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. Acai 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 pharmacological 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
protection in the blood (biologically relevant, as RBC play critical roles in antioxidant how well a substance protects against oxidative stress is 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 (H2O2),

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

<table>
<thead>
<tr>
<th></th>
<th>chemical assays</th>
<th>cell-based assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>assay principles</td>
<td>based on known chemical reactions</td>
<td>based on interaction between added compounds and complex enzymatic reactions in biological system</td>
</tr>
<tr>
<td>extraction of active ingredients</td>
<td>between limited number of reagents</td>
<td>assay must take place in physiological saline solution; limited use of solvents</td>
</tr>
<tr>
<td>use of alcohol-based solvents</td>
<td>some flexibility</td>
<td>yes, if properly diluted, tested for tolerance, and altered cellular behavior (depends on assay)</td>
</tr>
<tr>
<td>dimethyl sulfoxide (DMSO) as solvent</td>
<td>yes, with appropriate controls, if no interference with chemical reactions in assay; DMSO is a free radical scavenger (17)</td>
<td>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)</td>
</tr>
<tr>
<td>data analysis and interpretation</td>
<td>quantitative</td>
<td>qualitative</td>
</tr>
<tr>
<td>expectation of linear dose- responses</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>applicability of area-under-curve</td>
<td>yes (11)</td>
<td>no</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>enzyme</th>
<th>role for normal cell physiology</th>
<th>RBC</th>
<th>PMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>glutathione peroxidase</td>
<td>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)</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>glutathione reductase</td>
<td>maintains glutathione in its reduced state</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>catalase</td>
<td>breakdown of hydrogen peroxide to oxygen and water</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>superoxide dismutase</td>
<td>catalyzes dismutation of superoxide to oxygen and hydrogen peroxide</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>myeloperoxidase</td>
<td>produces hypochlorous acid (HOCI) from hydrogen peroxide (H2O2) and chloride anion (Cl- ) during the neutrophil’s respiratory burst; oxidizes tyrosine to tyrosyl radical using hydrogen peroxide</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>cyclooxygenase COX-1</td>
<td>contain both cyclooxygenase and peroxidase properties; catalyzes the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>constitutively expressed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyclooxygenase COX-2</td>
<td>contain both cyclooxygenase and peroxidase properties; catalyzes the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>induced upon inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hemoglobin/deoxyhemoglobin</td>
<td>scavenging and transport of O2, CO2, NO^-</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>

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).
Antioxidants in solution inhibit an oxidative chemical reaction. (A) Those antioxidants able to enter red blood cells protect the cells from oxidative damage. (B) Several different compounds in a natural product may have different/opposing effects.

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.

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% CO₂ 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 H₂O₂ 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 H₂O₂-challenged cells were analyzed by flow cytometry. Data was collected in triplicate for controls and in duplicate for each sample concentration. The mean fluorescence intensity (MFI) of red blood cells was compared between untreated, H₂O₂-treated, and cells pretreated with test products. A reduction in MFI in samples pretreated with test products prior to challenge with H₂O₂ 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% CO₂ 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 H₂O₂ 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 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, H₂O₂-treated, and cells pretreated with test products. A reduction in MFI in samples pretreated with test products prior to challenge with H₂O₂ 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 H₂O₂ 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%.

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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 açaí were previously published (22) and are reprinted here with the journal’s permission. Data were obtained for all four natural products from ORAC<sub>hydrophilic</sub> testing using fluorescein as the fluorescent probe and 2,2'-azobis(2-amidinopropane) dihydrochloride as a peroxyl radical generator (71). ORAC<sub>lipophilic</sub> 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, 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 OptiAçaí from *E. oleracea* (açaí), 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).

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

<table>
<thead>
<tr>
<th>Natural Product</th>
<th>ORAC&lt;sub&gt;hydrophilic&lt;/sub&gt; (µmol TE/g)</th>
<th>ORAC&lt;sub&gt;lipophilic&lt;/sub&gt; (µmol TE/g)</th>
<th>TAC (µmol TE/g)</th>
<th>NORAC (µmol TE/g)</th>
<th>HORAC (µmol GAE/g)</th>
<th>SOD (unit/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunel</td>
<td>18</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18</td>
<td>0.59</td>
<td>1.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>OptiAçaí&lt;sup&gt;b&lt;/sup&gt;</td>
<td>997</td>
<td>30</td>
<td>1027</td>
<td>34</td>
<td>52</td>
<td>1614</td>
</tr>
<tr>
<td>HA Joint Formula</td>
<td>2219</td>
<td>139</td>
<td>2350</td>
<td>372</td>
<td>123</td>
<td>33</td>
</tr>
<tr>
<td>EpiCor</td>
<td>614</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>614</td>
<td>54</td>
<td>214</td>
<td>2200</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND = not detected. <sup>b</sup> The antioxidant capacity for OptiAçaí was previously published in ref 22.

**Comparison of CAP-e Data to the Effect on ROS Formation in Human Polyomorphonuclear (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 H₂O₂ treatment resulted in product-specific effects on ROS production.

ImmuneI used for this study had a moderate ORAC<sub>hydrophilic</sub> 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).

Açaí has a high ORAC<sub>hydrophilic</sub> 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<sub>hydrophilic</sub> 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<sub>hydrophilic</sub> 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
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 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.

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.

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
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 immortal-
ized 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,
yet the ORAC value alone did not do each product justice with
regard to their overall biological effect. The product
Immune had the lowest ORAC value among the four products
tested but performed best in the CAP-e assay at low doses. In
addition, Immune 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 com-
ounds 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. Interest-
ingly, 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 hy-
droxyl 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 bioavail-
ability at the cellular level. Standards regarding assay proce-
dures, choice of cellular models, and the appropriate use and
interpretation of nonlinear dose—response in cellular models
are needed to ensure more consistency in reporting of natural
products in biological systems.

We have found the following sequential, multifaceted
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,
dichlorodihydrofluorescein diacetate; ORAC, oxygen radical absorbance
capacity assay; PMN, polymorphonuclear cells; RBC, red blood
cells; ROS, reactive oxygen species.

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