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Antioxidant Activity of the Flavonoid Hesperidin in Chemical and Biological Systems

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The antioxidant hesperidin, a major flavonoid in sweet orange and lemon, was evaluated using chemical and biological systems. The chemical assay evaluates the hesperidin capacity to sequester 1,1-diphenyl-2-picrylhydrazyl (DPPH·). Biological studies were done using the eukaryotic cells of superoxide-dismutase proficient and deficient strains of *Saccharomyces cerevisiae* treated with hesperidin and the stressing agents hydrogen peroxide or paraquat (methylviologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride). Hesperidin was able to reduce significantly the level of the free radical DPPH· with similar efficacy of trolox (positive control). When the yeast cells were exposed to the flavonoid hesperidin before the stressing agents, there was a significant increase in the survival of all strains. Paraquat induced higher catalase and superoxide dismutase than did hydrogen peroxide, which only increased catalase activity. Previous addition of hesperidin to these treatments was able to reduce significantly both enzymatic levels. These observations clearly demonstrate that hesperidin provides strong cellular antioxidant protection against the damaging effects induced by paraquat and peroxide hydrogen.

KEYWORDS: Hesperidin; flavonoids; superoxide dismutase; catalase; *Saccharomyces cerevisiae*

INTRODUCTION

Flavonoids are natural substances with variable phenolic structures. More than 4000 flavonoids have been identified and grouped according to their molecular structures. The best described property of almost every group of flavonoids is their capacity to act as antioxidants able to scavenge free radicals and reactive oxygen species (*1*, *2*), which are associated with several forms of tissue damage and disease, including cancer and atherosclerosis, as well as with aging. For protection against free radicals, aerobic organisms have evolved intricate and interrelated processes, which include the enzymes superoxide dismutase (SOD) and catalase (CAT). SOD catalyzes the dismutation of the superoxide anion ($O_2^{\cdot -}$) to oxygen and hydrogen peroxide (H_2O_2), and catalase converts hydrogen peroxide to water and molecular oxygen (*3*).

Hesperidin (**Figure 1**), a member of the flavanone group of flavonoids, can be isolated in large amounts from the rinds of some citrus species [e.g., *Citrus aurantium* L. (bitter orange), *Citrus sinensis* L. (sweet orange), and *Citrus unshiu* Marcov. (satsuma mandarin)] and has been reported to have antiallergenic, anticarcinogenic, antihypotensive, antimicrobial, and vasodilator properties (*4*). Studies with rats treated with orange juice (*5*) and mandarin juice (*6*) have show a 22% reduction in colon cancer and a 29% decrease in lung cancer, respectively. These effects have been attributed to the fact that juices contain high levels of the flavonoid hesperidin. Satsuma mandarin (*C. unshiu*) juice enriched with hesperidin has also been shown to significantly reduce the incidence of colon cancer in rats (*7*). In a hamster model of atherosclerosis, orange juice was able to significantly inhibit atherosclerosis and lower cholesterol and triglycerides (*8*). In view of the worldwide consumption of citric juice, it is important to study the antioxidant activity of hesperidin.

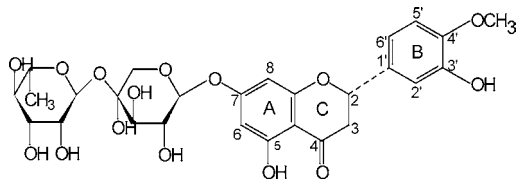

Figure 1. Chemical struture of hesperidin [3′,5,7-trihydroxy-4′-methoxyflavanone-7-(6-α-L-rhamnopyranosyl)-β-D-glucopyranoside] (*4*).

The antioxidant capacity of drugs can be evaluated using chemical methods, which are easy to execute and have high reproducibility. One of the most used methodologies is the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH·) scavenger capacity (*9*−*11*). Nevertheless, such methods do not represent what happens *in vivo* (*12*). Assays using living cells have proven to be very useful in the routine testing of various products, being fast, sensitive, reproducible, as well as producing reliable results in terms of the identification of biological and antioxidant activity (*12*−*14*).

