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Comparison of Chemical and Cell-Based Antioxidant Methods for Evaluation of Foods and Natural Products: Generating Multifaceted Data by Parallel Testing Using Erythrocytes and Polymorphonuclear Cells

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The objective of this study was to compare three tests frequently used for evaluation of antioxidant potential in natural products: (1) oxygen radical absorbance assay (ORAC), (2) cell-based antioxidant protection in an erythrocyte model (CAP-e), and (3) reactive oxygen species formation in polymorphonuclear cells (ROS PMN). The methods were applied to four natural products, all containing antioxidants capable of entering and protecting cells in the CAP-e assay. The magnitude of this effect was not directly correlated to the ORAC value of each product. Furthermore, the products showed different effects in the ROS PMN assay. Açai provided strong inhibition of ROS formation, indicating anti-inflammatory properties. In contrast, Immunel and EpiCor mildly enhanced ROS formation, suggesting activation of the innate immune response. HA Joint Formula showed a complex, nonlinear dose-response in the ROS PMN assay. This illustrates that complex natural products may have similar antioxidant properties but different effects on human cells. Cell-based antioxidant protection is addressed best in the CAP-e assay, since some natural products contain compounds that may provoke cellular signaling in other cell types. The PMN cell type is a useful model for assessment of overall anti-inflammatory versus immune supportive properties of a product. The sequential use of the three methods serves to bridge analytical and biological testing methods.

KEYWORDS: Antioxidant; method; erythrocyte; polymorphonuclear; cell-based antioxidant protection assay (CAP-e); oxygen radical absorbance capacity (ORAC) assay

INTRODUCTION

Free radicals arise during normal metabolism, are produced during immune activity, and are introduced by many environmental factors such as pollution, smoke, and sunlight. Antioxidant mechanisms in blood, cells, and tissue fluids play an important role in neutralizing the normal level of oxidative damage caused by free radicals. During chronic inflammatory conditions, combined with the absence of sufficient dietary antioxidants, the oxidative damage is accelerated, causing further dysregulation involving inflammatory reactions that contribute to many degenerative diseases and aging (*1*). A growing body of research is focused on nutritional and pharmaceutical prevention of chronic inflammatory conditions, as these have been associated with obesity (*2*), immune dysfunction (*3*), cardiovascular disease (*4*), declining cognitive function (*5*), and cancer (*6, 7*).

As continuing research examines the effect of antioxidants on health, the testing for antioxidant protection has become a powerful focus in the dietary and natural products industry. Researchers associated with the natural product industry have pushed for a standardized method for measuring antioxidant capacity in natural products (*8, 9*). A large number of methods have been developed to evaluate the antioxidant capacity in foods, including nutritional supplements, vitamins, minerals, and extracts of various natural products of plant, fungal, animal, and bacterial origins. The natural products industry has taken many steps in creating standardized tests to measure antioxidant levels in (1) nutritional and natural products and (2) blood samples before and after consuming such products. One of the most popular and best standardized chemical antioxidant methods is the oxygen radical absorbance capacity (ORAC) test (*10–12*). This test is widely used for evaluation and

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Table 1. Comparison of Chemical and Cell-Based Assays in Vitro

	chemical assays	cell-based assays
assay principles	based on known chemical reactions between limited number of reagents	based on interaction between added compounds and complex enzymatic reactions in biological system
extraction of active ingredients	some flexibility	assay must take place in physiological saline solution; limited use of solvents
use of alcohol-based solvents	yes, if no interference with chemical reactions in assay	yes, if properly diluted, tested for tolerance, and altered cellular behavior (depends on assay)
dimethyl sulfoxide (DMSO) as solvent	yes, with appropriate controls, if no interference with chemical reactions in assay; DMSO is a free radical scavenger (*17*)	no, alters bioavailability (*14–16*), thereby defeating the purpose of testing bioavailability in vitro; DMSO is anti-inflammatory (*18*) and can exaggerate mitochondrial ROS formation (*19*)
data analysis and interpretation	quantitative	qualitative
expectation of linear dose- responses	yes	no
applicability of area-under-curve	yes (*11*)	no

Table 2. Enzymes Involved in Redox Reactions in Red Blood Cells (RBC) and Polymorphonuclear Cells (PMN)

enzyme	role for normal cell physiology	RBC	PMN
glutathione peroxidase	reduces lipid hydroperoxides to their corresponding alcohols; reduces free hydrogen peroxide to water; protects integrity of cellular membranes; function depends on selenium; protects nucleated cells from oxidative stress-induced programmed cell death (apoptosis)	X	X
glutathione reductase	maintains glutathione in its reduced state	X	X
catalase	breakdown of hydrogen peroxide to oxygen and water	X	X
superoxide dismutase	catalyzes dismutation of superoxide to oxygen and hydrogen peroxide	X	X
myeloperoxidase	produces hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride anion (Cl^-) during the neutrophil's respiratory burst; oxidizes tyrosine to tyrosyl radical using hydrogen peroxide		X
cyclooxygenase COX-1	contain both cyclooxygenase and peroxidase properties; catalyzes the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins		constitutively expressed
cyclooxygenase COX-2	contain both cyclooxygenase and peroxidase properties; catalyzes the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins		induced upon inflammation
hemoglobin/deoxyhemoglobin	scavenging and transport of O_2, CO_2, NO^-	X	

comparison of the antioxidant capacity in natural products and extracts and has been successfully applied to bioavailability studies, showing increased antioxidant capacity in serum in test subjects after consumption of antioxidant-rich foods (*13*).

