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Antioxidative Activity of Propolis Extract in Yeast Cells

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ABSTRACT: The antioxidative activities of propolis and its main phenolic compounds, caffeic acid, *p*-coumaric acid, ferulic acid, and caffeic acid phenethyl ester, were investigated in the yeast *Saccharomyces cerevisiae*. After 1 h of exposure of the yeast cells, their intracellular oxidation was measured using 2′,7′-dichlorofluorescein. Yeast cells exposed to 96% ethanolic extracts of propolis in DMSO (EEP) showed decreased intracellular oxidation, with no significant differences seen for the individual phenolic compounds. However, cellular uptake was seen only for a moderately polar fraction of EEP (E2) and caffeic acid phenethyl ester. The EEP antioxidative activity thus resulted from this E2 fraction of EEP. The influence of EEP was also investigated at the mitochondrial proteome level, by analyzing its profile after 1 h of exposure of the yeast cells to EEP and E2. Changes in the levels of antioxidative proteins and proteins involved in ATP synthesis were seen.

KEYWORDS: propolis, phenolic compounds, caffeic acid, *p*-coumaric acid, ferulic acid, caffeic acid phenethyl ester, antioxidative activity, yeast, *Saccharomyces cerevisiae*, proteomics

INTRODUCTION

To prevent oxidative-stress-related diseases, different bee products, such as propolis, have generated considerable interest. Propolis is the strongly adhesive and resinous substance that is collected by the bees, transformed, and used to seal holes in their honeycombs, to smooth the internal walls, to protect the entrance of the beehive against intruders, and to prevent the decomposition of creatures that have been killed by the bees after an invasion of the beehive. Bees collect the resin from cracks in the bark of trees and leaf buds. This resin is masticated by the bees, thus adding salivary enzymes, and the partially digested material is mixed with beeswax and used in the beehive.[1,2]

Propolis has been reported to have anticancer, anesthetic, antimycotic, anti-inflammatory, antioxidant, antiseptic, astringent, antiviral, bacteriostatic, choleric, and spasmolytic properties.[3,4] It is believed that the various pharmacological activities of propolis can be attributed to its phenolic compounds and, in particular, the caffeic acid, caffeic acid derivates, and flavonoids in propolis are of interest for their known antioxidative activities.[4,5]

With the exception of a few studies of the antioxidative activity of propolis in vivo,[6−9] almost all such studies have been carried out in vitro,[4,5,10,11] with the main aim of defining the relationship between the antioxidative activity of propolis and its phenolic compounds.

On the basis of in vitro studies, we cannot assume that the antioxidant compounds in various bioactive substances will show the same activities in the cell. Hence, for any antioxidative evaluation, it is also necessary to understand the pharmacodynamics, which is in the case of phenolic compounds less well-known. Therefore, many studies are being carried out on the molecular and genetic interactions of phytochemicals and other bioactive substances in food and dietary supplements.

In the present study, the antioxidative activity of propolis was investigated in vivo using the yeast *Saccharomyces cerevisiae* in stationary phase. This lower eukaryote *S. cerevisiae* is an appropriate model organism for the study of fundamental eukaryotic cellular processes, such as their stress responses and metabolic pathways.[12−16] Furthermore, in their stationary phase, yeast cells resemble cells of multicellular organisms according to several aspects: (1) most of their energy comes from mitochondrial respiration; (2) the cells are in the G_0 phase; and (3) damage accumulates over time.[17]

To our knowledge, none of the in vivo studies on propolis have investigated the relationships between its antioxidative activity and its phenolic composition. The aim of the present study was to define the antioxidative activities and cellular uptake of propolis and its main phenolic compounds in the yeast cell. To determine whether these yeast cells respond to propolis exposure by changes in protein level, proteome analysis was also carried out, using two-dimensional (2-D) electrophoresis.

MATERIALS AND METHODS

Yeast Strain and Cultivation. The yeast *S. cerevisiae* ZIM 2155 was obtained from the Culture Collection of Industrial Microorganisms (ZIM) of the Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.

The yeast was cultivated in yeast extract (10 g/L; Biolife), peptone (20 g/L; Biolife), glucose (20 g/L; Merck) (YEPD) medium at 28 °C and 220 rpm, until stationary phase. The cells were then centrifuged for 3 min at 4000*g*, washed once with phosphate-buffered saline (PBS) (Merck), and suspended in PBS at a concentration of 1×10^8 cells/mL. The cells were further incubated at 28 °C and 220 rpm for 96 h.

Solid-Phase Extraction (SPE). The present study used a 96% ethanolic extract of propolis of Slovenian origin with solid-phase extraction used to clean this original extract and also to separate it into two elution fractions. Here, 200 μL of the original 96% ethanolic extract of propolis (or for the separation, the "cleaned" 96% ethanolic extract of propolis) was mixed with 200 μL of 20 mM ammonium formate and then added to a Strata-X SPE cartridge (Phenomenex) that had previously been conditioned with 2 mL of methanol (Merck) followed

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by 2 mL of 20 mM ammonium formate. After the sample was loaded, the cartridge was washed with 2 mL of 20 mM ammonium formate in 15% methanol/water (v/v) and vacuum-dried for 3 min.

