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Evaluation of Red Chicory Extract as a Natural Antioxidant by Pure Lipid Oxidation and Yeast Oxidative Stress Response as Model Systems

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ABSTRACT: The search for renewable and abundant sources of antioxidants has recently focused on agricultural byproducts, especially promising due to their natural origins and low costs. In particular, plant raw materials are sources of important compounds such as dietary fiber, carotenoids, tocopherols, and polyphenolics, which are mostly discarded during harvesting and processing. Among these vegetal crops, red chicory is attractive because of the large quantity of its byproducts (residues as leaves and stems); moreover, there is no information on its role as a food and feed ingredient. In this study, red chicory leaf residue was evaluated as a natural substitute for synthetic antioxidants for the food and feed industry. After lyophilization, a red chicory extract (RC) was characterized for its phenolic profile and its oxidative stability as compared to BHT. RC was shown to reduce lipid peroxidation of different oils in the Rancimat test. In addition, the antioxidant property of RC was studied in a model system by evaluating the *Saccharomyces cerevisiae* response to oxidative stress by means of gene expression. In this analysis, the RC extract, added to the yeast culture prior to oxidative stress induction, exhibited a pleiotropic protective effect on stress responsive genes.

KEYWORDS: red chicory, natural antioxidants, lipid peroxidation, *Saccharomyces cerevisiae*, oxidative stress

INTRODUCTION

There is an emerging interest in the use of naturally occurring antioxidants for the preservation of feeds and foods for both their technological relevance and their positive impact on consumer health. In this context, several foregoing studies have pointed out strength relationships between diet and chronic diseases: Phytochemicals present in fruits and vegetables may have antioxidant effects, thus protecting them from oxidative damage arising from metabolic and exogenous sources.[1−3]

Lipid oxidation is not an exclusive concern for the food industry but also occurs in feeds and feedstuffs, resulting in rancidity of fats, degradradation of proteins, vitamins, and pigments, and cross-linking of lipids and other macromolecules into non-nutritive polymers with a serious decrease in the nutritional value. The addition of exogenous antioxidants is a well-known strategy; although hundreds of them have been tested, only a few chemicals have shown the qualifications necessary to make them suitable for use in preventing undesirable oxidations in food and feed.

An antioxidant must have the following qualifications: (a) it must be effective in preserving animal and vegetable fats, vitamins, and other food and feed qualities subject to oxidative damage; (b) it must be nontoxic to man and to animals; (c) it should be effective at very low concentrations; and (d) it must be low enough in cost to be economically convenient.

Among the chemical compounds that have been investigated thus far, BHA and BHT have been demonstrated to be the most efficacious in both the food and feed industries. As these antioxidants are suspected to be promoters of carcinogenesis,[4] the public concern about their safety demands the evaluation of alternative substances. The growing interest in the replacement of synthetic food antioxidants by natural ones has led to multiple investigations in the field of new antioxidants. The search for cheap, renewable, and abundant sources of antioxidants has focused on raw materials of residual origin, especially promising due to their low costs.[5] Moreover, the residuals can be valuable resources for those new business models that are already competing in the emerging market of eco-products and/or healthy foods. Agricultural byproducts contain a variety of biologically active substances, which mostly go to waste, but these are also promising sources of compounds as dietary fibers, carotenoids, tocopherols, and polyphenolics. Among the bioactive compounds, phenolics play an important role because, in contrast to most carotenoids and vitamins, they are not chemically synthesized and need to be extracted from plant material. Moreover, recycling of the byproduct has been supported by the observation that polyphenols are located specifically in the peels.[6] In this respect, grape skin extracts are being marketed for their anthocyanin and procyanidin contents.[7] Studies on different chicory varieties have demonstrated that they are an interesting source of natural antioxidants.[8] Moreover, chicory is promising because of the large availability of its byproducts. Indeed, before commercialization, large amounts of wastes and residues (leaves, stems, etc.) are produced, which can reach 40−50% of the harvested material, and there is no information on their role as food and feed ingredients.[9]

The aim of this study is to evaluate red chicory (RC) extract as a natural antioxidant for the food and feed industries. In this work, the chemical composition and oxidative stability of a lyophilized RC extract were investigated. Moreover, the RC extract effect on eukaryotic stress response by means of *Saccharomyces cerevisiae* expression (SCE) analysis has been studied as a

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first approach to assess its antioxidant property in an eukaryotic model system.