Going beyond the ORAC test for antioxidant testing currently requires either more complex cellular testing systems or full clinical trials focused on serum antioxidant capacity, lipid peroxidation, and associated physiological measurements. The expense of full clinical trials makes selected panels of cell-based assays attractive as intermediate testing methods. The transition from chemical to cell-based assays involves different solubility issues and restricted use of solvents, particularly DMSO, which alters bioavailability (*14–16*) and possesses antioxidant and anti-inflammatory properties (*17–19*) (**Table 1**).

The use of erythrocytes, or red blood cells (RBC), in testing how well a substance protects against oxidative stress is biologically relevant, as RBC play critical roles in antioxidant protection in the blood (*20*). The ability of RBC to scavenge reactive oxygen and nitrogen species represents a direct antioxidant and anti-inflammatory protection to the body. The cells are living, energy-producing cells and represent the most abundant cell type in the blood circulation where they outnumber white blood cells by at least 100-fold. Using RBC as a cell model reduces the confounding contribution of cellular signaling. The assay is not affected by mitochondrial production of reactive oxygen species (ROS), as RBC do not have mitochondria but instead generate cellular energy by other metabolic pathways. Many of the redox enzymes present in RBC are the same as the enzymes present in polymorphonuclear (PMN) cells (**Table 2**).

We have developed the CAP-e assay (*21*) as a cell-based antioxidant protection assay using erythrocytes to address the question of whether antioxidants in complex natural products enter the cytosol and contribute to the reduction of oxidative damage within the cell (**Figure 1**). The assay measures the effects in the cytosol only, as the reporter dye we use in the test is only functional after penetrating into the intracellular space (i.e., the cytosol) where it undergoes chemical modification, resulting in its retention within the cell. The assay allows for semiquantification specifically of those antioxidants that are capable of penetrating into live cells. We used this test as a baseline for further cell-based testing using primary pro-inflammatory PMN cells, to allow for a more definitive assessment of the complex properties of natural products in vitro.

Examples of other existing cell-based assays for measuring effects on inflammatory cell types includes testing on freshly isolated human PMN cells (*22, 23*). This cell type is an important part of our innate immune defense and is capable of rapid production of ROS in response to both oxidative damage and pro-inflammatory stimuli. The PMN cell can respond to compounds in natural products extracts in three distinct ways; the data obtained from a PMN-based assay represent a total summary of these mechanisms often operating in parallel (**Figure 1**). Thus, data obtained from a PMN-based assay may be interpreted better in the light of a preceding RBC-based assay, such as the CAP-e.

MATERIALS AND METHODS

Reagents. The following buffers and reagents were obtained from Sigma-Aldrich (St. Louis, MO): phosphate-buffered saline (PBS), RPMI-1640 culture medium, hydrogen peroxide 30% solution (H_2O_2),

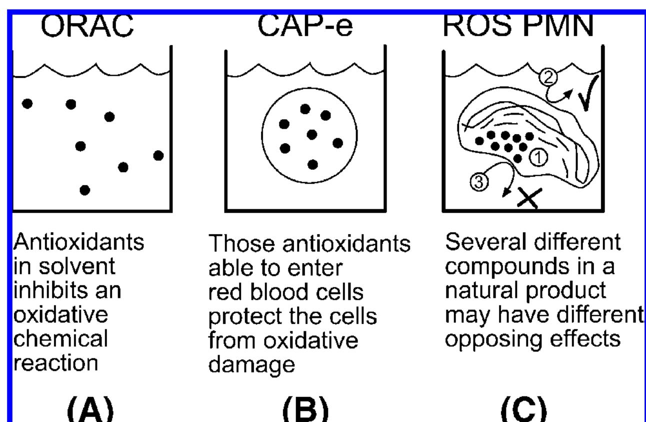

Figure 1. Three different but synergistic testing principles are shown. (**A**) The oxygen radical absorbance capacity (ORAC) assay is a chemical test, in which interference with specific chemical reactions are measured. (**B**) The cell-based antioxidant protection in erythrocytes (CAP-e) assay reflects whether antioxidants can enter into and protect live cells from oxidative damage. (**C**) The reactive oxygen species in polymorphonuclear cells (ROS PMN) assay monitors the combined effect of a test product on an inflammatory cell type, and the data reflects a combination of at least three different mechanisms: (1) Antioxidants penetrate into the cell and neutralize free radicals, similar to the CAP-e assay; (2) Anti-inflammatory compounds mediate cell signaling at the cell surface, reprogramming the PMN cell to a less inflammatory behavior, resulting in a reduction in formation of ROS; and (3) Pro-inflammatory compounds capable of supporting innate immune functions mediate a signal at the cell surface, resulting in an increase in the PMN cell function, thus increasing the production of ROS.

dimethyl sulfoxide (DMSO), Histopaque 1077, and Histopaque 1119. Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR), a subdivision of Invitrogen (Carlsbad, CA).

Natural Products. One animal-based, one microbial-based, one plant-based, and one mixed natural product were obtained directly from the following individual suppliers: A colostrum whey-based extract, Immunel (Sterling Technologies Inc., Brookings, SD), the high-metabolite yeast culture-based product, EpiCor (Embria Health Sciences, Ankeny, IA), freeze-dried Amazonian palm berry Açai (*Eutherpe oleracea Mart.*) (K2A LLC, Provo, UT), and H.A. Joint Formula (HAJF) (Purity Products, Plainview, NY).