For the cleaning of the propolis, the cartridge was eluted with 2 mL of 96% ethanol (Merck). Then the ethanol was removed by evaporation and replaced with dimethyl sulfoxide (DMSO) (Fluka). For the separation of the cleaned propolis into eluates E1 (polar fraction, retention time < 30 min) and E2 (moderately polar fraction, retention time > 30 min), the cartridge was eluted with 2 mL of 30% ethanol followed by elution with 2 mL of 96% ethanol. The ethanol was removed from both eluates by evaporation and replaced with 200 μL of DMSO.

Treatment of Yeast Cells. The cleaned 96% ethanolic extract of propolis in DMSO (EEP) was added to the yeast cell suspensions following their 96 h of incubation in PBS, at a concentration of the phenolic compounds of 0.05 g/L (expressed as grams of chlorogenic acid per liter of EEP).

As well as this treatment with EEP, the yeast cells were treated with ferulic acid (Sigma), caffeic acid (Sigma), *p*-coumaric acid (Sigma), and caffeic acid phenethyl ester (CAPE) (Sigma), a combination of phenolic acids (ferulic acid, caffeic acid, *p*-coumaric acid), eluate E1, and eluate E2. These phenolic compounds were also dissolved in DMSO and added to the yeast cell suspensions following their 96 h of incubation in PBS, at a concentration of 0.05 g/L.

After a further 1 h of incubation at 28 °C and 220 rpm, the samples were taken for further analyses: measurements of intracellular oxidation, cellular uptake, and analysis of the mitochondrial proteome (this last only for EEP and eluate E2).

Determination of Intracellular Oxidation. Intracellular oxidation was estimated using 2′,7′-dichlorofluorescein (H_2DCF), which reacts with oxidants, thus revealing the presence of reactive oxygen species (ROS). This was given to the cells as 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), which easily penetrates the plasma membrane and is hydrolyzed inside the cells by nonspecific esterases. The nonfluorescent H_2DCF can then be oxidized to fluorescent 2′,7′-dichlorofluorescin (DCF), which is measured fluorometrically.[18]

The cells from the 2 mL incubations were sedimented by centrifugation (14000*g*, 5 min) and washed three times with 50 mM potassium phosphate buffer (pH 7.8). The cell pellets were finally resuspended in 9 volumes of 50 mM potassium phosphate buffer (to 10%, v/v) and incubated at 28 °C for 5 min. The ROS-sensing dye H_2DCFDA (Sigma) was added from a 1 mM stock solution in 96% ethanol (Merck), to a final concentration of 10 μM. After a 20 min incubation at 28 °C and 220 rpm, the fluorescence of yeast cell suspension was measured, using a Safire II microplate reader (Tecan). The excitation and emission wavelengths of DCF were 488 and 520 nm, respectively.

The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of the relative fluorescence intensity ± SE. Individual comparisons were made using Duncan's multiple-range test[19] on a total of six samples. A value of $p < 0.05$ was considered to indicate a significant difference between groups.

Determination of Cell Energy Metabolic Activity. Cell energy metabolic activity was determined by BacTiter-Glo Microbial Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, after 1 h of incubation, 100 μL of cell suspension at a concentration of 1×10^7/mL and 100 μL of BacTiter-Glo reagent were placed in a 96-well microplate and mixed. After 5 min, luminescence was measured using the Safire II microplate reader (Tecan). The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of the relative luminescence intensity ± SE.

Cell Viability Determination. Cell viability was measured as colony-forming units (CFU). After 1 h of incubation, CFU were determined by plating cell suspension on yeast extract (10 g/L; Biolife), peptone (20 g/L; Biolife), glucose (20 g/L; Merck), agar (20 g/L; Biolife) (YEPD) medium, and then after a 2 day incubation at 28 °C, the number of colonies was counted. The experiments were performed in biological duplicates, and within each three technical replicates were done. Data are expressed as the mean of CFU/mL ± SE.