MATERIALS AND METHODS

Reagents. Phenolic acid standards were purchased from Sigma-Aldrich (Milano, Italy). Anthocyanins were obtained from Extrasynthese (Genay Cedex, France). Vegetables were purchased from a local market in Legnaro (Padova, Italy). All of the aqueous solutions were prepared with Milli-Q water (Millipore, Bedford, MA) and, when necessary, were passed through a column of Chelex-100 (Bio-Rad, Richmond, CA) to minimize the concentration of heavy metal ions.

Extract. About 200 g of vegetables was randomly sampled from 1 kg of raw material (*Cichorium intybus* var. *silvestre* cv. 'Chioggia'). The leaves were washed, weighed, cut in small pieces, and crushed with liquid nitrogen to obtain a fine powder that was put in a nylon bag (porosity = 5 μm). The pressurized liquid extraction was carried out for 1.5 h in an automatic equipment (NM LAB/M Depurex 88, Limena, Padova, Italy) with 460 mL of ethanol/water solution (50:50 v/v), pH 3, previously deoxygenated by flushing with nitrogen. To calculate the extract concentration (g L^{-1}), the volume of sample was measured. The ratio of extraction was 0.4. The freshly prepared extract was freeze-dried in aliquots of 2 mL in 10 mL glass vials at −40 °C (Edwards Mini Fast 1700). The dark vials, filled with nitrogen gas and closed with rubber caps, were stored at 4 °C until the analytical measurements.

HPLC Analyses. The RC lyophilized extracts were analyzed by a Thermo-Finnigan series liquid chromatography (San Jose, CA) equipped with a photodiode array detector UV 6000LP, and a Hewlett-Packard computer system was used. All samples were filtered on a 0.45 μm cartridge and directly injected.

Hydroxycinnamic and hydroxybenzoic acids were separated using an LC-18 Supelco-sil column at 25 °C with a flow rate of 1.5 mL min^{-1} under isocratic conditions.[10] The absorbances at 275 and 330 nm were used as reference wavelengths. The mobile phase consisted of an aqueous solution containing 0.15% acetic acid and 0.18% *n*-butanol. Phenolic acid standards were chromatographed singly and in mixture. The compounds were identified according to their retention times. Each reported value is the average of three repeated analyses.

Anthocyanins. Separation was performed on an LC-18 Supelco-sil (5 μm), 250 mm × 5 mm i.d., fitted with a Pelliguard LC-18 (Supelco, Bellefonte, PA). The binary solvent system consisted of an aqueous solution of 1% phosphoric acid, 10% acetic acid, and 6% acetonitrile (eluent A) and 100% acetonitrile (eluent B). Applied were the following conditions: isocratic 100% A for 20 min, then linear increment from 0 to 10% B in 10 min, and isocratic 100% B for 10.[11] The flow rate was 1.3 mL min^{-1}, and sample aliquots of 20 μL were injected. The column oven temperature was set to 20 °C. The diode array spectra were obtained in the wavelength range of 250−600 nm in steps of 2 nm. Detection was at 520 nm. Standards were used to identify peaks by retention time and cochromatography. Diode array spectral characteristics were matched to standards and to library spectra.

Demalonation. Demalonation of anthocyanins in RC extract was carried out as reported in ref 12. Samples were diluted in a solution of methanol/acetic acid (95:5 v/v), stored at 25 °C, and analyzed at various times (0, 5, 10, and 15 days) according to the anthocyanin procedure analysis by HPLC previously described.

