antioxidant activity of dietary supplements, phytochemicals, and foods than the chemistry methods in common usage (*23*). The CAA assay addresses this need for a biologically relevant protocol. In this method (**Figure 2**) the probe, DCFH-DA, is taken up by HepG2 human hepatocarcinoma cells and deacetylated to DCFH. Peroxyl radicals generated from ABAP lead to the oxidation of DCFH to fluorescent DCF, and the level of fluorescence measured upon excitation is proportional to the level of oxidation. Pure phytochemical compounds and fruit extracts quench peroxyl radicals and inhibit

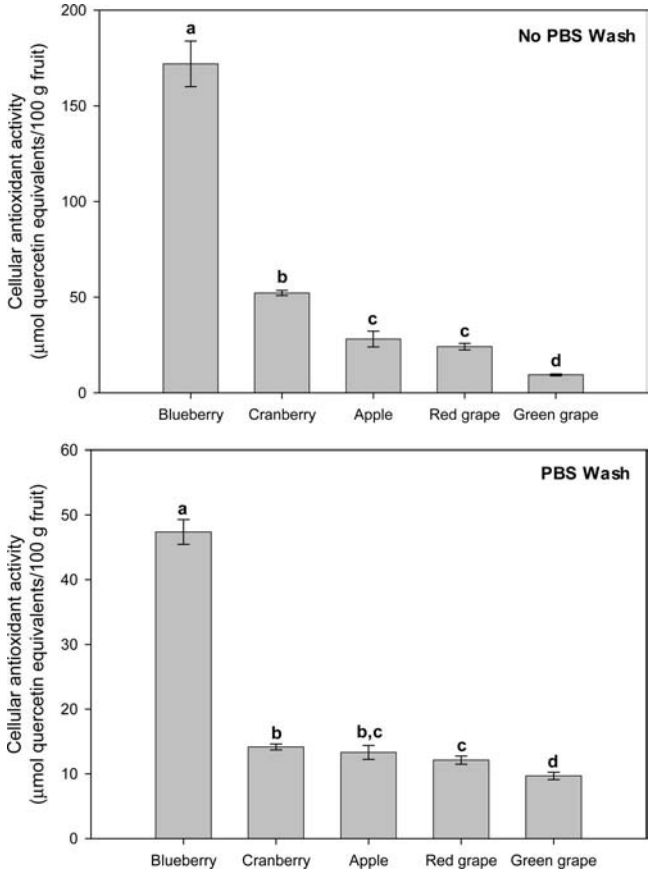

Figure 7. Cellular antioxidant activity (CAA) of selected fruits (mean ± SD, $n = 3$). Bars with different letters are significantly different ($p < 0.05$).

Table 4. Comparison of Antioxidant Quality of Fruit Extracts Using the Cellular Antioxidant Activity (CAA) Assay (Mean ± SD, $n = 3$)

	CAA[a] (μmol of QE/100 μmol of total phenolics)	
fruit	no PBS wash	PBS wash
blueberry	8.70 ± 0.19 a	1.82 ± 0.07 A
cranberry	3.36 ± 0.09 b	0.914 ± 0.03 CD
apple	3.07 ± 0.45 b	1.45 ± 0.12 B
red grape	1.67 ± 0.12 c	0.839 ± 0.044 D
green grape	1.04 ± 0.05 d	0.973 ± 0.057 C

[a] Values with no letters in common are significantly different ($p < 0.05$).

the generation of DCF. Thus, the CAA assay uses the ability of peroxyl radicals, reactive products of lipid oxidation, to induce the formation of a fluorescent oxidative stress indicator in the cell culture and measures the prevention of oxidation by antioxidants.

DCFH-DA as an Indicator of Oxidation. Keston and Brandt (*33*) first reported the use of DCFH oxidation to measure hydrogen peroxide levels in a cell-free system. DCFH-DA was first "activated" by alkali removal of the diacetate moiety. When added to hydrogen peroxide and peroxidase solutions, DCFH was oxidized to form fluorescent DCF, and the fluorescence measurements were proportional to the concentration of hydrogen peroxide. Several years later an assay to measure respiratory burst H_2O_2 in phorbol myristate acetate (PMA)-stimulated polymorphonuclear leukocytes was developed (*34*). Cells loaded with DCFH-DA fluoresced after PMA stimulation, and the fluorescence could be quantified by flow cytometry. A DCFH-DA oxidation mechanism in cells was proposed: nonpolar DCFH-DA diffused through the cell membrane, and once within the cell it was deacetylated by cellular esterases, forming DCFH, which was trapped within the cell due to its more polar nature. H_2O_2 generated by PMA stimulation, possibly in combination with cellular peroxidases, then oxidized DCFH to DCF, a polar fluorescent compound that was also trapped with the cell. Spontaneous deacetylation of DCFH-DA does not seem to be a problem, as it is slow under cell-free conditions (*20, 35*). Cellular uptake of DCFH-DA is rapid, and final concentrations are relatively stable, as cultured bovine aorta endothelial cells exposed to 11 μM DCFH-DA in the medium reached maximum intracellular levels of the probe within 15 min and the level remained constant for 1 h (*35*).