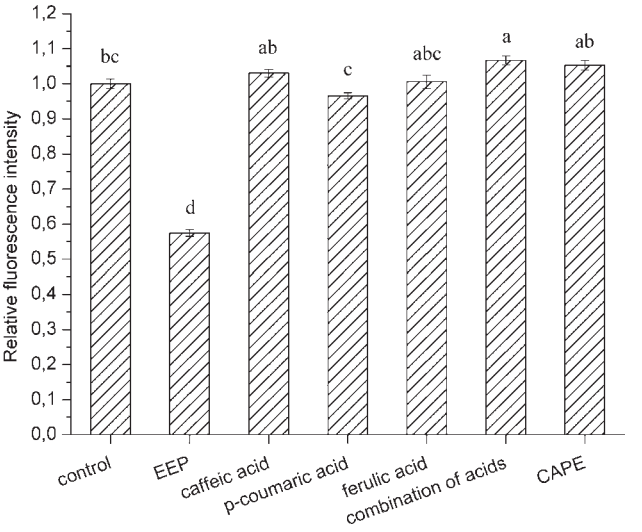

Figure 1. Intracellular oxidation in the yeast *S. cerevisiae* after 1 h of exposure to 96% ethanolic extract of propolis in DMSO (EEP) or to its main phenolic compounds, including caffeic acid phenethyl ester (CAPE), or to a combination of phenolic acids (caffeic, *p*-coumaric, and ferulic acid) at a concentration of 0.05 g/L, in comparison to the control. Data are the mean ± SE, with values not sharing a common letter (a−d) being significantly different (Duncan's multiple-range test; $p < 0.05$).

Cellular Uptake. To study the cellular uptake of phenolic compounds, the phenolic profile was determined both in the PBS and in the yeast cell suspensions 1 h after the addition of EEP or of the particular phenolic compounds. Thus, 1 h after the addition of the test compounds, the PBS and yeast cell suspensions were centrifuged (4000*g*, 3 min). The supernatants obtained were filtered (pore size: = 0.2 μm) and analyzed using liquid chromatography with diode array detection (LC-DAD), as below, to obtain the phenolic profile. All of the experiments were performed in duplicate.

LC-DAD Analysis. The samples were diluted 20-fold with 1% HCOOH in 50% methanol, filtered through a 0.22 μm PTFE filter, and analyzed by LC-DAD. The LC system consisted of an Agilent 1100 model G1312A binary pump and a model G1330B autosampler (Agilent Technologies). This reversed-phase HPLC separation was carried out using a Gemini C18 column (150 mm × 2.0 mm internal diameter; 3 μm particle size), which was protected by a Gemini C18 security guard cartridge (4.0 mm × 2.0 mm internal diameter) (Phenomenex). The mobile phase comprised aqueous 1% HCOOH (A) and acetonitrile (B), and the following gradient was used: 0−5 min, 10% B; 5−50 min, 10% −60% B; 50−52 min, 60%−80% B; 52−60 min, 80% B; 60−70 min, 80%−10% B; 70−80 min, 10% B. The column was maintained at 25 °C, with an injection volume of 20 μL and a flow rate of 0.2 mL/min.

Extraction of Mitochondrial Proteins. To analyze the mitochondrial proteome, 20 mL of yeast cell suspension was centrifuged at 4000*g* for 3 min. The pellet obtained was washed once with PBS and used for the extraction of the mitochondrial proteins using Cytosol/Mitochondria Fractionation kits (Calbiochem), according to the manufacturer's instructions, with a little modification. The yeast cells were disrupted by vortexing with zirconia/silica beads (BioSpec Products), five times for 1 min each, with 1 min intervals for cooling on ice.

