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Sequestering Ability of Butylated Hydroxytoluene, Propyl Gallate, Resveratrol, and Vitamins C and E against ABTS, DPPH, and Hydroxyl Free Radicals in Chemical and Biological Systems

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The antioxidant capacity of butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-*p*-cresol), propyl gallate (3,4,5-trihydroxybenzoic acid *n*-propyl ester), resveratrol (*trans*-3,4′,5-trihydroxystilbene), and vitamins C (L-ascorbic acid) and E [**(**+**)**-α-tocopherol] was studied in chemical and biological systems. The chemical assays evaluated the capacity of these antioxidants to sequester 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS•) and 1,1 diphenyl-2-picrylhydrazyl (DPPH•). A new colorimetric method to determine hydroxyl radical scavenging is also described. The biological tests use the eucaryotic cells of *Saccharomyces cerevisiae* treated with the antioxidants in the presence of the stressing agents apomorphine, hydrogen peroxide, and paraquat dichloride (methylviologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride). The results in chemical systems showed that all of the antioxidants were able to significantly inhibit the oxidation of β-carotene by hydroxyl free radicals. The assays in yeast showed that the antioxidant activity of the tested compounds depended on the stressing agent used and the mechanism of action of the antioxidant.

KEYWORDS: Butylated hydroxytoluene; propyl gallate; resveratrol; L-ascorbic acid; α-tocopherol; 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); *Saccharomyces cerevisiae*; 1,1-diphenyl-2-picrylhydrazyl

INTRODUCTION

It has been suggested that free radicals are responsible for lipid oxidation, which is the major chemical change involved in the deterioration of foods during processing and storage (*1*−*4*). Control of lipid oxidation is often achieved by adding antioxidants to food during processing (*5*), such antioxidants including the synthetics butylated hydroxytoluene (BHT; 2,6-di-*tert*-butyl-*p*-cresol) and propyl gallate (PG; 3,4,5-trihydroxybenzoic acid *n*-propyl ester) and the naturally occurring vitamins C (L-ascorbic acid) and E [**(**+**)**-α-tocopherol]. Although synthetic antioxidants have been widely used in the food industry, there are some arguments about the safety and adverse effects of these substances when used as food additives (*6*, *7*). As discussed in the review by Halliwell and Gutteridge (*8*), natural antioxidants such as vitamins and polyphenols may have important roles in the prevention of diseases associated with free radicals. Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a polyphenol present principally in grapes and a number of other plant species (*9*, *10*), which has been associated with reduced cardiovascular disease (*11*) and cancer risk (*12*) and which may be one of the compounds responsible for the health benefits of drinking red wine (*13*−*16*).

Although antioxidant capacity can be evaluated using chemical methods (*17*−*20*), which are easy to execute and have high reproducibility, such methods do not represent what happens in living cells (*21*). Assays using in vitro living cells have proved to be very useful in the routine testing/sampling of various products, tests based on this methodology being rapid, sensitive, reproducible, and cheap as well as producing reliable results in terms of the identification of biological activity (22).

The objective of the work described in this paper was to measure and compare the antioxidant activity of butyl hydroxytoluene, propyl gallate, resveratrol, and vitamins C and E using chemical assays to determine the capacity of these substances to sequester 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS•), 1,1-diphenyl-2-picrylhydrazyl (DPPH•), and hydroxyl free radicals and also to test the antioxidants in a biological system composed of *Saccharomyces cerevisiae* cells.

MATERIALS AND METHODS

Chemical Measurement of Radical-Scavenging Activities. The antioxidants BHT (2,6-di-*tert*-butyl-*p*-cresol), PG (3,4,5-trihydroxybenzoic acid *n*-propyl ester), resveratrol (*trans*-3,4′,5-trihydroxystilbene), and vitamin E [**(**+**)**-α-tocopherol] were dissolved in a 2:3 ethanol/water mixture, and vitamin C (L-ascorbic acid) was dissolved in distilled water. All of the antioxidants were prepared at a concentration of 1 mM immediately prior to use.

Scavenging of ABTS• was measured using the total antioxidant status kit (RANDOX Laboratory, NX 2332) containing 610 mol/L

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ABTS, 6.1 mol/L peroxidase−metamyoglobin, and 250 mol/L hydrogen peroxide, with and without the addition of the antioxidants. The standard for the antioxidant kit (6-hydroxy-2,5,7,8-tetramethylchroman-2 carboxylic acid) was used as a control, and the antioxidants were tested at 0.025 mM. The generation of ABTS$^{\bullet}$ was measured spectrophotometrically at 600 nm and expressed as a percentage of the scavenging achieved by the ABTS$^{\bullet}$.