Oxidative Stability. Oxidative stability is expressed in hours and determined by AOCS Official Method Cd 12b-92,[13] using a Rancimat apparatus (Metrohm, model 743, Herisau, Switzerland), which measured conductometrically the formation of volatile acids produced by the free radical chain reaction. The determination was made at 110 °C with a 20 L h^{-1} air flow, using 3 g, respectively, of corn, peanut, soybean oils, 0.15 or 1.50 mg g^{-1} BHT (Sigma, St. Louis, MO), or RC dissolved in a water/ethanol solution and 60 mL of distilled water in flasks containing electrodes. The volatile decomposition products were detected with a conductivity cell. The induction time (IT) for each model system was assessed in triplicate. The antioxidant activity index (AAI) was calculated from the measured ITs, according to the following formula by Forster and co-workers:[14] AAI = IT of oils with antioxidant/IT of pure oils.

Antioxidant Analysis in Yeast Model System. *Yeast Strains and Growth Media.* The yeast strain used in this study is *S. cerevisiae* NRRL Y-12632^T. Yeasts were routinely grown on YPD medium [10 g L^{-1} yeast extract; 20 g L^{-1} protease peptone; 20 g L^{-1} glucose (Oxoid, Hampshire, U.K.), pH 6.0−6.2] or YPD agar medium (15 g L^{-1} agar).

Yeast Cell Cultures and Stress Induction. RC extract was rehydrated in 50% ethanol at a concentration of 200 mg mL^{-1}. Yeast cells at early stationary phase (30 h YPD liquid preculture) were reinoculated in 50 mL of fresh YPD medium (initial cell concentration was 10^5 cells mL^{-1}), containing or not the RC extract (final concentrations of 0, 100, and 400 mg L^{-1}). Incubation was performed at 28 °C for 1.5 h, 160 rpm, to adapt the cells to the new medium, thus allowing the antioxidant to operate its protecting activity. All of the cultures, treated or not with RC, were then split: half was subjected to oxidative stress (2.5 mM H_2O_2 at 28 °C, 160 rpm, for 15 min); the other half was just incubated for 15 min and used as a control. After H_2O_2 exposure, cells were quickly collected for RNA extraction. Cell viability was analyzed by plating, in triplicate, on YPD agar medium, after proper dilution. To choose the doses of the oxidant used in the treatments, cells were exposed to H_2O_2 increasing concentrations and then spotted adjacently on YPD agar plates. The concentration chosen (2.5 mM) was the lowest that affected cell growth as compared to yeasts not exposed to stress (data not shown).

RNA Extraction and Reverse Transcription. Total RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) from samples containing approximately 10^7 cells, resuspended in 400 μL of Trizol, and broken by vortexing for 4 min with 300 μL of glass beads. The total volume was adjusted to 1 mL with Trizol solution; RNA extraction was performed following the protocol provided by the manufacturer. A maximal amount of 10 μg of total RNA was then purified from contaminants using the RNeasy kit (Qiagen, Hilden, Germany) following the "Cleanup protocol" in the manufacturer's instructions. The RNA concentration was determined by spectrophotometric analysis; RNA quality was tested by electrophoresis on 1.5% agarose gels under denaturing conditions (2% formaldehyde v/v, 20 mM MOPS, 5 mM N sodium acetate, and 1 mM EDTA, pH 7.0). One microgram of RNA was treated with DNaseI (Fermentas International Inc., Burlington, Canada) according to the manufacturer's instruction. After DNaseI thermal inactivation, cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer's instructions using both 0.025 μg μL^{-1} poliT(16) primers (MWG-biotech) and 0.025 μg μL^{-1} random hexamers (Promega, Madison, WI) using 0.5 μg of total RNA.

Gene Expression Analysis by Real-Time Quantitative Polymerase Chain Reaction (PCR). A list of genes previously described as involved in adaptation and preadaptation to oxidative stress in *S. cerevisiae*[15,16] was drawn by screening expression profiles in response to stress in *Saccharomyces* (available at www.sgd.org). Five genes (together with three housekeeping reference genes) were selected for real-time PCR expression analysis; Table 1 shows genes chosen for this work, and metabolic functions and involved pathways are summarized in GO terms listed in the second column (for each gene ontology category of interest, at least one gene was chosen). All of the real-time PCR reactions were performed in an i-Q thermal cycler (Bio-Rad, Hercules, CA). A ready-to-use master-mix (iQ SYBR Green) containing a proofreading Taq polymerase, reaction buffer, dNTPs, and SybrGreen was used according to the manufacturer's instructions (Bio-Rad). Optimized reactions were performed in 0.5 mL of MicroAmp optical plates (Bio-Rad); each 25 μL reaction mixture contained the following: a 200 nM concentration of