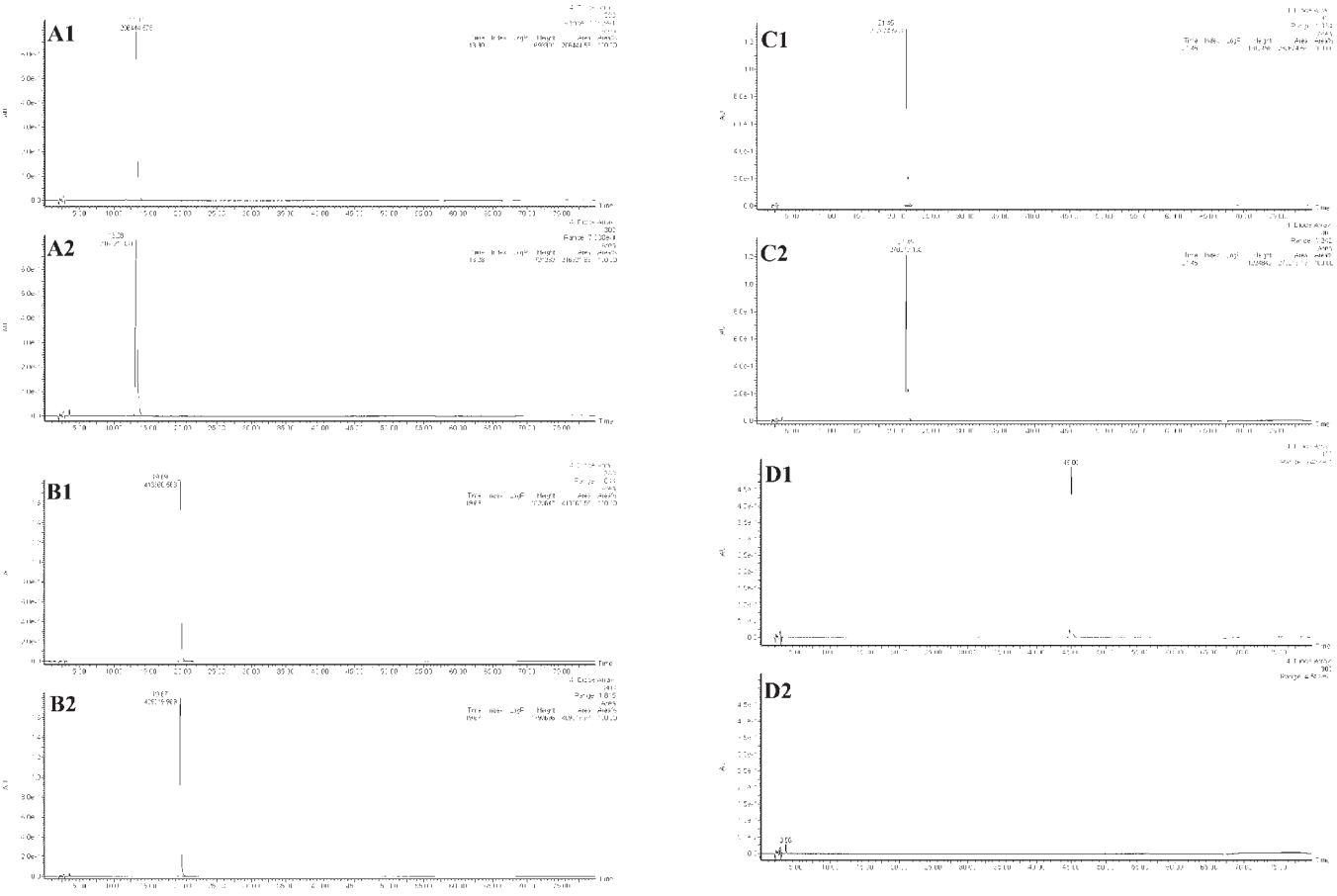

Figure 2. LC-DAD (300 nm) chromatograms of the phenolic compounds in the supernatants after centrifugation of suspensions of the yeast *S. cerevisiae* before (1) and after (2) their 1 h of exposure to caffeic acid (A), *p*-coumaric acid (B), ferulic acid (C), and caffeic acid phenethyl ester (CAPE) (D) at a concentration of 0.05 g/L.

The mitochondrial protein concentrations were determined according to the method of Bradford,[20] using bovine serum albumin (Sigma) as standard.

Two-Dimensional Electrophoresis. The 2-D electrophoresis was performed according to the method of Görg,[21] with minor modifications. The samples (100 μg of protein) were mixed with rehydration solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) immobilized pH gradient (IPG) buffer (pH 3−10), 18 mM dithiothreitol, and a trace of bromophenol blue] and applied to 13 cm pH 3−10 IPG strips (GE Healthcare). After rehydration, the first dimension of isoelectric focusing was carried out at 20 °C on a Multiphore II system (GE Healthcare). The following voltage program was applied: 0−300 V (gradient over 1 min), 300 V (fixed for 1 h), 300−3500 V (gradient over 1.5 h), and 3500 V (fixed for 5 h). Prior to the second dimension of the 2-D electrophoresis, the IPG strips were equilibrated in sodium dodecyl sulfate (SDS) equilibration buffer [75 mM Tris HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and a trace of bromophenol blue], containing 1% dithiothreitol, for 15 min, and containing 4.8% iodoacetamide for an additional 15 min. The second dimension (SDS−polyacrylamide gel electrophoresis) was carried out with the 12% running gels on a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments), at a constant 20 mA/gel for 15 min and then at a constant 40 mA/gel until the bromophenol blue reached the bottom of the gels. The 2-D gels were stained with SYPRO Ruby (Invitrogen). For each sample, two 2-D gels were run under the same conditions.

Protein Visualization and Image Analysis. After staining, the gels were analyzed using a CAM-GX-CHEMI HR system (Syngene). The gel image analysis was carried out using 2-D Dymension software, version 2.02 (Syngene). Duplicate gels for each sample were matched to provide an average gel sample. The spots were revealed and quantified on the basis of their normalized volumes, as the spot volume divided by the total volume over the whole set of gel spots. Expression changes (fold changes) were considered to be significant if the intensity of the corresponding spot reproducibly differed by >1.5-fold in a normalized volume as a comparison between the control and treated samples and if this was statistically significant (Student's *t* test).

Yeast Proteome Map and Protein Identification. To identify differentially expressed proteins, a previously obtained 2-D mitochondrial proteome map was used, where the protein identities were confirmed by LC−tandem mass spectrometry (MS/MS) using electrospray ionization−ion trap mass spectrometry, at the Cogeme Proteome Service Facility 1 (University of Aberdeen, U.K.; Istenič et al., unpublished results).