Scavenging of DPPH$^{\bullet}$ radical was measured using a modified Yamaguchi et al. (*23*) method in which the antioxidant solutions were added to Tris-HCl (100 mM) buffer, pH 7.0, containing 250 μM DPPH$^{\bullet}$ dissolved in ethanol to give final antioxidant concentrations of 0.002, 0.025, 0.100, and 0.200 mM; the tubes were maintained for 20 min in the dark, and the absorbency measured at 517 nm, the results being expressed as the amount of DPPH$^{\bullet}$ reduced by the different antioxidants. Controls used distilled water in place of antioxidant solution.

Measurement of hydroxyl radical scavenging was based on the reduction of the absorbance of β-carotene treated with OH radicals resulting from the interaction between hydrogen peroxide and ferrous ions. β-Carotene absorbs at 436 nm, whereas its reaction with the OH radical produces a cation radical (β-carotene$^{\bullet+}$), which is unable to absorb at this wavelength. In this new method, an ethanol solution was prepared containing 4 mM β-carotene, 1.8 mM hydrogen peroxide, and 8 mM ferrous sulfate, with and without the addition of antioxidant solutions at a final concentration of 0.025 mM. Absorbance was measured at 436 nm and the concentration of β-carotene calculated from a standard curve (0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 mM β-carotene), the results being expressed as percentage inhibition of β-carotene oxidation.

Evaluation of Antioxidant Capacity Using Yeast Cells. The yeast *S. cerevisiae* XV 185-14C (*MAT* α ade 2-1, arg 4-17, his 1-7, lys 1-1, trp 1-1, trp 5-48, hom 3-10) was provided by Dr. R. C. Von Borstel (Department of Genetics, University of Alberta, Canada). The stock solutions of stressing agents used were the alkaloid isoquinoline apomorphine (2 mM), the herbicide paraquat (PQ, methylviologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride, 0.16 M), and a solution of hydrogen peroxide (10.8 M) containing 4 mM ferrous sulfate. The concentration of hydrogen peroxide stock solutions was determined spectrophotometrically at 240 nm. All solutions were prepared in sterilized distilled water immediately prior to use.

To determine the antioxidant capacity of the antioxidants, suspensions were prepared containing 2×10^6 cells/mL of exponential phase *S. cerevisiae* cells, with and without antioxidants (0.025 mM) and stressing agents (apomorphine at 0.1, 0.2, and 0.3 mM; paraquat at 5, 10, and 15 mM; hydrogen peroxide at 11, 22, and 33 mM), which were incubated in a medium containing 1% yeast extract, 2% peptone, and 2% dextrose (YEPD) at 28 °C for 21 h with agitation. After incubation, samples were diluted in 0.9% sodium chloride solution, plated onto YEPD agar (without antioxidants or stressing agents), and incubated for 48 h at 28 °C, after which time colonies were counted and compared to the control plates (i.e., cell suspensions incubated without antioxidants or stressing agents), which were considered to represent 100% survival of yeast cells.

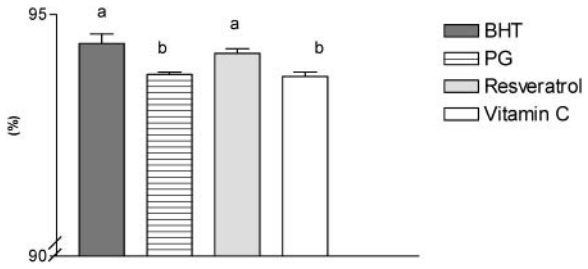
Statistical Analysis and Sources of Chemicals. At least three replicates were made for all assays and the data subjected to analysis of variance (ANOVA) and the Tuckey test using the SPSS 8.0 package. The antioxidants and ABTS and DPPH came from Sigma (St. Louis, MO); all other chemicals were from Merck (Darmstadt, Germany). Absorbance was measured in a Micronal B34211 spectrophotometer.

RESULTS AND DISCUSSION

Chemical Measurement of Radical-Scavenging Activities. BHT and resveratrol had slightly higher ABTS radical-scavenging capacity than PG and vitamin C (**Figure 1**), whereas vitamin E did not scavenge ABTS$^{\bullet}$ at the concentrations tested (data not shown). The ABTS kit used was originally developed to evaluate the antioxidant capacity of blood serum (*17*, *24*), but the results indicate that this kit can be used to test solutions of antioxidants in general.