Preparation of Natural Products Extracts. Natural products extracts were prepared in PBS in preparation for in vitro cell-based assays by adding 0.5 g of powder to 5 mL of PBS. This was mixed well by vortexing, placed on a rocker in the dark, and incubated at room temperature for 1 h. The remaining solids were removed by centrifugation at 2400 rpm for 10 min followed by filtration of the supernatants using a 0.22 μm low protein binding cellulose acetate syringe filter. The extracts were always used within 4 h of preparation, as longer storage periods could result in oxidative breakdown of the bioactive compounds that we were evaluating.

Purification of Peripheral Blood Polymorphonuclear Cells (PMN) and Red Blood Cells (RBC). Healthy human volunteers ($n = 12$) between the ages of 20 and 60 years served as blood donors after informed consent, as approved by the Sky Lakes Medical Center Institutional Review Board, was obtained. Peripheral venous blood samples were drawn into sodium heparin and layered onto a double-gradient of Histopaque 1077 and 1119. The vials were centrifuged for 25 min at 2400 rpm. The upper, PBMC-rich interface and the lower, PMN-rich interfaces were harvested using sterile transfer pipettes into new vials and washed twice in PBS without calcium or magnesium at 2400 rpm for 10 min. After the second wash, the PMN pellet was treated with 4 mL of distilled H_2O for 30 s to lyse red blood cells not removed by the gradient centrifugation. Immediately, 4 mL of 1.8% saline was added to restore the physiological isotonic condition. Subsequently, 4 mL of PBS was added, and the PMNs were centrifuged for 10 min at 2400 rpm.

Evaluation of Antioxidant Protection in a Red Blood Cell-Based Assay. The red blood cell is a convenient test model for examination of antioxidants that are able to enter living cells. Since the red blood cell is not able to signal in response to pro-inflammatory stimuli and is not able to produce reactive oxygen species, it provides a cleaner signal for antioxidant capacity of a test product than the PMN cell. Whole blood was layered onto Histopaque 1119 and centrifuged 25 min at 2400 rpm. All plasma, leukocytes, and Histopaque were removed. From the remaining packed red blood cells, 0.1 mL was transferred into 10 mL PBS without calcium or magnesium. Parallel samples of this red blood cell suspension were incubated at 37 °C, 5% CO_2 for 90 min, either untreated or with test products over a range of 5-fold serial dilutions from 10 to 0.016 mg/mL. A stock solution of DCF-DA, which becomes brightly green fluorescent upon exposure to free radicals, was prepared by adding 0.18 mL of DMSO to a 0.05 mg aliquot of DCF-DA and vortexing 3 times for 15 s. A working solution of DCF-DA was then prepared by adding 0.01 mL of stock to 10 mL of PBS. The red blood cells were washed twice in PBS, resuspended in the DCF-DA working solution, and incubated for 1 h at 37 °C. All samples, except for the untreated control samples, were then exposed to 167 mM H_2O_2 for a period of 45 min to induce severe oxidative stress. Samples were washed twice in PBS to remove the peroxide, transferred to cold PBS, and stored on ice in preparation for immediate analysis by flow cytometry. Intracellular levels of DCF-DA fluorescence intensity in untreated versus H_2O_2-challenged cells were analyzed by flow cytometry. Data was collected in quadruplicate for controls and in duplicate for each sample concentration. The mean fluorescence intensity (MFI) of red blood cells was compared between untreated, H_2O_2-treated, and cells pretreated with test products. A reduction in MFI in samples pretreated with test products prior to challenge with H_2O_2 signified a reduction in oxidative damage mediated by the test product. Experiments were repeated five times with similar results.

Assessment of Reactive Oxygen Species (ROS) Formation in PMN Cells. The evaluation of anti-inflammatory action of test products was done using freshly purified human PMN cells. The PMN were incubated at 37 °C, 5% CO_2 for 90 min, either untreated or with test products over a range of dilutions from 0.01 to 0.0001 mg/mL. The precursor dye DCF-DA was prepared by adding 0.18 mL of DMSO and 0.02 mL of a 20% solution of Pluronic F-127 in DMSO to a 50 μg aliquot of DCF-DA and vortexing 3 times for 15 s. A working solution of DCF-DA was then prepared by adding 0.01 mL stock to 10 mL PBS. The PMN cells were washed twice in PBS, resuspended in the DCF-DA working solution, and incubated for 1 h at 37 °C. All samples, except for the negative control samples, were then exposed to 167 mM H_2O_2 for 45 min to induce severe oxidative stress. Samples were washed twice in PBS to remove the peroxide, transferred to cold RPMI, and stored on ice. The DCF-DA fluorescence intensity was immediately analyzed by flow cytometry, using a FacsCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and the CellQuest Pro (Becton-Dickinson, San Jose, CA) and FlowJo (TreeStar, Ashland, OR) software. Data was collected in triplicate for controls and in duplicate for each sample concentration. The MFI of PMN cells was compared between untreated, H_2O_2-treated, and cells pretreated with test products. A reduction in MFI in samples pretreated with test products prior to challenge with H_2O_2 indicated a reduction in ROS production mediated by the test product. An increase in MFI in samples pretreated with test products prior to challenge with H_2O_2 indicated an increase in ROS production mediated by the test product. The experiments were repeated three times with consistent results.