The eukaryotic yeast *Saccharomyces cerevisiae* has been extensively studied both genetically and biochemically, and it

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Table 1. *S. cerevisiae* Strains Used in This Study and Their Relevant Genotypes

strain	genotype
EG103 (wild type)	*MATα leu2–3 112 his3-Δ1 trp1–289 ura3–52*
EG118 (*sod1*Δ)	*sod1*::URA3 all other markers as EG103
EG110 (*sod2*Δ)	*sod2*::TRP1 all other markers as EG103
EG133 (*sod1*Δ*sod2*Δ)	*sod1*::URA3*sod2*::TRP1 all other markers as EG103

is widely used in biological screening and testing. As with all aerobes, *S. cerevisiae* possesses a variety of antioxidant defenses, including a cytoplasmic Cu^{2+} and Zn^{2+} SOD (CuZnSOD, SOD1), coded for by the *SOD1* gene, a mitochondrial manganese SOD (MnSOD, SOD2) (*15*), coded for by the *SOD2* gene (*16*), a cytoplasmic catalase (coded for by the *CCT1* gene), and a peroxisomal catalase (coded for by the *CCA1* gene) (*17*−*19*).

The purpose of the work described in this paper was to evaluate the capacity of hesperidin to scavenge the DPPH· radical and to study its antioxidant activity in proficient and deficient SOD *S. cerevisiae* strains exposed to the stressing agents paraquat and hydrogen peroxide.

MATERIALS AND METHODS

Chemical Measurement of DPPH· Radical-Scavenging Activity. Scavenging of the DPPH· radical was measured using a modified Yamaguchi et al. (*20*) method in which the hesperidin or trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxilic acid) solutions, both diluted in dimethyl sulfoxide (DMSO), were added to Tris-HCl buffer (100 mM, pH 7.0) containing 250 μM of DPPH· dissolved in ethanol to give final antioxidant concentrations of 10, 25, 50, or 100 μM. The tubes were maintained for 20 min in the dark after which the absorbance was measured at 517 nm (Shimadzu UV-1700 spectrophotometer), and the results were expressed as the amount of DPPH· radical reduced by the antioxidants. Controls used distilled water in place of the antioxidant solutions.

Evaluation of Antioxidant Capacity Using Yeast Cells. The evaluation of the *in vivo* antioxidant actitivity of hesperidin was performed using SOD proficient and deficient *S. cerevisiae* yeast strains (**Table 1**), which were kindly provided by Dr. E. Gralla from University of California, Los Angeles, CA (*21*, *22*). The flavonoid hesperidin was prepared with a 10 mM solution in DMSO immediately prior to use. The stressing agents paraquat herbicide and hydrogen peroxide were prepared as 10 mM aqueous stock solutions.

To determine the antioxidant capacity of hesperidin, suspensions were prepared containg 2×10^6 cells/mL of exponential-phase *S. cerevisiae* cells with and without hesperidin (25 and 50 μM) and stressing agents (1 mM), which were incubated in a YPD medium (10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of dextrose). The effect of varying the order in which hesperidin and the stressing agents was added was also investigated by carrying out some assays with the antioxidant added first followed by the stressing agent and some assays in which this order was reversed. In all cases, the tubes were incubated for 6 h at 28 °C and 130 rpm. After incubation, samples were diluted in 0.9% (w/v) sodium chloride solution, plated onto YPD agar (YPD medium supplemented with 20 g/L of agar), and incubated for 72 h at 28 °C after which time colonies were counted and compared to the control plates, which were considered to represent 100% survival of the yeast cell.

Catalase and SOD Activities. For enzyme assays, the yeast cells were suspended in lysis buffer (50 mM of Tris-HCl, 150 mM of sodium chloride, and 50 mM of EDTA at pH 7.2) and ruptured using glass beads and alternate cycles of agitation and cooling (*23*). SOD activity was measured using the method of Misra and Fridovich. A total of 1 unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenochrome formation in 50% per gram of protein (*24*). The catalase assay was performed according to the method described by Maehly and Chance. A total of 1 unit of catalase decomposed 1 μmol of H_2O_2/mg of protein in 1 min at pH 7.4 (*25*). Total protein was determined using the Labtest Total Proteins Kit. Absorbance was measured in a Shimadzu UV-1700 spectrophotometer.

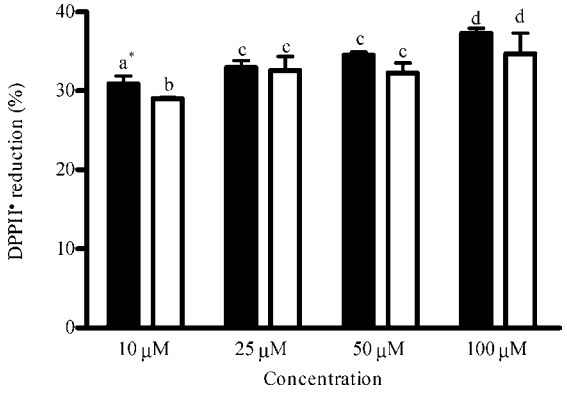

Figure 2. DPPH· concentration reduced by hesperidin (■) and trolox (□). The results represent the mean ± SD of three independent experiments. (*) Different letters are statistically different by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$).