In addition to H_2O_2, various other species have been found to oxidize DCFH to DCF in cell culture. In PC12, rat neuroendocrine cells, DCF can be generated from DCFH by treatment with peroxynitrite ($ONOO^-$), nitric oxide ($NO^\bullet$), dopamine, peroxyl radicals, and H_2O_2 (*36*). Xanthine oxidase, ferrous iron, superoxide, and hydroxyl radicals have also been implicated in DCFH oxidation in renal epithelial cells (*37*). In neutrophils, DCF was generated from DCFH by Arochlor A1242 (a polychlorinated biphenyl mixture that induces respiratory burst), H_2O_2, nitric oxide, and $FeSO_4$ (*38*). DCFH-DA has also been used as an indicator to measure oxidative stress due to exposure to irradiation in MCF10 human breast epithelial cells (*39, 40*). The wide array of ROS that are able to oxidize DCFH to fluorescent DCF makes it an attractive tool to measure general oxidative stress in cells.

There are a number of potential problems with the use of DCF as an indicator of oxidizing species. Exposure of DCFH-loaded cells to light should be minimized because DCF in the presence of reducing agents was photoreduced under conditions of visible irradiation (*41*). The resulting free radicals in the presence of oxygen can be generated continuously and contribute to oxidation. DCFH and DCF also may not be trapped intracellularly, as generally thought. When endothelial cells previously exposed to DCFH-DA were exposed to medium free of DCFH-DA, the levels of DCFH and DCF decreased intracellularly and increased extracellularly (*35*). Leakage of DCFH from mouse neuroblastoma N18 cells was also reported, and it was suggested that subsequent treatments should occur as quickly as possible after loading cells with the probe (*42*). Finally, DCFH oxidation decreased with increasing reduced glutathione levels in *Saccharomyces cereviseae* cells, showing that cellular antioxidant status can influence DCF response (*43*). Despite potential misinterpretation of results due to the above factors, DCFH-DA is useful as an indicator of general cellular oxidation levels in a well-defined protocol.

ABAP as a Generator of Peroxyl Radicals. ABAP is an azo radical initiator used as an oxidant source in many antioxidant activity protocols (*15, 16, 20, 44, 45*). It thermally decomposes to generate nitrogen gas and two carbon-centered radicals. These radicals can then react with each other or form peroxyl radicals by reacting with molecular oxygen. The half-life of ABAP at 37 °C in neutral water is about 175 h, and the rate of radical generation is constant for the first few hours (*46*). The peroxyl radicals are generated in the aqueous phase, where they can cause chain reactions and damage organs indiscriminately in vivo. ABAP has been shown to induce the formation of DCF in cell culture in a dose-dependent manner (*36*).

The use of azo compounds, such as ABAP, to form peroxyl radicals in biomimetic experiments has been criticized (*24, 47*). In particular, azo initiators form an abundance of peroxyl

radicals that do not have time to perpetuate chain reactions in the time employed in antioxidant activity assays, so their use overemphasizes the initiation phase of lipid oxidation and largely ignores the propagation and decomposition phases (*24*). Although ABAP is not a physiologically relevant compound, peroxyl radicals, which are generated by ABAP decomposition, are a major type of ROS in vivo, so it is a good tool for the examination of peroxyl radical-induced damage to membranes and other biological molecules and for studying the inhibition of these effects by antioxidants (*46*).

Suggested Standards for CAA Assay. To be able to compare data in the literature from different laboratories, the CAA method should be standardized. On the basis of our results, we strongly recommend that quercetin be used as a standard in this new assay for quantifying cellular antioxidant activity for the following reasons: (1) quercetin has high CAA activity compared to other phytochemicals (**Figure 6**); (2) the pure compound is easily and economically obtained; (3) quercetin and its conjugates are found widely in fruits, vegetables, and other plants; and (4) it is relatively stable.