As part of our initial testing, experiments were performed to reduce the contribution of pro-inflammatory signaling from the total results in the ROS PMN assay. This was done by inhibiting cytoskeletal movements involved in signaling and intracellular motility of vacuoles involved in ROS formation. EpiCor was tested in two parallel sets of tests, in which one set of duplicate tests was performed in culture medium allowing full functionality of the PMN cells, and the parallel set of duplicate tests were performed in culture medium, to which sodium azide was added at a final concentration of 0.02%.

Oxygen Radical Absorbance Capacity (ORAC) Assay. Samples of Immunel and HAJF were shipped to Brunswick Laboratories, Norton, MA, an independent contract laboratory specializing in standardized

Table 3. Antioxidant Capacity of Test Products As Evaluated by ORAC Tests

natural product	$ORAC_{hydrophilic}$ (μmol TE/g)	$ORAC_{lipophilic}$ (μmol TE/g)	TAC (μmol TE/g)	NORAC (μmol TE/g)	HORAC (μmol GAE/g)	SOD (unit/g)
Immunel	18	ND[a]	18	0.59	1.72[a]	ND[a]
OptiAçai[b]	997	30	1027	34	52	1614
HA Joint Formula	2219	139	2358	372	123	33
EpiCor	614	ND[a]	614	54	214	2200

[a] ND = not detected. [b] The antioxidant capacity for OptiAçai was previously published in ref *22*.

testing of natural products. The batch of EpiCor used for this project was the same as the product used for in vitro testing of immunomodulatory activity (*24*), but the results of the ORAC panel were not previously published. The ORAC values on açai were previously published (*22*) and are reprinted here with the journal's permission. Data were obtained for all four natural products from $ORAC_{hydrophilic}$ testing using fluorescein as the fluorescent probe and 2,2′-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator (*11*), $ORAC_{lipophilic}$ testing for lipid antioxidants capable of quenching peroxyl free radicals (*25*), HORAC testing for antioxidants capable of quenching hydroxyl free radicals (*26*), NORAC testing for antioxidants capable of quenching peroxynitrite, and SORAC testing for superoxide dismutase-like activity.

Statistical Analysis. Statistical analysis was performed using Microsoft Excel. Statistical significance was tested using Student's *t* test with a *p* value of less than 0.05 indicating a significant difference between data sets.

RESULTS

Chemical Antioxidant Tests. The selected products were analyzed for total antioxidant capacity in a panel of ORAC assays (**Table 3**). The products were selected to include products known to have various levels of protective activity in the CAP-e assay. As an example of an animal-based product, we chose an extract from bovine colostrum whey,

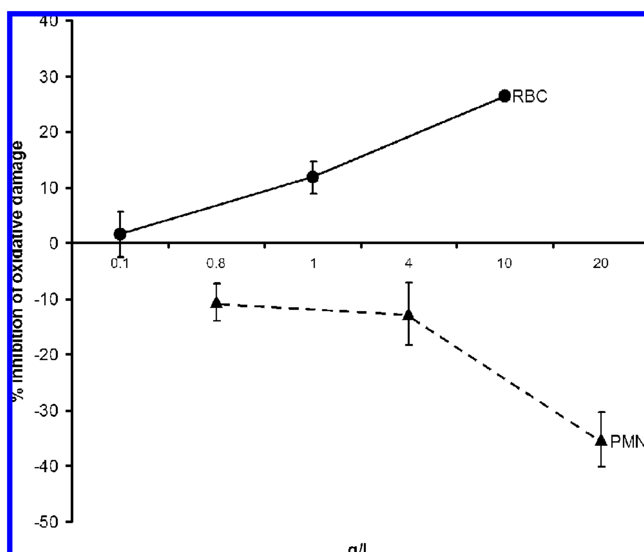

Figure 2. Comparison of the effects of the colostrum whey-based extract Immunel in two different cell-based assays: The cell-based antioxidant protection of red blood cells (RBC) versus polymorphonuclear (PMN) cells. The RBC-based CAP-e assay shows that Immunel contains antioxidants able to enter and protect live cells from oxidative damage. The PMN-based assay shows that Immunel triggers cellular signaling and ROS formation in PMN cells, as seen by the negative percent inhibition (i.e., an increase in ROS formation). Thus, even though the PMN-based assay is not suitable for demonstrating the antioxidant capacity of the complex product Immunel, the PMN-based assay demonstrates the immune modulating property of Immunel.

Immunel, known to provide a mild but significant effect in the CAP-e assay (*27*). As a plant-based extract, we used a freeze-dried berry product OptiAçai from *E. oleracea* (açai), on which we have previously reported a strong ROS inhibitory effect in the PMN-based assay (*22*). In addition, the joint health product HAJF was chosen. This product is a blend of hyaluronic acid with hydroxytyrosol, a polyphenol from olives. The yeast-based high-metabolite immunogen EpiCor was also included, based on its combination of high antioxidant content and immunomodulatory activity (*24*).