RESULTS AND DISCUSSION

Intracellular Oxidation and Cellular Uptake. The yeast cells were treated for 1 h with EEP at a concentration of 0.05 g/L. This had been determined in our previous study (Mavri et al., unpublished results) as a concentration that promoted a significant decrease (42%) in the intracellular oxidation of the cells treated with EEP (Figure 1), as compared to the control

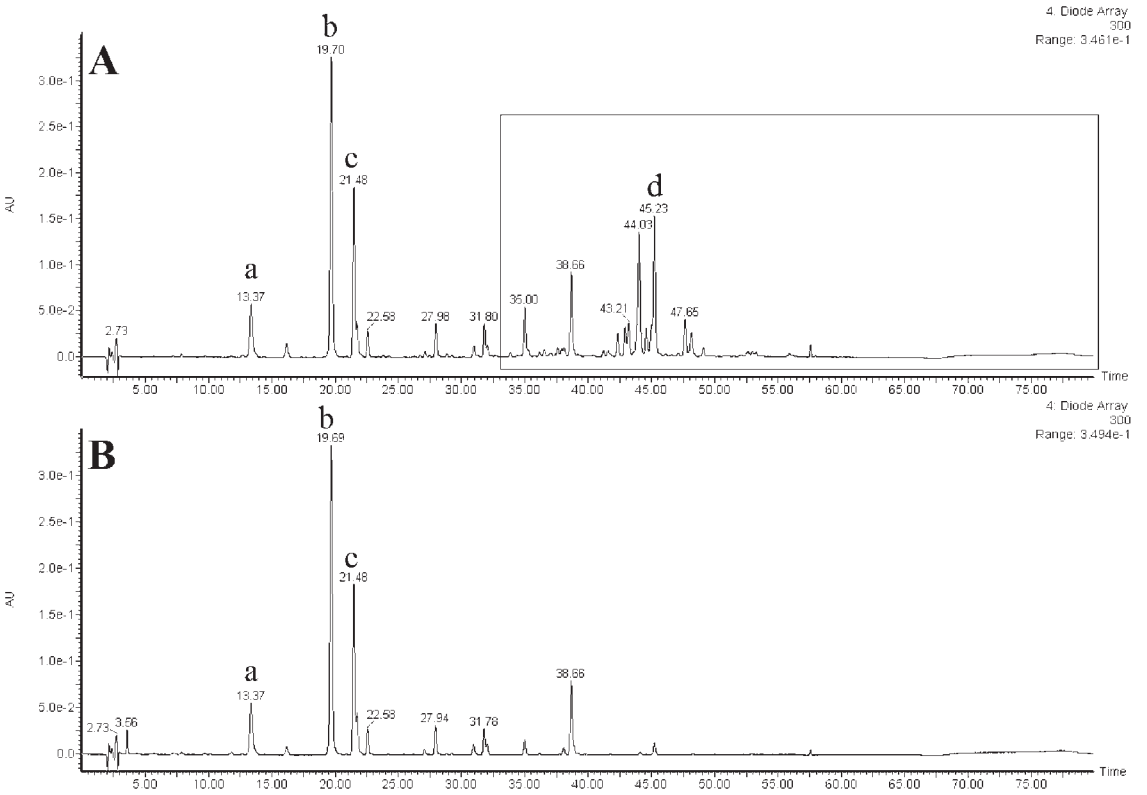

Figure 3. LC-DAD (300 nm) chromatogram of phenolic compounds in the supernatants after centrifugation of suspensions of the yeast *S. cerevisiae* before (A) and after (B) their 1 h of exposure to 96% ethanolic extract of propolis in DMSO (EEP) at a concentration of 0.05 g/L. The marked phenolic compounds are (a) caffeic acid, (b) *p*-coumaric acid, (c) ferulic acid, and (d) caffeic acid phenethyl ester (CAPE), and the frame shows phenolic compounds that entered the yeast cells.

(nontreated cells). Also, there were no effects on intracellular oxidation by DMSO at this concentration, whereas concentrations above 0.05 g/L DMSO caused increased intracellular oxidation (data not shown).

To test if the propolis has any effect on cell viability and activity, we measured cell viability as CFU and cell energy metabolic activity. Results showed that there is no change in CFU between control and treated cells [$(8.5 \pm 0.2) \times 10^7$ and $(7.6 \pm 0.6) \times 10^7$, respectively]. Additionally, in the cells treated with EEP, cell energy metabolic activity increased 2.3-fold compared to control (1.00 ± 0.04, control; 2.30 ± 0.05, treated sample). On the basis of these results it can be concluded that decreased intracellular oxidation reflects the antioxidative activity of propolis.