Statistical Analyses and Chemical Sources. At least three replicates were made for all assays, and data were subjected to analysis of variance (ANOVA) and the Tukey test using the SPSS 12.0 package. Hesperidin, DMSO, DPPH·, and trolox were purchased from Sigma, St. Louis, MO, and the Total Proteins Kit from Labtest (Labtest Diagnostica SA, catalog number 18, Brazil). All other chemicals were purchased from E. Merck, Damstadt, Germany.

RESULTS AND DISCUSSION

Scavenging of the DPPH· Radical. The chemical assay showed that hesperidin significantly reduced the level of the DPPH· in a similar way to the antioxidant trolox (positive control) with the exception of the lower concentration (10 μM), where it was reported that hesperidin has a higher capacity of reducing the DPPH· radical than trolox (**Figure 2**). These data indicate the antioxidant potential of the flavonoid in *in vitro* assays.

Evaluation of Antioxidant Capacity Using Yeast Cells. *S. cerevisiae* yeast cells were used as biological systems for assaying antioxidant capacity. Noncytotoxic concentrations (25 and 50 μM) of hesperidin were chosen for the test. When hesperidin (in both concentration assayed) was added first, it was able to protect all of the strain damages caused by the stressing agents paraquat or hydrogen peroxide (**Table 2**), but when added after the stressing agents, hesperidin did not protect cells from damage induced by paraquat and hydrogen peroxide (data not shown).

In the presence of NADPH + H^+, paraquat forms the paraquat free radical ($PQ^{\cdot+}$), which stimulates lipid peroxidation and can also reduce iron or react with oxygen to form the superoxide radical. The reduction of iron or the superoxide formation is dependent on the concentration of oxygen in the system, with high oxygen concentrations favoring superoxide formation (*3*). The presence of iron salts increases the capacity of paraquat to cause peroxidation and DNA damage, which suggests that paraquat can also generate extremely reactive hydroxyl radicals (*3*, *26*). Hydrogen peroxide causes cell damage by the production of hydroxyl radicals via the Haber-Weiss/Fenton reaction (*3*). Opposite to the free radicals $PQ^{+\cdot}$, $O_2^{-\cdot}$, and OH·, the hydrogen peroxide is very difusible within and between cells *in vivo*, causing damage in membranes, organelles, and also in cellular nucleus (*3*).

Table 2. Survival of SOD Proficient and Deficient *S. cerevisiae* Strains Treated and Untreated with the Antioxidant Hesperidin and/or the Stressing Agents Paraquat or Hydrogen Peroxide

	survival (%)[a]			
treatments	SOD1SOD2	*sod1*Δ	*sod2*Δ	*sod1*Δ*sod2*Δ
untreated cells (control)	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
1 mM paraquat	57.60 ± 0.88[b]	40.62 ± 2.46[b]	37.30 ± 3.56[b]	40.76 ± 5.60[b]
25 μM hesperidin and 1 mM paraquat	82.02 ± 0.32[c]	83.16 ± 3.00[c]	84.31 ± 1.08[c]	61.67 ± 1.65[c]
50 μM hesperidin and 1 mM paraquat	80.87 ± 1.94[c]	81.54 ± 3.90[c]	89.10 ± 3.08[c]	63.27 ± 2.59[c]
1 mM H_2O_2	49.25 ± 0.49[b]	49.42 ± 0.83[b]	45.55 ± 0.64[b]	49.47 ± 0.76[b]
25 μM hesperidin and 1 mM H_2O_2	77.00 ± 0.07[c]	74.57 ± 0.40[c]	77.80 ± 0.67[c]	69.93 ± 1.04[c]
50 μM hesperidin and 1 mM H_2O_2	85.68 ± 0.61[c]	86.90 ± 0.85[c]	81.78 ± 1.82[c]	85.77 ± 0.08[c]

[a] Average ± SD. [b] Significantly different from the untreated cells (control) by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$). [c] Significantly different from the stressing agents by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$).

