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Comparison of the Total Oxyradical Scavenging Capacity and Oxygen Radical Absorbance Capacity Antioxidant Assays

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ABSTRACT Epidemiological studies have shown that phytochemicals in fruits and vegetables may decrease the incidence of cancer. The antioxidant activity of phytochemicals may be partially responsible for the reduced cancer risk. In this study, the antioxidant activity of several phytochemicals was compared using two different antioxidant assays: the oxygen radical absorbance capacity (ORAC) assay, which measures the decrease in fluorescence decay caused by antioxidants, and the total oxyradical scavenging capacity (TOSC) assay, which measures the decrease in ethylene production caused by antioxidants. TOSC and ORAC values were measured for 11 different phytochemicals, and values were expressed as µmol of Trolox equivalents/mg. As expected, a correlation was seen between the TOSC values and the ORAC values (R² = 0.60). Quercitin, maritime pine bark extract (Pycnogenol®, Horphag Research Ltd., Geneva, Switzerland), grape skin extract, and green tea polyphenols had the highest overall antioxidant activity of the 11 phytochemicals measured. Lemon fruit and citrus bioflavonoids had the lowest overall antioxidant activity. Rutin and α-lipoic acid had low ORAC values but high TOSC values when compared to the other phytochemicals. The correlation between the in vitro TOSC and ORAC antioxidant assays suggests that both assays may be useful in identifying phytochemicals with high antioxidant activity.

KEY WORDS: antioxidant activity • grape seed • grape skin • green tea polyphenols • α-lipoic acid • phytochemicals • Pycnogenol • quercitin

INTRODUCTION

To assess the risk of chronic life-style diseases, many epidemiological studies have been completed. These studies show that diets high in fruits and vegetables significantly reduce the occurrence of chronic diseases.1-3 It has been suggested that the antioxidants in fruits and vegetables may help to prevent the onset of atherosclerosis and carcinogenesis by quenching free radicals, which are capable of damaging DNA and cell membranes.4-6 Antioxidants can also affect intracellular signaling pathways. For example, many antioxidants, such as green tea polyphenols, quercitin, and α-lipoic acid, inhibit inflammation, angiogenesis, and metastasis.7,8-13 In addition, quercitin, green tea, and grape seed extract can modulate cellular proliferation, cell cycle arrest, and apoptosis.1,2,4,9,14-16 Green tea has also been implicated in the metabolism of carcinogens.14,16-18

Several assays have been designed that can measure the antioxidant activity of phytochemicals. Many of these assays measure the inhibition of a test reaction by antioxidant compounds.19,20 However, not all antioxidant assays give the same antioxidant activity trends.21,22 Consequently, multiple antioxidant assays should be used to resolve questions regarding the true activity of antioxidants.23

In this study, 11 phytochemicals with significant antioxidant activity were identified by both the total oxyradical scavenging capacity (TOSC) assay (which uses gas chromatography) and the oxygen radical absorbance capacity (ORAC) assay (which uses a multifuorescence plate reader). In the TOSC assay, the thermal homolysis of 2,2'-azobis(2-methyl-propionamidine) (ABAP) dihydrochloride generates peroxyl radicals, which then oxidize α-keto-γ-(methylthio)butyric acid sodium salt (K MBA) to produce ethylene gas. The resulting ethylene gas production is measured by gas chromatography. Antioxidants, when present, quench peroxyl radicals and inhibit the ethylene production. The ORAC assay is widely accepted for measuring the antioxidant activity of phytochemicals.24 Using the ORAC assay, the free radical initiator 2,2'-azobis(2-amidinopropane) (AAPH) dihydrochloride decreases the fluorescence of a compound such as β-phycoerythrin or fluorescein. Antioxidants quench the AAPH radicals and slow down the fluorescence decay.25 Hence, the ORAC assay measures the interruption of free radical reactions by the absorption of peroxyl radicals.26 A sensitive and reliable way to determine the ORAC value of antioxidants was first developed in 1993 using β-
phycoerythrin. This assay was then automated with the help of the COBAS FARA™ II centrifugal analyzer (Hoffmann-La Roche, Basel, Switzerland). An additional improvement to the ORAC assay was developed and validated using fluorescein as the fluorescent probe instead of β-phycoerythrin. Fluorescein has several advantages: it is less expensive, does not interact with other compounds, and does not photo-bleach. To further increase the assay efficiency, a high throughput assay was designed using the Bio-Tek (Winsko, VT) Precision™ 2000 microplate fluorescence reader and 96-well plates. Our current high throughput protocol uses the BMG Labtech (Durham, NC) FLUOstar Optima fluorescence microplate reader.