CAA of Selected Phytochemicals and Fruits. There are two opportunities for compounds to exert their antioxidant effects in our CAA model. They can act at the cell membrane and break peroxyl radical chain reactions at the cell surface, or they can be taken up by the cell and react with ROS intracellularly. Therefore, the efficiency of cellular uptake and/or membrane binding combined with the radical scavenging activity likely dictates the efficacy of the tested compound. Among the pure phytochemicals examined in the CAA assay, quercetin, kaempferol, EGCG, myricetin, and luteolin showed the highest cellular antioxidant activities, exhibiting between 22 and 100% of the antioxidant activity of quercetin. These flavonoids were likely well-absorbed by the HepG2 cells, as quercetin, kaempferol, and luteolin were also shown to be absorbed and incorporated into Caco-2 cells (*48*), although there was no myricetin uptake in that study and EGCG was not examined. Other phytochemicals, such as ascorbic acid, gallic acid, caffeic acid, and catechin, had less than 10% of the activity of quercetin in the CAA assay.

The physical properties of flavonoids (and presumably other classes of phytochemicals) determine their interactions with the cell membrane (*49*). Hydrophobic flavonoids may become deeply embedded in membranes, where they can influence membrane fluidity and break oxidative chain reactions. More polar compounds interact with membrane surfaces via hydrogen bonding, where they are able to protect membranes from external and internal oxidative stresses. There is also some evidence that uptake in vivo may be related to the polarity of the compounds because the net transfer of flavonoids across the brush border of rat small intestine was found to be related to their lipophilicity, rather than their spatial conformation (*50*).

The hydrophobicity of compounds may be important, but it is not the only factor determining their effectiveness as antioxidants in cell culture, as there was no relationship between log *P* (octanol–water partitioning coefficients) and activity in our model (data not shown). This was supported by a study using PC12 cells treated with H_2O_2, which showed that the effectiveness of flavonoids to decrease oxidative stress as measured by DCFH oxidation was strongly associated with structural principles, not octanol–water partitioning behaviors (*51*). In the evaluation of quercetin and compounds structurally similar to quercetin, they found that the 3′,4′-hydroxyl groups in the B ring and a 2,3-double bond conjugated with a 4-oxo group in the C ring of quercetin conferred it with the most activity against H_2O_2 oxidation. We did not test enough phenolic compounds to determine the structure–function relationships that exist for the CAA protocol; however, the flavonoids with a 2,3-double bond and 4-oxo group, which include quercetin, kaempferol, myricetin, and luteolin, all had high activity. It is unknown why catechin, epicatechin, ferulic acid, and resveratrol had low efficacy in this model and why EGCG had such high activity. A more comprehensive screening of phytochemicals and their conjugates is necessary to determine the structural and physical properties that dictate effectiveness in the CAA assay.

Some compounds, such as quercetin, kaempferol, myricetin, EGCG, and luteolin, showed little, if any, difference in antioxidant efficacy whether or not a PBS wash was done between antioxidant and ABAP treatments, as measured by EC_{50} values for CAA. Gallic acid, ascorbic acid, caffeic acid, and catechin, on the other hand, displayed dramatically lower effects when a PBS wash was done. The comparisons in antioxidant activities using the protocols with and without a PBS wash may provide information on the degree of uptake and membrane association of the pure phytochemicals or the compounds present in the fruit extracts. When a PBS wash is employed, compounds must either be taken up by the cells or be closely associated with the cell membrane to have antioxidant effects, as the PBS will remove compounds that are only loosely associated with the membrane. The results imply that gallic acid, ascorbic acid, caffeic acid, and catechin adsorb more loosely to the cell membrane and are taken up less readily than the flavonols, luteolin, and EGCG.

In addition to the differences in activity using the two protocols, there were also differences in variation. When no PBS wash was done between treatments, the activity may have been higher, but the CV also tended to be higher (**Tables 1**–**3**). This was likely due to the interaction of the samples and oxidants with other factors in the residual medium on the cells. Washing the cells with PBS removed most of the interfering medium components and increased the consistency of the results. Other sources of variation may include differences in cell characteristics due to the passage number of the cells, deviation in the actual number of cells plated or surviving between experiments, and cell clumping. In addition, "cross-talk" and variation may have been decreased by using black 96-well plates instead of the clear plates employed. Differences in the content in cellular antioxidant defenses naturally present, such as glutathione, vitamin E, cysteine, phenolic amino acids, and proteins, may also contribute to the variation in CAA between experiments. However, using the area under the curve ratios of treated cells to controls should negate the effects of these compounds.