Table 3. Catalase Activities of SOD Proficient and Deficient *S. cerevisiae* Strains Treated and Untreated with the Antioxidant Hesperidin and/or the Stressing Agent Paraquat or Hydrogen Peroxide

	catalase activity [(μmol of H_2O_2 decomposition)$^{-1}$ (mg of protein)$^{-1}$ min][a]			
treatments	SOD1SOD2	*sod1*Δ	*sod2*Δ	*sod1*Δ*sod2*Δ
untreated cells (control)	15.47 ± 1.68[b]	27.04 ± 0.98[b]	40.45 ± 0.64[b]	42.51 ± 2.27[b]
1 mM paraquat	79.38 ± 1.78[c]	166.97 ±7.69[c]	103.50 ± 0.84[c]	146.44 ± 0.86[c]
1 mM H_2O_2	327.85 ± 3.61[c]	277.32 ± 6.51[c]	399.34 ± 1.07[c]	294.93 ± 1.16[c]
25 μM hesperidin	10.60 ± 3.92[c]	16.15 ± 2.05[c]	20.03 ± 1.13[c]	38.03 ± 2.04[c]
50 μM hesperidin	10.40 ± 2.23[c]	11.13 ± 0.28[c]	36.05 ± 3.84[c]	39.68 ± 1.73[c]
25 μM hesperidin and 1 mM paraquat	22.55 ± 2.43[b]	54.44 ± 0.14[b]	26.93 ± 0.48[b]	33.32 ± 0.02[b]
50 μM hesperidin and 1 mM paraquat	16.19 ± 0.95[b]	46.22 ± 0.10[b]	21.35 ± 0.74[b]	18.71 ± 2.90[b]
25 μM hesperidin and 1 mM H_2O_2	155.82 ± 1.87[b]	196.48 ± 5.13[b]	185.46 ± 5.71[b]	181.72 ± 2.28[b]
50 μM hesperidin and 1 mM H_2O_2	138.30 ± 5.40[b]	140.28 ± 2.57[b]	102.65 ± 3.61[b]	137.98 ± 0.68[b]

[a] Average ± SD. [b] Significantly different from the stressing agents by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$). [c] Significantly different from the untreated cells (control) by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$).

The mutant *S. cerevisiae* strains investigated by us, which were deficient in one (strains EG118 and EG110) or both (strain EG133) SOD enzymes, were more sensitive to paraquat than the wild-type strain (EG103) (**Table 2**). This indicates that SOD enzymes are extremely important for defense against the reactive oxygen species generated by paraquat and that the superoxide radical plays an important role in the cytotoxicity of paraquat. The cytotoxic effect of hydrogen peroxide was similar for all strains, indicating that the detoxify capacity of hydroxyl radical was the same for all of the SOD proficient and deficient strains.

The antioxidant capacity of flavonoids is directly related to their structure (*27*, *28*), and in the case of hesperidin, the presence of a hydroxyl group at position 3′ of ring B (**Figure 1**) may be responsible for the capacity of hesperidin to scavenge the hydroxyl radicals generated from hydrogen peroxide. It is already known that the ability to scavenge superoxide is due to a hydroxyl group at position C-4′ of ring B (*28*, *29*). Although hesperidin has no hydroxyl at this site, our results show that this flavonoid significantly increased the survival of cells exposed to paraquat (**Table 2**). It has previously been shown that the ring B C-4′ methyl substitution of hesperidin can activate the ring B C-3′ hydroxyl, making hesperidin a more active scavenger to the superoxide radical (*30*).

Therefore, hesperidin is able to protect the yeast cells from the damage induced both by paraquat and hydrogen peroxide, making this antioxidant a promising agent for the prevention of atherosclerosis and other pathologies in which free radicals are implicated, such as degenerative diseases, i.e., brain dysfunction, cataracts, and cancers (*3*).

Catalase Assay. The results of the enzymatic activity catalase are presented in **Table 3**. We found that catalase activities for both SOD proficient and deficient strains treated with paraquat and hydrogen peroxide increased in relation to the untreated cells.

The yeast *S. cerevisiae* has two catalases called A and T. Catalase A is located in the peroxisome, and the main physiological role of this enzyme appears to be to remove hydrogen peroxide produced by fatty acid β oxidation. The physiological role of the cytosolic catalase T protein is less clear; *CTT1* gene expression, which codified the enzyme, is, however, regulated by oxidative and osmotic stress. Although both catalases are clearly important for resistance toward H_2O_2, the catalase genes are only moderately inducible by hydrogen peroxide (*31*). Our results showed the catalase activity increased in the presence of both paraquat and hydrogen peroxide (**Table 3**), indicating that the peroxide hydrogen and/or the oxidative stress produced by it are able to induce the antioxidant activity of the catalase enzyme. In relation to paraquat, it is possible that this compound could generate hydrogen peroxide before hydroxyl radicals (*3*, *26*) or that the oxidative stress produced by $PQ^{\cdot+}$ and $O_2^{\cdot-}$ radicals has provoked an increase in the catalase activity.