Comparison of CAP-e Data to the Effect on ROS Formation in Human Polymorphonuclear (PMN) Cells. To illustrate the differences between the cell-based assays (**Figure 1**), we compared the effects of the four selected test products in both the CAP-e and the ROS PMN assays. For the ROS PMN assay, freshly purified human PMN cells were used for assessment of modulatory effects on this inflammatory cell type in vitro. Pretreatment of freshly isolated healthy human neutrophils with the four natural products before the induction of ROS by H_2O_2 treatment resulted in product-specific effects on ROS production.

Immunel used for this study had a moderate $ORAC_{hydrophilic}$ value of 18 μmol TE/g, but antioxidants in Immunel were available to live cells. Even more important, the data from the CAP-e test suggested that the antioxidant compounds in Immunel were retained within the cells, providing higher-than-expected antioxidant protection at lower doses. In addition, Immunel provided a mild but significant increase in the formation of ROS by PMN cells, indicative of the support Immunel provided to the innate immune system (**Figure 2**).

Açai has a high $ORAC_{hydrophilic}$ value of 997 μmol TE/g, and a significant amount of those antioxidants were able to enter and protect live cells in the CAP-e assay (**Figure 3**). In addition, açai inhibits ROS formation by PMN cells, even at extremely low doses. When comparing the CAP-e data to the ROS PMN data for similar doses of açai, it became clear that the antioxidants alone could not account for the strong anti-inflammatory effect of açai on PMN cells.

HAJF, containing hydroxytyrosol from olives, had a very high $ORAC_{hydrophilic}$ of 2219 μmol TE/g. HAJF performed very well in the CAP-e assay (**Figure 4**), indicating that the antioxidants in HAJF were highly able to enter and protect live cells. HAJF showed an inverted dose-response in the ROS PMN assay; that is, the lower doses were more efficient at preventing ROS formation by PMN cells than higher doses. We interpret the inverse dose-response as the combined effect of hyaluronan, known to activate cells in the innate immune response, and the strong antioxidant capacity of hydroxytyrosol.

EpiCor had a relatively high $ORAC_{hydrophilic}$ value of 614 μmol TE/g, and a significant amount of those antioxidants could enter and protect live cells, as shown by the CAP-e assay (**Figure 5**). However, the PMN assay showed that EpiCor at lower doses was able to induce a mild increase in

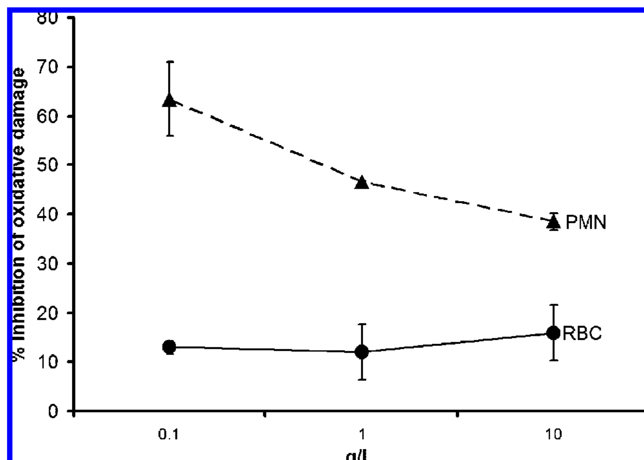

Figure 3. Comparison of the effects of the Amazonian palm berry açai in two different cell-based assays: The cell-based antioxidant protection of red blood cells (RBC) versus polymorphonuclear (PMN) cells. The freeze-dried açai contains antioxidants capable of entering and protecting live cells in vitro. However, when a careful comparison across lower doses of açai was performed in parallel using both the red blood cell (RBC)-based CAP-e assay and the polymporphonuclear (PMN) cell-based assay, it was shown that the simple antioxidant content was not sufficient to explain the reduction in ROS formation in the PMN assay. We conclude that the data from the PMN assay reflected strong anti-inflammatory signaling of PMN cells by açai.

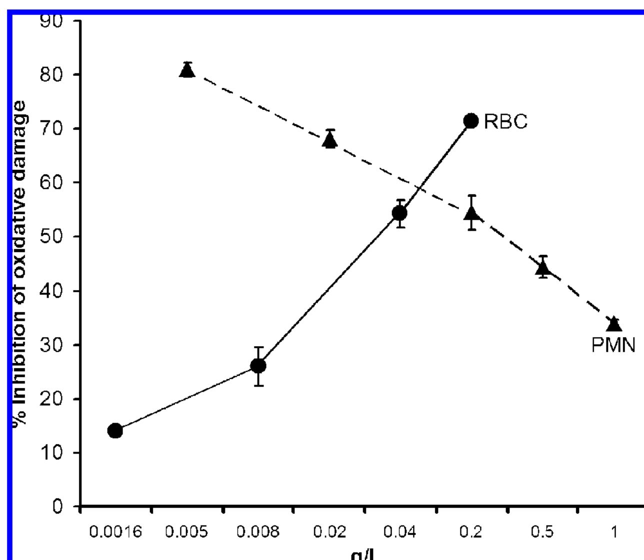

Figure 4. Comparison of the effects of HAJF in two different cell-based assays: The cell-based antioxidant protection of red blood cells (RBC) versus polymorphonuclear (PMN) cells. HAJF showed a simple linear dose- response in the RBC-based CAP-e assay, indicating that HA Joint Formula contains antioxidants capable of entering and protecting live cells from oxidative damage in vitro. However, an opposite dose-response was seen in the PMN-based cellular assay, indicating opposing effects between different compounds in HAJF, probably involving (1) antioxidants, (2) activating compounds, and (3) anti-inflammatory signaling compounds.