The TOSC and ORAC assays each have unique advantages. The TOSC assay can distinguish between fast-acting and slow-acting antioxidants, measure both water- and lipid-soluble antioxidants, and measure the antioxidant status of biological tissues. On the other hand, the ORAC assay can accurately measure both the inhibition time and the inhibition degree of antioxidants to provide an accurate measurement of antioxidant activity. However, the increased sensitivity of the ORAC assay means that test compounds need to be approximately 10 times less concentrated than those tested by the TOSC assay. The ORAC assay is also sensitive to temperature variations (a problem solved by pre-heating the 48 well plates) and cannot measure a complete range of biologically relevant radicals, since it only uses peroxyl radicals. In addition, fluorescein is pH sensitive, so its pH must be kept above 7.

In this study, we compared the antioxidant values of 11 phytochemicals using the TOSC and ORAC assays.

MATERIALS AND METHODS

The phytochemicals analyzed in this study were (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark theraplant, (6) quercetin, (7) maritime pine bark extract (Pycnogenol® Harphag Research Ltd., Geneva, Switzerland), (8) grape skin extract, (9) rutin, (10) α-lipoic acid, and (11) green tea polyphenols. Each compound was prepared for the TOSC and ORAC assays by adding 0.05 g to a total of 1.5 mL of deionized water (1.5 mL of dimethyl sulfoxide was used for α-lipoic acid). In addition, 1:10 and 1:100 dilutions of the samples were prepared using deionized water for the TOSC assay, and 1:1,000 dilutions of the samples were prepared using deionized water for the ORAC assay.

**TOSC assay**

KMBA, ABAP, and Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were obtained from Sigma-Aldrich (St. Louis, MO). Then, in 10-mL glass vials, 100 μL of potassium phosphate buffer (100 mM), 100 μL of KMBA (20 mM), and 10 μL of sample were added, and deionized water was added up to 900 μL total volume. Next, the vials were sealed and incubated at 37°C. After 5 minutes, the reaction was initiated by injecting 100 μL of ABAP (200 mM) into the vials.

Samples were assayed by an HP5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) with a 6-foot Supelco (Bellevonte, PA) Porapak Q column. Temperatures were set to 160°C for the injector port, 60°C for the oven, and 220°C for the flame ionization detector. Mobile-phase helium (30 mL/minute) was used as a carrier gas.

At 12 minutes following the ABAP injections, a 1-mL aliquot of gas from each vial was injected into the gas chromatograph. This was repeated seven times at 12-minute intervals. The area under the resulting peaks was integrated by ChemStation software (Agilent Technologies, Santa Clara, CA) to determine the ethylene production.

**Computation of TOSC values**

The amount of ethylene measured at each time point was plotted, and the resulting ethylene production graphs showed an increase in ethylene production over time. The total amount of ethylene produced for each control and sample was then obtained for each 96-minute run. TOSC values for each sample (including the sample dilutions) were calculated using the formula of Winston et al. TOSC = 100 − (area under sample curve/area under control curve × 100).

The TOSC values for the samples and for the sample dilutions were averaged, and these values were plotted versus the amount of sample on a concentration scale, with the samples having a relative concentration of 100 and the sample dilutions having relative concentrations of 10 and 1. A relative TOSC value for each sample was calculated by extrapolation of the best-fit regression line to obtain the expected TOSC value for the least concentrated sample dilution. The expected TOSC value for the least concentrated sample dilution was then scaled to obtain the expected TOSC value for 1 mg of sample. The expected TOSC value for 1 mg of sample was then divided by the TOSC value for a 1 μM concentration of Trolox (a water-soluble vitamin E analog) (previously measured at 4.37) to obtain the values in μmol of Trolox equivalents/mg.

**ORAC assay**

The ORAC assay was performed with some modifications from previously employed methods. Trolox and fluorescein sodium salt were obtained from Sigma-Aldrich. AAPH dihydrochloride was purchased from Waco Chemicals USA (Richmond, VA). All assays were performed on a FLUOstar Optima fluorescence microplate reader equipped with multiple injectors that had fluorescence filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Clear polystyrene 48-well plates were used, and each well contained a reaction mixture of 150 μL of AAPH, 400 μL of fluorescein, and 40 μL of sample. Phosphate buffer (75 mM, pH 7.4) was used as the blank, and a Trolox dilution series (50, 25, 12.5, and 6.25 μM) were used to produce a standard. The phos-
phate buffer was kept at 37°C for 30 minutes to decrease variability.25

During cycle 2, 400 μL of fluorescein solution was injected into the respective wells at a final concentration of 14 μM. During cycle 4, the reaction was initiated by injecting 150 μL of AAPH into the respective wells at a final concentration of 4.8 mM. Fluorescence decay was measured every 3.5 minutes, up to 35 cycles.