As the antioxidative activity of propolis is believed to be related to its phenolic compounds,[4,5] we also tested the antioxidative activities of a series of its main phenolic compounds: caffeic acid in our previous study (Mavri et al., unpublished results) and, in the present study, *p*-coumaric acid, ferulic acid, and CAPE, the levels of which in EEP were determined by LC-MS as being higher compared to other compounds (CAPE, 501.32 μg/mL; *p*-coumaric acid, 465.26 μg/mL; caffeic acid, 359.67 μg/mL; ferulic acid, 225.56 μg/mL) (Mavri et al., unpublished results). The yeast cells were thus treated with these particular compounds and with a combination of phenolic acids, each at 0.05 g/L. The individually tested compounds did not show any antioxidative activities compared to the control [control, 1.00 ± 0.01; caffeic acid, 1.03 ± 0.01; *p*-coumaric acid, 0.97 ± 0.01; ferulic acid, 1.01 ± 0.02; combination of acids, 1.07 ± 0.01; CAPE, 1.05 ± 0.01 (Figure 1)].

Therefore, the following question arose: did these compounds even enter the cells? To test the uptake of these particular compounds into these yeast cells, their levels were determined using LC-DAD, before and after exposure of the cells. These data showed that only CAPE entered these yeast cells, whereas for the other compounds there were no changes in their levels before and after exposure (Figure 2). Interestingly, CAPE did not decrease the intracellular oxidation (Figure 1); indeed, considering its polarity, it would have remained in the cell membrane and, therefore, did not show any effects on intracellular oxidation. It is known that phenolic compounds can interact with lipids. These interactions seem to be rather unspecific, based essentially on physical adsorption. Adsorption is the process of accumulation at an interface and should be distinguished from absorption, which implies the penetration of one component, for example, the polyphenol molecule, throughout the membrane.[22]

Furthermore, cellular uptake was also examined for EEP, which demonstrated that only a fraction of the phenolic compounds entered these yeast cells (Figure 3). On the basis of this observation, using solid-phase extraction, EEP was separated into two eluates according to polarity: a polar eluate, E1, and moderately polar eluate, E2.

EEP used in this study was chemically characterized by Mavri et al. (unpublished results). It consists of the following components, from more to less polar: gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, myricetin, luteolin, quercetin, formononetin, caffeic acid benzyl ester, pinobanksin, apigenin, kaempferol, caffeic acid isoprenyl ester, CAPE, pinobanksin-3-*O*-acetate, kaempferide, chrysin,

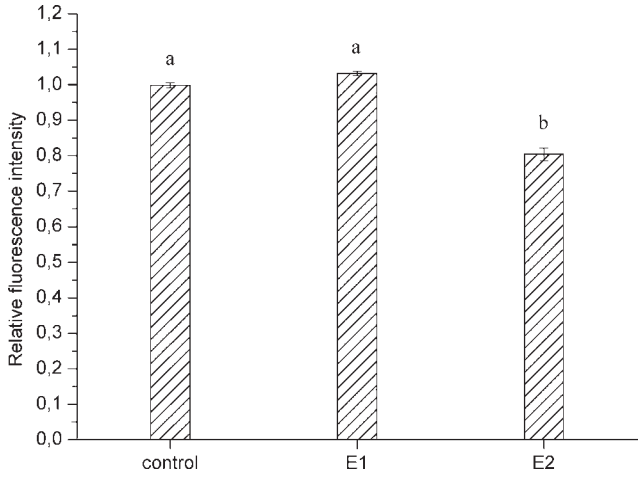

Figure 4. Intracellular oxidation in the yeast *S. cerevisiae* after 1 h of exposure to eluates E1 and E2 from EEP, in comparison to the control. Data are the mean ± SE, with values not sharing a common letter (a, b) being significantly different (Duncan's multiple-range test; $p < 0.05$).

pinocembrin, galangin, and caffeic acid cinnamyl ester. Eluate E1 is mostly composed of hydroxycinnamates, whereas in eluate E2 flavonoids are mostly present.

Then the yeast cells were treated separately with both eluate E1 and eluate E2, and their intracellular oxidation was measured. Eluate E2 corresponded to the fraction that entered the cells, which decreased (20%) the intracellular oxidation, in contrast to eluate E1, with which no cellular uptake or changes in oxidant levels were seen (control, 1.00 ± 0.01; eluate E1, 1.03 ± 0.01) (Figure 4). Difference in the decrease of intracellular oxidation for EEP and eluate E2 can be explained by varieties in preparation procedure. Namely, to prepare eluate E2 solid-phase extraction was used once more compared to EEP, resulting in a greater loss of phenolic compounds.

Mitochondrial Proteome Analysis. The influence of EEP and eluate E2 at the proteome level was investigated using 2-D electrophoresis. Although the effects of both of these treatments on the total proteome were not significant (data not shown), intensive changes were found for the mitochondrial proteome. The main source of ROS in yeast cells is the mitochondrial respiratory chain.[23] Therefore, it is not surprising that the first changes at the proteome level in the presence of exogenous antioxidants are observed in the mitochondria. To analyze the mitochondrial proteome, a subcellular proteomic approach was used.