The capacity of each of the antioxidants to scavenge DPPH$^{\bullet}$ is shown in **Figure 2**, where it can be seen that BHT, PG, and vitamin C all reduce (with no statistical differences between them) the level of DPPH$^{\bullet}$ and that this decrease is related to the concentration of the antioxidant. As was as seen with ABTS$^{\bullet}$, vitamin E did not scavenge DPPH$^{\bullet}$, a result that agrees with the work of Sánchez-Moreno et al. (*25*), who showed that the capacity of vitamin E to scavenge DPPH$^{\bullet}$ is very low and probably related to the aqueous system in which the test is carried out. Resveratrol was efficient at scavenging when present at concentrations up to 0.1 mM. Similar results having been obtained by Wang et al. (*19*) and Yamaguchi et al. (*23*), who demonstrated that at high concentrations resveratrol forms a dimer (of unknown toxicity) which lacks the capacity to scavenge DPPH$^{\bullet}$.

BHT and resveratrol showed the greatest capacity to scavenge hydroxyl radicals, with PG and vitamins C and E being less effective at scavenging these radicals and showing no statistically significant differences in scavenging hydroxyl radicals (**Figure 3**). In contrast to the assays with ABTS$^{\bullet}$ and DPPH$^{\bullet}$, which were carried out in aqueous solution, the assays using β-carotene employed ethanol as solvent, and this may explain the free radical scavenging ability of vitamin E seen in the β-carotene assays, although other experiments are needed to validate this new method, which may be very useful in evaluating liposoluble compounds.



* different letters are statistically different by the Tuckey test (p<=0,05).

Figure 1. Percentage of ABTS$^{\bullet}$ scavenging by the antioxidants (0.025 mM).

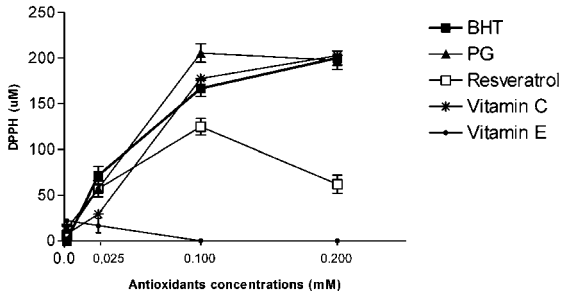
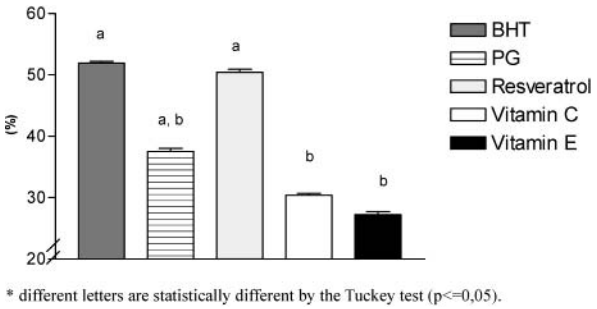


Figure 2. DPPH$^{\bullet}$ concentration reduced by the antioxidants (initial solution containing 250 μM DPPH$^{\bullet}$).



* different letters are statistically different by the Tuckey test (p<=0,05).

Figure 3. Inhibition of β-carotene oxidation by antioxidants (0.025 mM) in the presence of H_2O_2 and Fe^{2+}.

Table 1. Percentage Survival of *S. cerevisiae* Cells Treated with the Stressing Agents Apomorphine, Paraquat, and Hydrogen Peroxide and the Antioxidants Butylated Hydroxytoluene, Propyl Gallate, Resveratrol, and Vitamins C and E