ROS formation by PMN cells. Subsequently, EpiCor was tested in the PMN assay using two parallel culture conditions: (a) regular culture medium allowing the PMN cells full functionality; and (b) culture medium containing sodium azide to abrogate cytoskeletal movements involved in cellular signaling and intracellular transport involved in ROS formation. By using the culture conditions in which the signaling

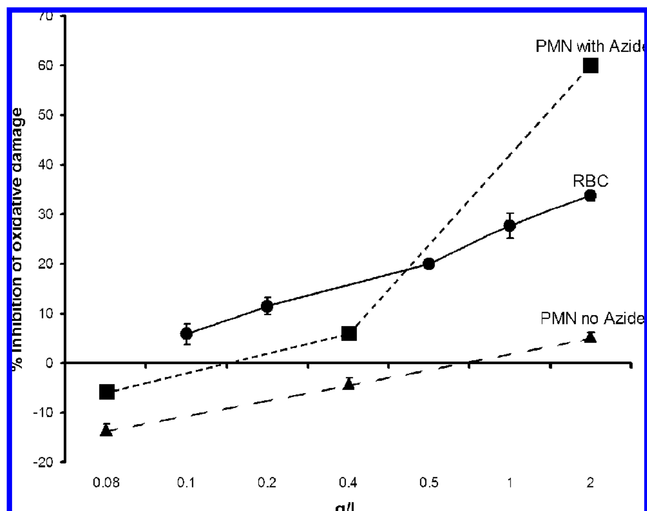

Figure 5. Comparison of the effects of EpiCor in three different cell-based assays: The cell-based antioxidant protection of red blood cells (RBC) versus polymorphonuclear (PMN) cells. The RBC-based CAP-e assay showed that EpiCor contains antioxidants able to enter and protect live cells in vitro, and the data demonstrate a clear and linear dose-response. The PMN-based assay was performed in two parallel manners: (a) The exposure of the PMN cells to EpiCor was performed in culture medium, allowing the cells to react to signaling compounds present in the EpiCor extract; (b) The exposure of the PMN cells to EpiCor was performed in culture medium containing 0.05% sodium azide to block cytoskeletal movements, thus abrogating many aspects of cellular signaling and intracellular transport mechanisms involved in formation of reactive oxygen species (ROS). The comparison between these two data sets on PMN cells shows that when the PMN cells are able to perform cellular signaling, the PMN cells respond to EpiCor by increasing their immune reactivity and producing ROS. When cellular signaling is inhibited, some antioxidant protection can be demonstrated in the PMN-based cell model. The advantage of the CAP-e assay over the PMN-based assay is that RBC do not produce ROS, neither as part of their immune function nor as part of mitochondrial metabolism; and thus, the RBC provides a much simpler and more conclusive cell-based model for antioxidant testing in vitro.

was inhibited, the data showed that EpiCor also provided protection from oxidative damage in the PMN assay, at least as well as in the CAP-e assay.

DISCUSSION

Both the dietary and nutraceutical industries are rapidly moving beyond simple measures of nutrient content toward a more complex understanding of the functionality of foods and supplements in biological systems. The industry is moving away from basing claims solely on methods based in analytical chemistry and embracing methods based on biological systems in vivo and in vitro. A continued forum for discussion is in high demand to support the creation of new standards for the measurement of the effect of natural products in nonchemical testing systems.

With regard to antioxidants and products with anti-inflammatory properties, each available chemical tests has their strengths and weaknesses because each is based upon detecting antioxidant interference with well-known, but limited, chemical reactions. Moving into cell-based testing systems in vitro, as well as to testing in whole organisms in vivo, eliminates many of the limitations of the chemical interaction based assays. This has both positive and negative consequences. The positive aspect

is that the results are more relevant for biological systems. One of the down-sides is that a singular numerical value is no longer easily obtainable. However, it is more important to apply numerous assays to gain a comprehensive understanding of a product's performance than to use a single numerical value to describe a product.

The choice of immortalized cell lines for the purpose of antioxidant testing is not a desirable approach. Some immortalized cell lines show altered functional responses to oxidative stress, such as the human hepatocellular carcinoma line HepG2, which shows increased expression of catalase mRNA in response to oxidative stress (*28*). Most immortalized cell lines, including the HepG2 cell line, are hyperdiploid and perform asymmetrical cell divisions, often rendering a proportion of the cells in culture dysfunctional and in various stages of cell death. The process of programmed cell death (apoptosis) leads to some production of ROS by the cell's mitochondria (*29*). A number of berry extracts have been found to induce apoptosis in several types of tumor cell lines (*30–32*). If an antioxidant-rich natural product reduces ROS production in such a cell model (*33*), the interpretations are far from simple. One possible interpretation is that the product possibly protects tumor cells, which is not a desirable conclusion.

In this study, we used three methods sequentially to examine antioxidant and anti-inflammatory properties of four selected natural products. The products have very different ORAC values, and yet the ORAC value alone did not do each product justice with regard to their overall biological effect. The product Immunel had the lowest ORAC value among the four products tested but performed best in the CAP-e assay at low doses. In addition, Immunel supported the innate immune response by providing a mild but significant enhancement of PMN function, as seen by a negative percent inhibition of ROS formation (**Figure 2**). The induction of ROS formation by PMN cells should not be interpreted on its own as a damaging effect but should be seen as an activation of one of our innate immune defense mechanisms against bacterial invaders. It is interesting that while such an effect may be beneficial, the resulting risk of oxidative damage may be buffered by antioxidants in the same complex natural product.