Computation of ORAC values

The area under the fluorescence kinetic curve (AUC) for each sample was calculated as:

\[ AUC = (0.5 + f_0 / f_4 + f_0 / f_4 + f_0 / f_4 + \ldots + f_0 / f_4) \times CT \]

where \( f_0 \) = initial fluorescence reading at cycle 4, \( f_i \) = fluorescence reading at cycle \( i \), and CT = cycle time in minutes.25 Net AUC values for the samples were determined by subtracting the AUC of the blank from that of each of the samples. The net AUC values for the samples were compared to the net AUC values for the Trolox standard, and the final results were expressed as μmol of Trolox equivalents/mg.

RESULTS

The antioxidant activity of the 11 phytochemicals was measured using the TOSC and ORAC assays. The TOSC assay was repeated five times for each phytochemical, and the ORAC assay was repeated eight times. The values were expressed as μmol of Trolox equivalents/mg and then scaled to percentages for each assay to facilitate interassay comparisons, with the minimum value for each assay converted to 0% and the maximum value for each assay converted to 100% (Fig. 1). Quercitin, Pycnogenol, grape skin extract, and green tea polyphenols had the highest overall antioxidant activity as measured by the TOSC and ORAC assays. Lemon fruit and citrus bioflavonoids had the lowest overall antioxidant activity (Fig. 1).

After measurement of the antioxidant activity of the selected phytochemicals by the TOSC and ORAC assays, the average antioxidant activity as measured by each of the two assays was then compared to determine the correlation between the assays (Fig. 2). Quercitin, Pycnogenol, grape skin extract, and green tea polyphenols had high TOSC values and high ORAC values. Rutin and α-lipoic acid had high TOSC values but low ORAC values. Lemon fruit and citrus bioflavonoids had low TOSC values and low ORAC val-

![FIG. 1. Antioxidant activity of 11 phytochemicals measured by the TOSC assay (□) and by the ORAC assay (▪). Minimum and maximum values obtained for each assay were scaled to 0% and 100%, respectively, to facilitate interassay comparisons. The phytochemicals are numbered as follows: (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark therapant, (6) quercitin, (7) Pycnogenol, (8) grape skin extract, (9) rutin, (10) α-lipoic acid, and (11) green tea polyphenols. Values reflect the mean and standard deviation.](image-url)
ues. Pomegranate, grape seed extract, and pine bark thera-
plant had moderately high TOSC values and moderately
high ORAC values. The $R^2$ was 0.60. When three potential
outliers (rutin, $\alpha$-lipoic acid, and green tea polyphenols)
were removed from the comparison between the two assays,
the $R^2$ improved to 0.94 (Fig. 3).

The data for each phytochemical as measured by the an-
tioxidant assays are provided in tabular format (Tables 1
and 2). Table 1 lists the antioxidant activity values for the eight
phytochemicals whose antioxidant activity shows a high de-
gree of correlation between the two assays. Table 2 lists the
antioxidant activity values for the remaining three phyto-
chemicals whose antioxidant activity shows less correlation
between the two assays. In Tables 1 and 2, TOSC and ORAC
values are expressed in $\mu$mol of Trolox equivalents/mg.
Green tea polyphenols had the highest TOSC value of $3.78 \times
10^3$ $\mu$mol of Trolox equivalents/mg, as well as the highest
ORAC value, at $1.01 \times 10^3$ $\mu$mol of Trolox equivalents/mg.
$\alpha$-Lipoic acid had the second highest TOSC value of $3.38 \times
10^3$ $\mu$mol of Trolox equivalents/mg, as well as the second
highest ORAC value, at $2.32 \times 10^4$ $\mu$mol of Trolox equiv-
alents/mg. Lemon fruit had the lowest TOSC value of $1.03 \times
10^2$ $\mu$mol of Trolox equivalents/mg and the lowest ORAC
value of $3.89 \times 10^1 \mu$mol of Trolox equivalents/mg.