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Table 1. Genes Selected for This Study and PCR Primers Used for Expression Quantification

gene	gene ontology terms	primer	sequence (5′−3′)	annealing temperature (°C)	PCR product size (bp)
ACT1	cytoskeleton cell wall organization	ACT1-Qfw	AATGCAAACCGCTGCTCAATCTTCTTCA	60.0	145
		ACT1-Qrv	AATACCGGCAGATTCCAAACCCAAAACAG		
FBA1	glycolysis gluconeogenesis	FBA1-Qfw	CTCCATTGCTGCTGCTTTCGGTAACTGT	60.0	153
		FBA1-Qrv	GAACCACCGTGGAAGACCAAGAACAATG		
TEF1	protein translation	TEF1-Qfw	ACGCCATTGAACAACCATCTAGACCAACTG	60.3	153
		TEF1-Qrv	CAGCTGGGGCGAAAGTAACAACCATACC		
CTA1	response to reactive oxygen species catalase activity	CTA1-Qfw	CAGTACCACGGTTCCTTACCAAGAAGC	60.0	165
		CTA1-Qrv	CCATCTCTGATAGCGGGATTGAAAAAT		
CTT1	response to reactive oxygen species catalase activity	CTT1-Qfw	TGATTCCGTTCTACAAGCCAGACTTTTC	60.4	136
		CTT1-Qrv	GTATTGGGAATCACCTTTGGAGTATGGAC		
GSH1	response to hydrogen peroxide glutathione biosynthesis	GSH1-Qfw	TCTATGCTCGACGTTTGCCATGACAAGATAC	60.0	143
		GSH1-Qrv	TTCAAATATGGAGAAGCTGGTGTTGCCTCTAA		
HSP26	response to stress, heat shock protein	HSP26-Qfw	GACCAAATAACTATGCTGGCGCTCTTTA	60.3	133
		HSP26-Qrv	AAAATATCAACTGGAACTGCGACACTTCTA		
SOD1	response to stress superoxide dismutase activity	SOD1-Qfw	GGTAACGTAAAGACGGACGAAAATGGT	60.0	155
		SOD1-Qrv	TTCAAAGATTCTTCAGTGTCACCCTTACCTA		

each primer, 1× iQ SYBR Green supermix, and 5 μL of cDNA (various dilutions). PCR primers (Table 1) were designed using Primer Select software (DNAstar, Madison, WI) and synthesized by MWG-Biotech (HPSF purified). For each gene, a standard curve was determined with yeast genomic DNA, and yeast DNA was extracted and purified using a MasterPure Yeast DNA Purification Kit by Epicentre (Madison, WI) according to the manufacturer's instructions. Five putative housekeeping genes (*ACT1*, *PDA1*, *FBA1*, *TEF1*, and *APE2*) have been amplified from all cDNA samples, and raw threshold cycle data have been treated by the Genorm online tool http://medgen.ugent.be/~jvdesomp/genorm/.[17] This allowed us to find the most stable genes in the data set (data not shown) and the optimal number of housekeeping genes for normalization, thus according to current constraints for real-time PCR data[18] as recently proposed also for *S. cerevisiae*.[19,20] Actin (*ACT1*), fructose-bisphosphate aldolase (*FBA1*), and translational elongation factor EF-1 α (*TEF1*) have been chosen as reference genes; data have been normalized to the three genes simultaneously using the iQ5 software provided by Bio-Rad.[17] Indeed, the suitable number of housekeeping genes that resulted was 3 (stability cutoff, 0.15) and the stability of *ACT1*, *FBA1*, and *TEF1* was confirmed in all samples for both stressed and non-stressed conditions. The study was carried out using two biological repetitions (independent yeast cultures), and each time real-time PCR was performed in three technical repetitions. Both experimental errors were finally considered in the global standard deviation.