Both açai and HAJF showed a more linear dose-response in the CAP-e assay, but they each performed differently in the ROS PMN assay. açai drastically reduced the PMN response across a wide dose range, and therefore, we expect that açai possesses strong anti-inflammatory properties in vivo. HAJF showed a more complex effect of PMN cells, indicating that different compounds in the product support different aspects of PMN cell biology. HAJF showed an opposite dose-response in the PMN-based cellular assay (i.e., more effect at lower doses) possibly indicating opposing effects between different compounds in HAJF, likely involving (1) antioxidants, (2) activating compounds, and (3) anti-inflammatory signaling compounds, as outlined in **Figure 1C**. Using the EpiCor yeast extract in the ROS PMN assay under two parallel culture conditions, we were able to show that EpiCor had immunomodulatory effects on PMN cells. These effects prevented detection of EpiCor's antioxidant capacity in the PMN-cell based assay, unless cytoskeletal rearrangement was inhibited by sodium azide, thus abrogating many aspects of cellular signaling and intracellular transport necessary for ROS formation. Under those conditions, the PMN-based assay could demonstrate the antioxidant capacity of EpiCor, in parallel to the RBC-based CAP-e assay. Interestingly, at the highest dose, the inhibition of ROS formation by PMN cells was greater than what could be accounted for by the protection from hydroxyl radicals in the CAP-e assay. It is possible that EpiCor provides a better protection from peroxyl free radicals, and work is in progress to adapt the CAP-e assay to include protection from peroxyl free radicals. However, the possibility also exists that EpiCor may be sending an anti-inflammatory signal to the PMN cells, thus serving to illustrate all three aspects of PMN responses to compounds in natural products, as outlined in **Figure 1**.

The CAP-e assay has use beyond in vitro evaluation of natural products. The assay was applied to serum samples in a double-blinded, placebo-controlled, clinical pilot study examining the antioxidant uptake after consumption of the açai-rich juice MonaVie Active (*34*). Using the CAP-e assay in parallel to an assay for serum lipid peroxidation, we showed that consumption of the juice resulted in a statistically significant improvement of serum antioxidant status within 2 h after consumption. A single laboratory validation manuscript is currently being prepared to document the CAP-e method for broader use in the natural products industry, including parallel testing with hydroxyl and peroxyl free radical generators, adaptation to an accelerated testing system (*21*), dose-response of a panel of antioxidant standards, and the effects of excipients on bioavailability at the cellular level. Standards regarding assay procedures, choice of cellular models, and the appropriate use and interpretation of nonlinear dose−responses in cellular models are needed to ensure more consistency in reporting of natural products in biological systems.

We have found the following sequential, multifacetted strategy highly useful for the testing of natural products: (1) ORAC, (2) CAP-e, and (3) ROS PMN tests. When these tests were used sequentially, a foundation of understanding was generated, providing a more comprehensive understanding of antioxidant, anti-inflammatory, and immunomodulatory effects. The four test products used in this paper serve to illustrate different ways to look at complex antioxidant-containing natural products. The data demonstrates the frequent nonlinearity of data in biological systems where the total observed effect depends on the interaction between compounds in the test products and the nature of the cell model and cannot be analyzed by the more simple approaches used in analytical chemistry.

ABBREVIATIONS USED

CAP, cell-based antioxidant protection assay; DCF-DA, dichlorofluorescein diacetate; ORAC, oxygen radical absorbance capacity assay; PMN, polymorphonuclear cells; RBC, red blood cells; ROS, reactive oxygen species.

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LITERATURE CITED

(1) Flora, S. J. Role of free radicals and antioxidants in health and disease. *Cell. Mol. Biol.* **2007**, *53*, 1–2.

(2) Martínez, J. A. Mitochondrial oxidative stress and inflammation: An slalom to obesity and insulin resistance. *J. Physiol. Biochem.* **2006**, *62*, 303–306.

(3) Larbi, A.; Kempf, J.; Pawelec, G. Oxidative stress modulation and T cell activation. *Exp. Gerontol.* **2007**, *42*, 852–858.