DISCUSSION

The measurement of antioxidant activity in vitro is the
first step towards identifying the most promising phyto-
chemicals for further in vivo testing. High antioxidant ac-
tivity has previously been reported for the compounds
quercitin and rutin, suggesting that they may be promising
antioxidants.\textsuperscript{19,31,34,35} The results of this study show
quercitin to have moderately high antioxidant activity (Fig.
1). Several explanations exist for the moderately high an-
tioxidant activity seen in this study for quercitin. For ex-
ample, quercitin is planar, with a completely conjugated
electron system, a free 3-OH group, and increased flavonoid
radical stability.\textsuperscript{19,31,36,37}

$\alpha$-Lipoic acid has previously been reported to be a potent
scavenger of hydroxyl radicals, as well as an effective per-
oxyl radical scavenger.\textsuperscript{12} The results of this study using per-
oxyl radicals are inconclusive for $\alpha$-lipoic acid and for rutin
(Figs. 1 and 2). The TOSC assay suggests that they have
high antioxidant activity, while the ORAC assay suggests
that they have low antioxidant activity.\textsuperscript{32} The cause of the
discrepancy between the two assays is not known. One pos-
sibility is that $\alpha$-lipoic acid was the only hydrophobic phy-
tochemical analyzed and had to be dissolved in dimethyl

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**FIG. 2.** Moderate correlation between average TOSC assay values and average ORAC assay values for 11 phytochemicals. ORAC values are in $\mu$mol of Trolox equivalents/mg and are the average of several replicates using the ORAC assay. TOSC values are in $\mu$mol of Trolox equivalents/mg and are the average of several replicates using the TOSC assay. $R^2 = 0.60$. The phytochemicals are numbered as follows: (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark theraplnet, (6) quercitin, (7) Pycnogenol, (8) grape skin ex-
tract, (9) rutin, (10) $\alpha$-lipoic acid, and (11) green tea polyphenols.
FIG. 3. High correlation between average TOSC assay values and average ORAC assay values for eight phytochemicals. ORAC values are in μmol of Trolox equivalents/mg and are the average of several replicates using the ORAC assay. TOSC values are in μmol of Trolox equivalents/mg and are the average of several replicates using the TOSC assay. \( R^2 = 0.94 \). The phytochemicals are numbered as follows: (1) lemon fruit, (2) citrus bioflavonoids, (3) pomegranate, (4) grape seed extract, (5) pine bark therapeutant, (6) quercetin, (7) Pycnogenol, and (8) grape skin extract.

sulfoxide. Rutin, however, was water-soluble and was also measured as having a high TOSC and low ORAC value.

This study found pomegranate to have moderately low antioxidant activity, while Pycnogenol had moderately high antioxidant activity (Fig. 1). Pomegranate contains three major anthocyanidins (pelargonidin, cyanidin, and delphinidin), as well as catechins and ellagic acid, which is why its low antioxidant activity was surprising. The water-soluble Pycnogenol (from fresh pine bark of *Pinus maritima*), however, consisted of condensed flavonoids (procyanidins and proanthocyanidins), as well as catechin, epicatechin, and taxifolin, which may explain why it exhibited higher antioxidant activity.

The *in vitro* assays mentioned in this study (Fig. 1) consistently suggest that quercitin, Pycnogenol, grape skin extract, and green tea polyphenols have much higher antioxidant activity than Trolox, a hydrophilic vitamin E analog. Alternatively, lemon fruit and citrus bioflavonoids have only low antioxidant activity.

More variability in antioxidant activity values can be seen for the TOSC assay than for the ORAC assay (Fig. 1). This variability may be due to manual error in the injection process into the gas chromatograph. As a result, the TOSC results may be more qualitative than quantitative (Agilent Technologies technical support, 2003). In contrast, the ORAC assay was performed using an automated fluorescence plate reader. Despite such fluctuations, the ranking of antioxidants by antioxidant activity was similar among replicates.

Establishing specific numbers for the antioxidant activity of phytochemicals is difficult, since the antioxidant activity measured for a phytochemical depends greatly on the conditions under which its corresponding fruit or vegetable is cultivated. Specific numbers for antioxidant activity also differ depending upon the assay being employed. A recent study of sorghum and sorghum products compared the ORAC assay with the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and 1,1-diphenyl-2-picrylhydrazyl assays. The ORAC values measured in the study were generally three to four times higher than the 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) values, even though all of the values were expressed as μmol of Trolox equivalents/g of sample. This led the authors to conclude that “the relative rank in activity of different samples across methods is more relevant than absolute values for comparing activities.”