RESULTS

Determination of Phenolic Content and Oxidative Stability of RC. To increase the utility of RC liquid extract, it was freeze-dried. One gram of raw material yielded 30 mg of lyophilized powder. Moreover, lyophilization did not affect the RC phenolic profile. In this regard, our data (Table 2) are in agreement with those of Rossetto and co-workers[11] using Chioggia RC liquid extract.

Table 2. Phenolic Composition of RC

polyphenol	μg g^{-1} FW	CV[a]
gallic acid	11.7 ± 1.0	8.5
protocatechuic acid	471.1 ± 21.1	8.7
chlorogenic acid	703.0 ± 15.0	4.9
p-hydroxybenzoic acid	13.8 ± 1.2	8.6
vanillic acid	4.4 ± 0.2	0.4
syringic acid	8.1 ± 0.3	3.7
caffeic acid	14.5 ± 1.1	7.5
cyanidin-3-*O*-glucoside	14.4 ± 1.3	9.0
cyanidin-3-*O*-rutinoside	8.8 ± 0.4	4.5
pelargonidin-3-*O*-glucoside	15.4 ± 1.0	6.4
peonidin-3-*O*-glucoside	15.2 ± 1.2	7.8
cyanidin-3-malonylglucoside	177.3 ± 9.2	5.1
pelargonidin	1.8 ± 0.1	5.5
malvidin	1.1 ± 0.1	4.5

[a] The coefficient of variation % (CV) is <10%.

In the phenolic composition, expressed as μg/g of fresh weight (FW), the presence of a large amount of hydroxycinnamic and benzoic acids can be seen. Among the phenolics identified, chlorogenic acid was the main component followed by protocatechuic acid and various glycosylated forms of cyanidin, which represented the main fractions of total anthocyanins. The

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Table 3. Effect of RC and BHT at Low and High Dosage (0.15 and 1.50 mg g^{-1}) on Different Oils' Oxidative Rancidity Measured by Rancimat Test

sample	IT[a] (h)	AAI
corn oil	9.67 ± 0.11 d	1.00
BHT (low)	10.43 ± 0.56 bc	1.07
BHT (high)	12.16 ± 0.4 a	1.25
RC (low)	10.20 ± 0.06 b	1.05
RC (high)	10.61 ± 0.08 c	1.09
soybean oil	8.29 ± 0.31 bd	1.00
BHT (low)	8.91 ± 0.42 b	1.07
BHT (high)	10.83 ± 0.63 a	1.30
RC (low)	9.12 ± 0.31 b	1.10
RC (high)	9.67 ± 0.1 c	1.16
peanut oil	5.24 ± 0.66 d	1.00
BHT (low)	6.42 ± 0.08 b	1.22
BHT (high)	8.61 ± 0.41 a	1.64
RC (how)	6.11 ± 0.01 c	1.16
RC (high)	6.49 ± 0.15 b	1.24

[a] a, b, c, and d: $P < 0.05$. IT refers to the time (h) at the break point of the two extrapolated straight parts of the curve obtained by Rancimat apparatus. AAI = IT of oils with antioxidant/IT of oils (control).

presence of malonyl ester of cyanidin 3-glucoside in the HPLC chromatograms was confirmed by demalonation experiments as reported by Du Pont and co-workers.[12]

The same amounts of RC and BHT were also analyzed for their antioxidant capacity expressed as AAI value (Table 3). The results of Rancimat test showed that the high dose of BHT had a significantly higher antioxidant power than RC. In contrast, for low BHT dose, an oil-dependent effect was observed. The AAI value of BHT low dose was achieved with a high dose of RC extract in a peanut oil experiment; when using soybean oil, a low level of RC extract was needed ($p < 0.05$). Moreover, in corn oil, no significant differences were detected between BHT low levels, and both RC amounts were employed.

Antioxidant Analysis in Yeast Model System. The impact of RC extracts on *S. cerevisiae* oxidative stress defense system was investigated by real-time quantitative PCR expression analysis of the stress responsive genes: *CTT1*, *CTA1*, *GSH1*, *SOD1*, and *HSP26*.