Subcellular proteomics has the advantage not only of relating proteins to functional compartments within eukaryotic cells but also of reducing the complexity of a whole cell or a tissue protein extract, which can often prevent satisfactory proteomic analysis. Namely, it allows the identification of novel and low-abundance proteins that can otherwise remain masked when total cellular extracts are investigated.[24]

In the case of this EEP exposure, this promoted a reduced level of α subunit of mitochondrial F_0F_1-ATP synthase (Atp1) ($R = -1.547$; $p = 0.022$) (Figure 5). This F_0F_1-ATP synthase is a multisubunit membrane-associated protein complex that catalyzes the phosphorylation of ADP to ATP at the expense of a proton motive force that is generated by the electron transport chain. In some organisms, this enzyme can also work in the reverse direction, by hydrolyzing ATP and generating an electrochemical proton

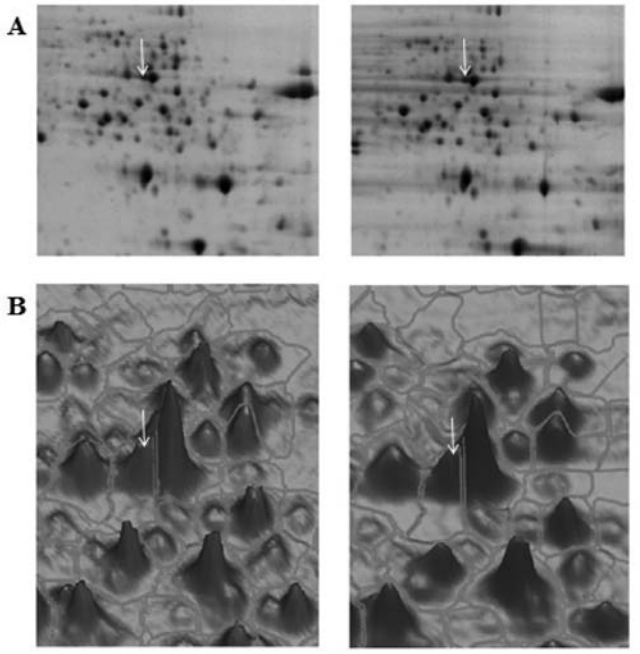

Figure 5. Comparison of mitochondrial protein profiles of control (left) and EEP-treated (right) yeast cells: (A) arrow, spot representing the α subunit of mitochondrial F_0F_1-ATP synthase (Atp1); (B) statistically significant differences in this spot ($R = -1.547$, $p = 0.022$) as shown in the 2-D Dymension gel image analysis software. Arrows indicate spots with levels that are statistically significantly different (Student's *t* test).

gradient across a membrane, to support locomotion or nutrient uptake. F_0F_1-ATP synthase can be divided into two parts: a soluble globular F_1 catalytic section, where, among others, the α subunit is located, and a membrane-bound F_0 proton-translocating section. F_0F_1-ATP synthase has been suggested to be a good molecular target for drugs in the treatment of various diseases and in the regulation of energy metabolism.[25]

Bioactive compounds can affect proteins through interactions with these proteins. In the case of phenolic compounds, it has been shown that these compounds often target F_0F_1-ATP synthase.[25−27] Gledhill et al.[27] showed that some phenolic compounds can inhibit the rotary mechanism of F_1-ATPase by binding to a site where the upper extremity of the central stalk fits into the hydrophobic annular sleeve of the "bearing" formed by the loop regions below the "crown". In the case of resveratrol, the residues are either within 4 Å of the inhibitor and form hydrophobic interactions, or they are linked to it via hydrogen bond networks.

Whereas in most studies interactions of bioactive compounds with proteins are indicated, changes in gene expression are also observed.[28] Bioactive compounds can alter mRNA and protein levels by altering the activities of transcription factors or by binding to cell receptors, which can result in changed activities of enzymes, including phosphatases and kinases.[29] The expression levels of subunit e of F_0F_1-ATP synthase have been shown to be highly sensitive to diverse physiological changes and stresses.[25] Indeed, it is already known that the intracellular balance of oxidants and antioxidants is a key factor in the regulation of the expression of certain genes.[29]

In the present study, decreased levels of Atp1 were seen in these yeast cells exposed to EEP, which can be explained directly