The ORAC values for the 11 phytochemicals were approximately 19 times higher than the TOSC values (Tables 1 and 2); however, the ranking of antioxidants by antioxidant activity was similar. Several explanations exist for the
TABLE 1. ANTIOXIDANT ACTIVITY VALUES FOR EIGHT PHYTOCHEMICALS WHOSE ANTIOXIDANT ACTIVITY AS MEASURED BY THE TOSC AND ORAC ASSAYS SHOWS HIGH CORRELATION

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>TOSC</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon fruit</td>
<td>103</td>
<td>3,885</td>
</tr>
<tr>
<td>Citrus bioflavonoids</td>
<td>446</td>
<td>7,410</td>
</tr>
<tr>
<td>Pomegranate</td>
<td>1,543</td>
<td>22,530</td>
</tr>
<tr>
<td>Grape seed extract</td>
<td>2,294</td>
<td>37,125</td>
</tr>
<tr>
<td>Pine bark therapeutant</td>
<td>2,608</td>
<td>59,483</td>
</tr>
<tr>
<td>Quercitin</td>
<td>3,059</td>
<td>63,750</td>
</tr>
<tr>
<td>Pycnogenol</td>
<td>3,154</td>
<td>53,970</td>
</tr>
<tr>
<td>Grape skin extract</td>
<td>3,246</td>
<td>63,668</td>
</tr>
</tbody>
</table>

The phytochemicals were measured for antioxidant activity using the TOSC and ORAC antioxidant activity assays. TOSC values are the average of several replicates using the TOSC assay, and ORAC values are the average of several replicates using the ORAC assay.

discrepancy in the observed absolute values. First, different radical sources were used for the two assays (ABAP was used for the TOSC assay, while AAPH was used for the ORAC assay). Second, the ORAC samples were at least 10 times less concentrated than the TOSC samples because of the enhanced sensitivity of the ORAC assay. Slight differences from the less concentrated ORAC assay samples may have been magnified when the ORAC values were scaled up to μmol of Trolox equivalents/mg.

The in vitro antioxidant activity values measured in this study (Tables 1 and 2) should not be assumed to reflect the activity of the antioxidants in vivo. In vivo results do suggest that those who consume more fruits and vegetables have higher antioxidant values in their plasma (as measured by the ORAC assay). However, synergy frequently occurs between multiple antioxidants in vivo, leading to greater than expected results.

TABLE 2. ANTIOXIDANT ACTIVITY VALUES FOR THREE PHYTOCHEMICALS WHOSE ANTIOXIDANT ACTIVITY AS MEASURED BY THE TOSC AND ORAC ASSAYS SHOWS LESS CORRELATION

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>TOSC</th>
<th>ORAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>2,727</td>
<td>25,440</td>
</tr>
<tr>
<td>α-Lipoic acid</td>
<td>3,381</td>
<td>23,235</td>
</tr>
<tr>
<td>Green tea polyphenols</td>
<td>3,777</td>
<td>100,688</td>
</tr>
</tbody>
</table>

The phytochemicals were measured for antioxidant activity using the TOSC and ORAC antioxidant activity assays. TOSC values are the average of several replicates using the TOSC assay, and ORAC values are the average of several replicates using the ORAC assay.

The potential in vivo antioxidant activity of the phytochemicals measured in this study may also be affected by the bioavailability of the antioxidants consumed, as well as by their metabolites. Low bioavailability may lead to lower than expected antioxidant activity in vivo. For example, some flavonoids must first be degraded into smaller compounds before they can be absorbed across the intestinal wall. The potential in vivo antioxidant activity of phytochemicals may also be decreased by nearby proteins, which can mask the total antioxidant capacity.

Additional research is needed to determine how antioxidants modulate a wide range of effects in vivo. In addition, in vitro assays such as those described in this study are needed to help determine which compounds should be studied in vivo. As hypothesized, the in vitro assays in this study generally ranked the antioxidant activity of the phytochemicals in the same order (Figs. 1 and 2). Some intriguing discrepancies, however, were seen between the TOSC and ORAC assays (Figs. 1 and 2). This suggests that in some cases the results from both assays may be useful in determining an antioxidant’s true activity. Further research is needed to determine why the two assays suggest different antioxidant activity values for some compounds, particularly in the case of rutin and α-lipoic acid (Table 2). Despite the discrepancies seen between the TOSC and ORAC assays, the results of this study suggest that quercitin, Pycnogenol, grape skin extract, and green tea polyphenols are excellent antioxidants worthy of continued investigation (Fig. 2).

REFERENCES