When only hesperidin was added to the culture medium, we found that there was, in most cases, a reduction in catalase activity as compared to the controls, which had received no antioxidant or stressing agent (**Table 3**). This may have been due to the scavenging activity of hesperidin, which is able to reduce the reactive oxygen species generated during normal cell metabolism, reducing the need for catalase biosynthesis. We also found that, when hesperidin was added before the stressing agents paraquat or hydrogen peroxide, there was a reduction in catalase activity in relation to that occurring in the paraquat and hydrogen peroxide alone. These results confirm the antioxidant activity of hesperidin, which had probably reacted with the reactive oxygen species generated by the stressing agents and decreased the need for catalase.

SOD Assay. The enzymatic activity values of SOD are presented in **Table 4**. As expected, hydrogen peroxide did not induce SOD activity (data not shown), this enzyme was induced

Table 4. SOD Activities of SOD Proficient and Deficient *S. cerevisiae* Strains Treated and Untreated with the Antioxidant Hesperidin and/or the Stressing Agent Paraquat

treatments[b]	SOD activity (units of SOD/mg of protein)[a]		
	SOD1SOD2	*sod1*Δ	*sod2*Δ
untreated cells (control)	0.626 ± 0.04	0.220 ± 0.02	0.275 ± 0.01
1 mM paraquat	1.070 ± 0.04[c]	0.520 ± 0.03[c]	0.550 ± 0.02[c]
25 μM hesperidin	0.293 ± 0.02[d]	0.153 ± 0.07[d]	0.155 ± 0.03[d]
50 μM hesperidin	0.423 ± 0.03[d]	0.131 ± 0.06[d]	0.167 ± 0.01[d]
25 μM hesperidin and 1 mM paraquat	0.155 ± 0.02[d]	0.139 ± 0.01[d]	0.149 ± 0.01[d]
50 μM hesperidin and 1 mM paraquat	0.283 ± 0.07[d]	0.093 ± 0.10[d]	0.010 ± 0.01[d]

[a] Average ± SD. [b] There was no significant induction of SOD when yeasts were treated with H_2O_2. [c] Values statistically different from the untreated cells (control) by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$). [d] Values statistically different from the paraquat treatment by the analysis of variance (ANOVA) and Tukey pos-test ($p \leq 0.05$).

by the superoxide radical (*3*). When only hesperidin was added to the culture medium, we found that there was a reduction in SOD activity as compared to the controls with no antioxidant or paraquat. Paraquat treatment showed a significant rise in SOD activity in all strains EG103, EG118, and EG110 (**Table 4**). Therefore, it appears that, as previously described (*3*), paraquat produces higher levels of superoxide.

The literature describes CuZnSOD as the major enzyme involved in removing superoxide anions from the cytoplasm (and possibly also the peroxisome), while the physiological role of MnSOD appears to protect mitochondria from the superoxide generated during respiration and seems to have a little role in countering the toxicity of the superoxide anions generated by exogenously added redox cycling compounds (*31*). Nevertheless, in our experiments, the increases in CuZnSOD and MnSOD activities were similar (**Table 4**), indicating the importance of both cytosolic and mitochondrial SOD enzymes in the detoxification of the superoxide generated by paraquat.

It was found that when hesperidin was added prior to paraquat SOD activities were lower for the wild-type EG103 and for the deficient EG118 and EG110 strains, as compared with the paraquat controls. This indicates that, as discussed above, hesperidin has important antioxidant properties and probably is able to scavenge the superoxide radical generated by paraquat and, thus, decrease the need of the cells to produce SOD. Even so, it should be remembered that all of the cellular defense systems, both enzymatic and nonenzymatic, operate together at different levels to ensure an efficient response to oxidative stress (*23*). Further studies on other endogenous defense mechanisms for reducing oxidative stress in yeast, such as the antioxidant enzymes glutathione peroxidase and glutathione reductase and the cellular content of glutathione, could complement the data presented in this paper.

Although other studies are needed, the results presented in this paper show the significant antioxidant activity of hesperidin both *in vitro* and *in vivo* assay, a flavonoid found in species of oranges and lemons, which are eaten in many different countries. Experiments using male and female mice have shown that hesperidin is nontoxic, easily assimilated, nonaccumulative, and causes no allergic reactions. This antioxidant has also been reported to have an antimutagenic effect in rats against *N*-methyl-*N*-amylnitrosamine (*4*). In light of our findings, hesperidin can be considered as a potentially active compound for use in conditions where reactive oxygen species are implicated, although clinical studies are needed to establish the usefulness of this natural product in the treatment or prevention of human disease.

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