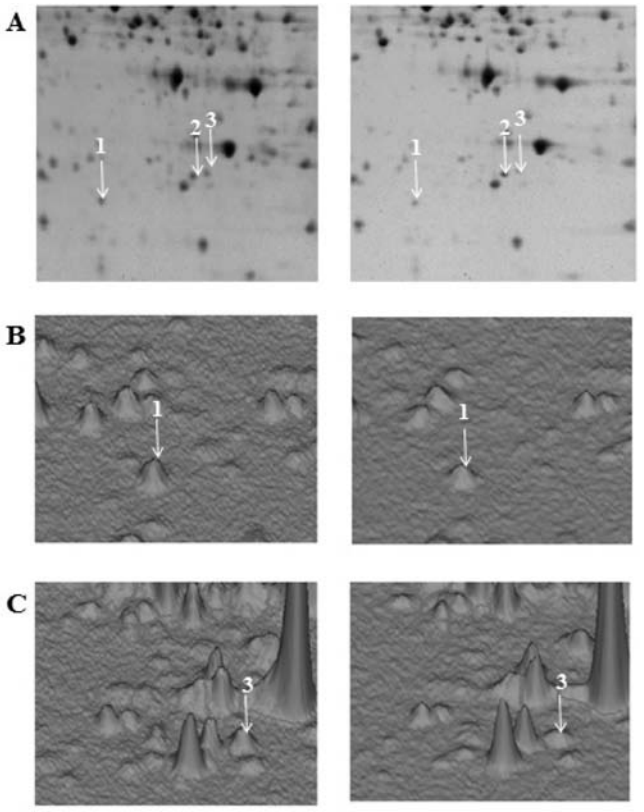

Figure 6. Comparison of mitochondrial protein profile of control (left) and EEP eluate-E2-treated (right) yeast cells: (A) spot 1, Mn SOD; spot 2, peroxiredoxin (Prx1); spot 3, probably an isoform of Prx1; (B) statistically significant differences in Mn SOD ($R = -1.784$, $p = 0.096$) and (C) in spot 3 ($R = -1.740$, $p = 0.026$), as shown in the 2-D Dymension gel image analysis software. Arrows indicate spots with levels that are statistically significantly different (Student's *t* test).

via the binding of the phenolic compounds to Atp1, to cause its modification. It has to be noted that by using a proteomic approach, such chemical interactions can be disrupted due to the use of denaturants in the sample extraction buffer, which can result in the restoring of a protein to its former state or, also indirectly, in the following: (1) binding of the phenolic compounds to transcription factors can modify the regulation of transcription of Atp1 and (2) post-translational modification of Atp1 can occur through, for example, kinases and phosphatases, to cause a shift in p*I*.

In the cells exposed to eluate E2, a reduced level of Mn SOD was seen ($R = -1.784$; $p = 0.096$), along with protein spot 3 ($R = -1.740$; $p = 0.026$) (Figure 6). Spot 3 might be an isoform of peroxiredoxin (Prx1) that has changed its p*I* due to post-translational modifications, such as, for example, phosphorylation. Prx1 has a thioredoxin peroxidase activity with a role in the reduction of hydroperoxides.[30] Both of these proteins (Mn SOD and Prx1) belong to endogenous antioxidant defense systems, and their decreased levels might be due to reduced intracellular oxidation after exposure to exogenous antioxidants (eluate E2) (Figure 5).

These reduced levels of endogenous antioxidant proteins and reduced intracellular oxidation indicate that eluate E2 is likely to be acting directly as a radical scavenger, and not indirectly as a pro-antioxidant. This latter would lead to decreased intracellular oxidation due to increased levels of endogenous antioxidant proteins.[31] Similarly, Jamnik et al.[32] showed a reduced level of Cu/Zn SOD in the yeast *S. cerevisiae* when exposed to bee royal jelly, which decreased intracellular oxidation. On the other hand, changes in mitochondrial proteome of yeast cells exposed to this fraction (Figures 5 and 6) indicate that these compounds might be involved in cellular metabolism or interactions with transcription factors or appropriate proteins.

In the present study, we have focused on propolis. As most of the information relating to the antioxidative activity of propolis arises from in vitro studies, we here investigated the antioxidative activity of propolis extract in cells at both the cellular and proteome levels using the yeast *S. cerevisiae* as our model organism. Only a moderately polar fraction of the EEP was shown to enter these yeast cells and to decrease their intracellular oxidation. Changes were also found at the mitochondrial proteome level, including for antioxidative proteins and proteins involved in ATP synthesis. Further investigations of the phenolic compounds in this moderately polar fraction of EEP will be carried out to provide a better understanding of these antioxidative activities of propolis in vivo.

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ABBREVATIONS USED

CAPE, caffeic acid phenethyl ester; DMSO, dimethyl sulfoxide; EEP, 96% ethanolic extracts of propolis in DMSO; E1, polar fraction of EEP; E2, moderate polar fraction of EEP; PBS, phosphate-buffered saline; DCF, 2′,7′-dichlorofluorescin; LC-DAD, liquid chromatography with diode array detection; Atp1, α subunit of mitochondrial F_0F_1-ATP synthase; Prx1, peroxiredoxin; SOD, superoxide dismutase.

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