(4) Victor, V. M.; Rocha, M. Targeting antioxidants to mitochondria: A potential new therapeutic strategy for cardiovascular diseases. *Curr. Pharm. Des.* **2007**, *13*, 845–863.
(5) Swerdlow, R. H. Treating neurodegeneration by modifying mitochondria: Potential solutions to a "complex" problem. *Antioxid. Redox Signaling* **2007**, *9*, 1591–1603.
(6) Klaunig, J. E.; Kamendulis, L. M. The role of oxidative stress in carcinogenesis. *Annu. Rev. Pharmacol. Toxicol.* **2004**, *44*, 239–267.
(7) Federico, A.; Morgillo, F.; Tuccillo, C.; Ciardiello, F.; Loguercio, C. Chronic inflammation and oxidative stress in human carcinogenesis. *Int. J. Cancer* **2007**, *121*, 2381–2386.
(8) Cao, G.; Prior, R. L. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **1998**, *44*, 1309–1315.
(9) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.
(10) Ou, B.; Huang, D.; Hampsch-Woodill, M. Method for assaying the antioxidant capacity of a sample. U.S. Patent 7132296 B2.
(11) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619–4626.
(12) Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273–3279.
(13) Prior, R. L.; Gu, L.; Wu, X.; Jacob, R. A.; Sotoudeh, G.; Kader, A. A.; Cook, R. A. Plasma antioxidant capacity changes following a meal as a measure of the ability of a food to alter in vivo antioxidant status. *J. Am. Coll. Nutr.* **2007**, *26*, 170–181.
(14) Gurtovenko, A. A.; Anwar, J. Modulating the structure and properties of cell membranes: The molecular mechanism of action of dimethyl sulfoxide. *J. Phys. Chem. B* **2007**, *111*, 10453–10460.
(15) Di, L.; Kerns, E. H. Biological assay challenges from compound solubility: Strategies for bioassay optimization. *Drug Discovery Today* **2006**, *11*, 446–451.
(16) Williams, A. C.; Barry, B. W. Penetration enhancers. *Adv. Drug Delivery Rev.* **2004**, *56*, 603–618.
(17) Santos, N. C.; Figueira-Coelho, J.; Martins-Silva, J.; Saldanha, C. Multidisciplinary utilization of dimethyl sulfoxide: Pharmacological, cellular, and molecular aspects. *Biochem. Pharmacol.* **2003**, *65*, 1035–1041.
(18) Remick, D. G.; Villarete, L. Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. *J. Leukocyte Biol.* **1996**, *59*, 471–475.
(19) Gibson, G. E.; Huang, H. M. Mitochondrial enzymes and endoplasmic reticulum calcium stores as targets of oxidative stress in neurodegenerative diseases. *J. Bioenerg. Biomembr.* **2004**, *36*, 335–340.
(20) Buehler, P. W.; Alayash, A. I. Redox biology of blood revisited: The role of red blood cells in maintaining circulatory reductive capacity. *Antioxid. Redox Signaling* **2005**, *7*, 1755–1760.
(21) Jensen, G. S. *Cell-based antioxidant protection assay*. U.S. Provisional patent application No.60/985,166.
(22) Schauss, A. G.; Wu, X.; Prior, R. L.; Ou, B.; Huang, D.; Owens, J.; Agarwal, A.; Jensen, G. S.; Hart, A. N.; Shanbrom, E. Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* mart. (açai). *J. Agric. Food Chem.* **2006**, *54*, 8604–8610.
(23) Patterson, K. M.; Yoon, I.; Jensen, G. S. Yeast culture has anti-inflammatory effects and specifically activates NK cells. *Comp. Immunol. Microbiol. Infect. Dis.* In press (doi:10.1016/j.cimid.2007.08.005).
(24) Jensen, G. S.; Hart, A. N.; Schauss, A. G. An anti-inflammatory immunogen from yeast culture induces activation and alters chemokine receptor expression on human natural killer cells and B lymphocytes in vitro. *Nutr. Res. (N. Y.)* **2007**, *27*, 327–335.
(25) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50*, 1815–1821.
(26) Ou, B.; Hampsch-Woodill, M.; Flanagan, J.; Deemer, E. K.; Prior, R. L.; Huang, D. Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. *J. Agric. Food Chem.* **2002**, *50*, 2772–2777.
(27) Patterson, K. M.; Carter, S. G.; Jensen, G. S. A novel fraction from bovine colostrum whey supports phagocytosis and natural killer cell activation while protecting cells from oxidative damage in vitro. Manuscript submitted for publication.
(28) Cuthbert, C.; Wang, Z.; Zhang, X.; Tam, S. P. Regulation of human apolipoprotein A-I gene expression by gramoxone. *J. Biol. Chem.* **1997**, *272*, 14954–14960.
(29) Jou, M. J.; Jou, S. B.; Chen, H. M.; Lin, C. H.; Peng, T. I. Critical role of mitochondrial reactive oxygen species formation in visible laser irradiation-induced apoptosis in rat brain astrocytes (RBA-1). *J. Biomed. Sci.* **2002**, *9*, 507–516.
(30) Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9339.
(31) Mertens-Talcott, S. U.; Lee, J. H.; Percival, S. S.; Talcott, S. T. Induction of cell death in Caco-2 human colon carcinoma cells by ellagic acid rich fractions from muscadine grapes (*Vitis rotundifolia*). *J. Agric. Food Chem.* **2006**, *54*, 5336–5343.
(32) Bermudez-Soto, M. J.; Larrosa, M.; Garcia-Cantalejo, J. M.; Espin, J. C.; Tomas-Barberan, F. A.; Garcia-Conesa, M. T. Up-regulation of tumor suppressor carcinoembryonic antigen-related cell adhesion molecule 1 in human colon cancer Caco-2 cells following repetitive exposure to dietary levels of a polyphenol-rich chokeberry juice. *J. Nutr. Biochem.* **2006**, *18*, 259–271.
(33) Wolfe, K. L.; Liu, R. H. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *J. Agric. Food Chem.* **2007**, *55*, 8896–8907.
(34) Jensen, G. S.; Patterson, K. M.; Barnes, J.; Carter, S. G.; Wu, X.; Scherwitz, L.; Beaman, R.; Endres, J. R.; Schauss, A. G. In Vitro and in vivo antioxidant and anti-inflammatory capacity of a juice containing the Amazonian palm berry, açai (*Euterpe oleracea Mart.*). Manuscript submitted for publication.

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