	cell survival (%) ± SD					
stressing agent (mM)	no antioxidant	BHT	propyl gallate	resveratrol	vitamin C	vitamin E
apomorphine						
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.1	63.63 ± 1.11	100.00 ± 0.00*	86.20 ± 6.64*	100.00 ± 0.00*	100.00 ± 0.00*	100.00 ± 0.00*
0.2	49.80 ± 1.27	100.00 ± 0.00*	74.03 ± 2.96*	100.00 ± 0.00*	100.00 ± 0.00*	68.20 ± 0.74*
0.3	41.25 ± 1.62	100.00 ± 0.00*	66.70 ± 2.12*	100.00 ± 0.00*	71.70 ± 3.67*	61.85 ± 1.76*
paraquat						
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
5	43.25 ± 4.66	85.80 ± 3.39*	51.53 ± 4.78	48.58 ± 2.52	100.00 ± 0.00*	44.58 ± 3.13
10	32.55 ± 1.01	52.50 ± 3.53*	34.70 ± 1.06	33.00 ± 0.14	87.70 ± 2.82*	37.07 ± 0.14
15	23.95 ± 3.75	44.87 ± 4.16*	28.49 ± 1.06	24.85 ± 1.34	35.50 ± 4.34*	28.31 ± 6.60
hydrogen peroxide						
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
11	48.27 ± 0.97	100.00 ± 0.00*	48.60 ± 0.14	100.00 ± 0.00*	96.48 ± 4.07*	100.00 ± 0.00*
22	41.67 ± 2.74	99.50 ± 0.70*	41.80 ± 0.14	100.00 ± 0.00*	94.08 ± 1.41*	86.60 ± 1.32*
33	31.80 ± 2.23	95.70 ± 7.4*4	33.65 ± 2.34	100.00 ± 0.00*	73.39 ± 1.42*	84.20 ± 2.16*

[a] *, significantly different by the Tuckey test ($p \leq 0.05$).

Evaluation of Antioxidant Capacity Using Yeast Cells. A system based on *S. cerevisiae* was used to compare chemical and biological systems for assaying antioxidant capacity, the highest non-cytotoxic concentration (0.025 mM) of antioxidants being used in this test. The results shown in **Table 1** indicate that all of the antioxidants tested were capable of protecting yeast cells against damage caused by apomorphine, an alkaloid capable of generating superoxide radicals $O^{-\bullet}{}_2$ and $OH^\bullet$ in the presence of metal ions (*26*−*29*). On the other hand, antioxidant activity against paraquat was shown only by butylated hydroxytoluene and vitamin C. Except for PG, all of the antioxidants showed activity against hydroxyl radicals generated by the action of ferrous ions on hydrogen peroxide.

The antioxidant mechanism of PG is based on its capacity to sequester metal ions, which can catalyze the formation of $OH^\bullet$ by the Haber−Weiss reaction (*6*). It is possible that the antioxidant action of PG with respect to apomorphine was mediated by removal of cations, thus inhibiting the formation of $OH^\bullet$ by the alkaloid. PG did not show antioxidant activity with respect to paraquat, something which may be explained by the fact that the stressing agent paraquat can generate the PQ radical and $O^{-\bullet}{}_2$ by mechanisms which are not mediated by metal ions. Also, tests with H_2O_2 and PG were not efficient because in this treatment the Fe^{2+} ions were added prior to the H_2O_2, generating $OH^\bullet$ which were then added to the yeast cells. On the other hand, in experiments in which Fe^{2+} ions were added separately to the system from the H_2O_2 (the β-carotene assay), PG showed important antioxidant activity. These results confirm the ability of PG to chelate divalent cations.

We found that BHT and vitamin C were able to protect the yeast cells against damage caused by all of the stressing agents tested, these results indicating that both BHT and vitamin C are able to act against $PQ^{\bullet+}$, H_2O_2, and $OH^\bullet$, corroborating the results obtained by other authors (*8*).

In our experiments, resveratrol and vitamin E (both of which have known antioxidant activity) were not effective at preventing the oxidative damage caused to yeast cells by paraquat. Resveratrol and vitamin E were the most lipophilic compounds tested, and this may have prejudiced their antioxidant action in the aqueous *S. cerevisiae* system because in the β-carotene assay (which used ethanol as the solvent) both of these compounds showed significant antioxidant activity.

These results clearly demonstrate that the antioxidants studied showed distinct antioxidant activity against the different free radicals tested. It should, however, be remembered that the expression of antioxidant activity in cells is more complex because it involves cellular permeability and the possible metabolism of the assayed compounds as well as complex endogenous cellular enzymatic antioxidant defense mechanisms. Even so, there are advantages in using biological systems because of what they may tell us about the mechanisms that occur in human beings, and because of this such assays merit special attention.

Although there is no doubt about the benefit of using antioxidants in foods, the data discussed in this paper demonstrate that the mechanism of action of each antioxidant should be investigated in detail to determine the relative advantages and disadvantages of different antioxidants. The good results obtained with the natural antioxidants, especially resveratrol, suggest that such compounds may be suitable to replace synthetic antioxidants such as BHT and PG without reducing antioxidant capacity or having the undesirable effects seen with these two last synthetic antioxidants (*30*−*32*).

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