Advantages of CAA Assay. It has been suggested that the following should be considered in choosing appropriate methods to measure antioxidant activity: physiologically relevant substrates; conditions that mimic biological systems; low levels of oxidants that represent all stages of lipid oxidation; measurement of different compounds at comparable concentrations and use of plant extracts where the phenolic composition is known; and quantification based on induction period, percent inhibition, rates of product formation/decomposition, or median effective dose (*24*). We think the cellular antioxidant activity assay presented here addresses many of those issues. A relatively low level of ABAP, 600 μM, is used to generate peroxyl radicals to initiate oxidation, and the use of excessive levels of antioxidants was avoided. We employ the area under the kinetic curve to calculate

cellular antioxidant activity, which takes into consideration both the oxidation lag time increases and degree of ROS scavenging by the antioxidants tested. The median effective dose is calculated, and expression of the results in micromoles of quercetin equivalents relates the activities to an inexpensive and ubiquitous phytochemical with biological activity. It also allows for direct comparisons of activities of different sample types and of results from other laboratories. The use of molarity instead of mass makes comparisons of antioxidant activity of compounds with different molecular weights more valid. Expression of results in quercetin equivalents per milligrams of phytochemical may be more accessible, but it does little to describe the relative efficacy of compounds. By describing antioxidant activity per micromole of phytochemical, molecules of compounds with different molecular weights and functional groups can be compared directly.

Popular antioxidant activity/capacity assays, such as ORAC (*15*), TRAP (*16*), TEAC (*18*), TOSC (*19*), PSC (*20*), and FRAP (*21*), all have the limitation of the inability to represent the complexity of biological systems. They measure chemical reactions only, and these reactions cannot be interpreted to represent activity in vivo, as they cannot account for the bioavailability, stability, tissue retention, or reactivity of the compounds under physiological conditions (*52*). Oxidation of DCFH to DCF has been used as an indicator of oxidative stress and its attenuation by phytochemicals and food extracts in cell cultures (*48*,*51*,*53*,*54*), but these assays are not designed to measure antioxidant activity and there is no consistency in the protocols used. Differences exist in the cell lines, types of oxidants, media, concentrations of reagents, treatment orders and times, and oxidative stress quantification methods. For results to be comparable among laboratories, a standardized method should be adopted.

The importance of using a more biologically relevant model in the determination of antioxidant activity is highlighted by the differences between the results of pure chemistry assays and those based in cell culture. Of the phytochemicals tested in our model, quercetin, catechin, and caffeic acid had the most activity in the ORAC assay (*55*); gallic acid, epicatechin, and EGCG were the most effective in the TEAC assay (*56*); quercetin, myricetin, and kaempferol were the best using the FRAP method (*57*); and EGCG, chlorogenic acid, and caffeic acid were the most efficacious in the PSC protocol (*20*). Not only are the results different from those yielded from our cellular antioxidant activity model, but they are also different from each other. Similarly, there is no consistency in the order of antioxidant activity of fruit extracts in different assays. In our model, the order of antioxidant activity was blueberry > cranberry > apple ≈ red grape > green grape. In the PSC and TOSC assays, the order of efficacy cranberry > apple > red grape was the same (*6*, *20*). However, oxidation of LDL by cupric ions was prevented best by cranberry, then blueberry, apple, green grape, and red grape (*58*); and in the ORAC assay, red grape had higher activity than apple (*59*). In a study that compared results from a cell-based model to those from a chemistry model using the same samples, the prevention of ABAP-induced DCFH oxidation in HepG2 cells by broccoli extracts was not correlated to ORAC, indicating that the chemical assay may not be a good measure of antioxidant activity in biological models (*53*).

Summary. We believe the CAA assay reported here is a great improvement over the "test tube" chemical methods used to evaluate the efficacy of pure phytochemical compounds, plant extracts, and dietary supplements. It is an assay for screening antioxidants that considers cellular uptake, distribution, and efficiency of protection against peroxyl radicals under physiological conditions. The CAA assay presented here answers the demand for the next step forward from chemistry assays to assess the potential bioactivity of antioxidants.

ABBREVIATIONS USED

ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; CAA, cellular antioxidant activity; CV, coefficient of variation; DCF, dichlorofluorescein; DCFH, dichlorofluoorescin; DCFH-DA, dichlorofluorescin diacetate; DPPH, 2,2-diphenylpicrylhydrazyl; EC_{50}, median effective concentration; EGCG, epigallocatechin gallate; FRAP, ferric reducing/antioxidant parameter; GAE, gallic acid equivalents; HBSS, Hanks' Balanced Salt Solution; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; PSC, peroxyl radical scavenging capacity; QE, quercetin equivalents; ROS, reactive oxygen specieds; TEAC, Trolox equivalent antioxidant capacity; TOSC, total oxyradical scavenging capacity; TRAP, total radical-trapping antioxidant parameter; WME, Williams' Medium E.

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