In nonstressed cells, the expression level of target genes was stable; RC extract did not affect their expression at any concentration (Figure 1). With regard to the response to stress, the genes *CTT1*, *CTA1*, *GSH1*, and *SOD1* showed a clear up-regulation after exposure to H_2O_2 (6.6−9.2-fold, as compared to control, Figure 1A), as expected. Results indicate that all of these genes displayed a significant attenuation of their up-regulation in H_2O_2-stressed cells if the RC extract was present (i.e., *CTA1* overexpression was halved, Figure 1A). In particular, the preincubation of yeast cultures with RC triggered lowering in expression of *CTA1*, *CTT1*, and *SOD1* at both concentrations tested (100 and 400 mg L^{-1}), whereas the effect was visible only at the highest dose for the gene *GSH1*. The gene *HSP26* showed a lower but significant H_2O_2-induced overexpression (2.6-fold, Figure 1B); nevertheless, it maintained the same expression level regardless of any dose of RC added to the medium: Up-regulation of this general stress responsive protein was not strongly involved in H_2O_2 protection in these conditions or affected by the RC extract.

DISCUSSION

Freeze-drying may be a strategy to increase the stability of natural extracts, which in their liquid form have a very short shelf life. From a technology perspective, freeze-dried products have advantages due to ease of storage, flexibility for dosing at multiple concentrations, and solubility characteristics. However, biomolecules can be adversely affected by the stresses of freezing and/or drying processes. In this regard, product comparison between lyophilized and liquid formulations showed no significant difference for RC phenolic profile. Among the phenolics identified, chlorogenic acid, which is formed between caffeic and quinic acid, was the main component. It has been shown that both chlorogenic acid and caffeic acid are strong antioxidants in vitro, and chlorogenic acid is widely recognized to be active because of its free radical scavenging properties. It inhibits peroxidation of linoleic acid[21] and acts as a cancer chemopreventive agent.[22] However, a study on fish oil stability[23] also revealed that chlorogenic acid was a weak antioxidant, and its antioxidant effectiveness did not substantially vary with its concentration. It is well-known that chlorogenic acid makes up 5−10% of the weight of coffee beans, and a 200 mL cup of Arabica coffee contains between 70 and 200 mg of chlorogenic acid. In this respect, our results showed that 200 g of RC (FW) had a similar concentration as a cup of coffee. Moreover, as reported by Rossetto and co-workers,[11] the high concentrations of anthocyans and, in particular, of cyanidin, together with their bioavailability, are very interesting to define RC as a low-cost food/feed ingredient.

The potential of using RC as a natural antioxidant was studied by pure lipid oxidation model (Rancimat method). It is a very rapid, standardized, and above all comparative method to investigate the effectiveness of antioxidant substance.[23] Experiments were performed with edible oils (peanut, soybean, and corn oils) and BHT to compare the antioxidant efficiency of RC with the most commonly used synthetic antioxidant material, but as reported by Nwuha and co-workers,[24] direct solubilization of RC extract in edible oils was not successful. To overcome the problems, RC extract was dissolved in a water/ethanol solution. The same amounts of BHT and RC were compared, taking into account that the natural extract was not law-limited in quantity (high level) as reported by Moure.[5] The legal limit of BHT according to Codex Alimentarius (available at www.codexalimentarius.netTail) or EU legislation (European Parliament and Council Directive No. 95/2/EC) was considered to choose the dosage (low level). The results of Rancimat test showed that at the largest doses tested, the antioxidant power of BHT was significantly higher than that of RC ($P < 0.05$), in agreement with other works, which found a certain (not always high) protection of oil oxidation by different natural extracts.[25−27] These results suggest that the amount of RC extract has comparable effects with low BHT levels, close to its legal limit in vegetable oil and fat. This finding indicates that RC extract may contribute in a significant way to control lipid peroxidation in different oils and, due to its organoleptic characteristics, may be suitable for incorporation into food/feed products without conferring the intense herb flavor, which limits the application of aromatic plants despite their high antioxidant activity.[28] However, a detailed study in other conditions also considering RC potential pro-oxidant activity, as reported by Papetti and co-workers,[29] could be tackled in future work.

In the last step of our work, we evaluated the capability of RC extracts to protect the yeast *S. cerevisiae* against sublethal

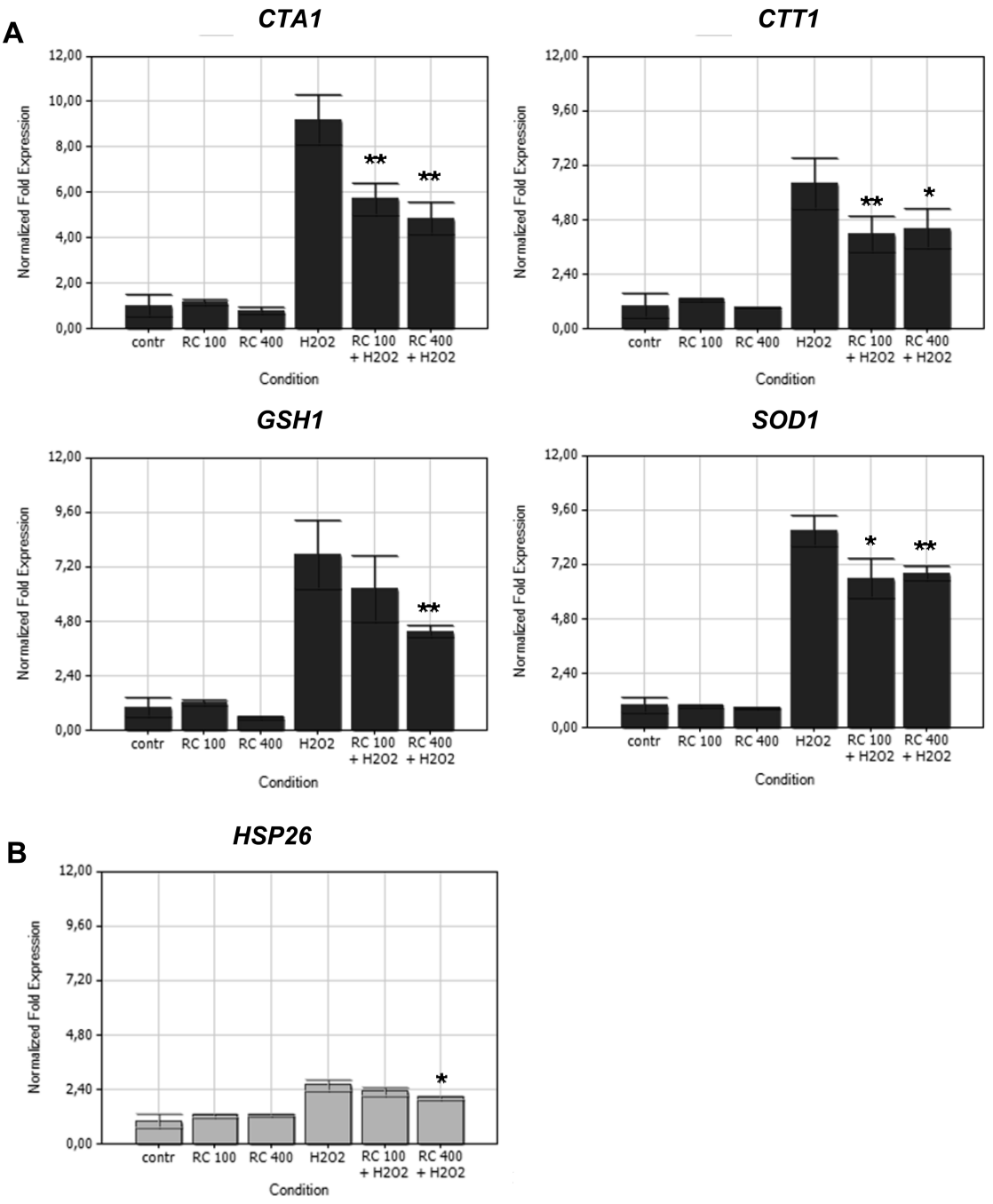

Figure 1. Expression levels of *CTT1*, *CTA1*, *GSH1*, *SOD1* (A) and *HSP26* (B) in *S. cerevisiae* cells. Samples are identified by RC content, if any (RC 100 or 400 mg L^{-1}) and/or by H_2O_2 presence; the control sample was not treated with RC or stressed with H_2O_2. For each data set, the control was chosen as a reference (expression level = 1). Error bars show standard deviations, including three technical repetitions for two independent biological samples. The relative normalized fold expression is calculated by the ΔΔCt method, using *ACT1*, *FBA1*, and *TEF1* as reference genes (see Materials and Methods). For statistical validation (*t* test), unstressed samples containing RC (RC100 and RC400) were compared to control, and stressed samples containing RC (RC100 + H_2O_2 and RC400 + H_2O_2) were compared to stressed reference sample (H_2O_2); significant variations are indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$).

oxidative stress, using this simple micro-organism as a functional and informative model for studying higher eukaryote stress.[30] This approach is likely to be particularly appropriate for studying complex matrices containing several antioxidant molecules, such as vegetal material and food byproducts, because it allows us to evaluate the pleiotropic effect of a compound on different stress responding genes. Indeed, each class of antioxidant molecules could exert its action by affecting a different pathway.[31−35] To investigate the impact of RC on the *S. cerevisiae* stress defense system, we quantified expression changes after H_2O_2 exposure of genes previously described as necessary for adaptation and preadaptation to oxidative stress in *S. cerevisiae*.[15,16] Indeed, hydrogen peroxide has been widely used for studying genome-wide expression changes in yeast due to oxidative stress,[15,36,37]

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and it is clearly involved in general responsive pathways. Moreover, H_2O_2 has been shown to be involved in protecting yeasts from impending stresses other than ROS,[16] particularly interesting for our study.

In this study, target genes have been chosen for exploring the activation of different pathways involved in oxygen detoxification:[37] a superoxide dismutase (*SOD1*), two catalases (*CTA1* and *CTT1*), a glutathione synthetase (*GSH1*), and a heat-shock protein (*HSP26*) have been quantified. Two of these genes (*CTT1* and *SOD1*) are regulated by the "environmental stress response" (ESR),[36,38,39] and one belongs to the "response to heat shock" (*HSP26*).[40] Moreover, the orthologues of some of these genes (*SOD1* and *GSH1*) are supposed to be influenced by the presence of antioxidant plant phenolics, such as flavonoids and procyanidins, in human cell lines.[33]

Oxidative stress triggered by H_2O_2 on yeast cells was clearly observed by up-regulation of genes *CTT1*, *CTA1*, *GSH1*, and *SOD1* in the nontreated sample. All of these genes displayed a significant attenuation of their up-regulation when RC was present, indicating that the yeast cell was sensing a lower stress.[16] This could be due both to the free radical scavenging activity of RC and to its modulatory actions in cells through actions at signaling pathways as recently shown for other plant phenolics.[35,41] Our results show that RC has a strong impact on the expression of all of the genes tested but *HSP26*. This result indicates that different stress responsive pathways are interested by RC action, whereas in a previous study,[32] a single gene was associated with the effect of pure antioxidant molecules such as catechin (*CTT1*) and resveratrol (*GSH1*). This pleiotropic effect suggests a wide range of protective effects for an antioxidant complex such as RC.

Moreover, the effect of RC extracts by themselves on yeast cells has been analyzed, because red *C. intybus* has been previously characterized for both its anti- and pro-oxidant activities ex vivo.[8,42] In our test, RC did not affect the expression of any target gene in nonstressed cells; this means that the tested dose did not exert any pro-oxidative effect. The results suggest the possibility of using the RC (at least up to the doses tested) without a pro-oxidative effect. A more thorough study of the mechanism of RC action, both in stressed and in nonstressed cells, will be an interesting task for a future work (including higher RC doses, different times of exposure prior to stress, and expression analysis of other genes involved in oxidative stress response, such as thioredoxins and glutathione transferases).

In conclusion, this study emphasizes by different approaches that RC extract might be interesting as a cheap, natural, and abundant food/feed additive for replacing synthetic antioxidants. Moreover, a pleiotropic effect of RC extract on yeast stress response could be a promising starting point for the validation of its antioxidant capacity in eukaryotic organisms.

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