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ARTICLE

A Mint Purified Extract Protects Human Keratinocytes from Short-Term, Chemically Induced Oxidative Stress

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Oxidative stress is strictly correlated to the pathogenesis of many diseases, and a diet rich in fruits and vegetables, or adequately integrated, is currently considered to be a protective and preventive factor. This study aimed to analyze the efficacy of a 1 h preincubation with the highest nontoxic dose of a characterized *Mentha longifolia* extract (80 μg/mL) in protecting human keratinocytes (NCTC2544) from chemically induced oxidative stress (500 μM H_2O_2 for 2, 16, and 24 h). As reference synthetic pure compounds rosmarinic acid (360.31 μg/mL), a major mint phenolic constituent, and resveratrol (31.95 mg/mL), a well-known antioxidant, were used. Cellular viability was significantly protected by mint, which limited protein and DNA damage, decreased lipid peroxidation, and preserved glutathione and superoxide dismutase activity in the shorter phases of oxidative stress induction, in extents comparable to or better than those of pure compounds. These data suggest that mint use as only a flavoring has to be revised, taking into consideration its enrichment in foodstuff and cosmetics.

KEYWORDS: Antioxidant; keratinocytes; mint; oxidative stress; rosmarinic acid; resveratrol

INTRODUCTION

The use of natural products with beneficial properties is as ancient as human civilization and, for a long time, minerals, plants, and animal products were the main sources of drugs (*1*). The Industrial Revolution and the development of chemistry resulted in a preference for synthetic products; the process of synthesis leads to more active, pure, safer, and cheaper drugs. In recent years, a trend turnaround led to the revaluation of natural products, on the basis of growing evidence that many extracts show an improved effectiveness with respect to synthetic monocompounds (*2*). This aspect is mainly explained with synergic effects of complex mixture bioactive constituents (*3*).

Mint presents a large variety of biological properties, such as antiallergenic, antibacterial, anti-inflammatory, antimycotic, antitumor, antiviral, gastrointestinal protective, hepatoprotective, and chemopreventive activities (*4*), most of which are attributable to its antioxidant activity. Mint leaves represent a rich source of polyphenolic compounds, including rosmarinic acid, eriotricin, luteolin, and hesperidin, although the chemical composition greatly varies with plant maturity, variety, geographical region, and processing conditions (*5*).

Oxidative stress, leading to reactive oxygen and nitrogen species (ROS and RNS, respectively) production, is more and more strictly correlated to the pathogenesis of many serious diseases (*6*, *7*). Epidemiological and experimental studies reveal a negative correlation between the consumption of a diet rich in fruits and vegetables and the risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation, and cancer (*8*). These beneficial activities are ascribed to the phenolics abundance, especially to flavonoid compounds (secondary metabolites), in such natural products.

Skin is one of the human organs more exposed to oxidative stress, mostly due to relatively high oxygen tension, ultraviolet light, and, on occasion, oxidizing chemicals; it constitutes the body's first line of defense (*9*). Keratinocytes represent the major cell type in the epidermis and are actively involved in regulating electrolytic balance and thermoregulation. Therefore, their inability to appropriately respond to the chemical and physical environment can induce physiological disorders (*10*). In particular, NCTC2544 keratinocytes are human undifferentiated and rapidly dividing cells that provide a good experimental model for the epidermal basal cell layer (*11*).

In the present study the protective activity of a mint purified extract (MINT PE), obtained by a modern, reproducible, high-yielding, and time-saving method, was evaluated on human skin keratinocytes after chemical induction of oxidative stress. As one of the major components of ROS, hydrogen peroxide (H_2O_2) has been extensively used as an inducer of oxidative stress for in vitro models (*12*). Because our aim was also to compare the efficacy of a complex natural extract with respect to pure synthetic compounds, rosmarinic acid, a secondary metabolite present in mint extract, and resveratrol, a polyphenol found in red wine, were employed as reference antioxidant compounds. After a preliminary

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in vitro evaluation, to assess their respective antioxidant activities (ABTS and DPPH), we analyzed the damage induced by cell treatment with 500 μM H_2O_2 for 2, 16, and 24 h (short-, medium-, and long-time exposure, respectively) after 1 h of preincubation with the different antioxidant compounds. To evaluate cell damage and protection we analyzed different end points and, in particular, report results on cell viability (MTT), protein damage (HSP70 induction), DNA damage (γ-H2AX histone phosphorylation), glutathione content (GSH), activity of the foremost antioxidant enzymes, such as glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation induction (TBARS).

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO), except when differently indicated. Cell culture materials were purchased by International PBI (Milan, Italy).

All of the antioxidant stock solutions (50 mg/mL) were freshly prepared in ethanol (EtOH) 70%, avoiding sonication, and kept in the dark on ice. Further dilutions were prepared in cell culture medium.

Plant Material, Extract Preparation, and Standardization. Seeds of *Mentha longifolia* were received from the National Centre for Plant Genetic Resources at the Plant Breeding and Acclimatization Institute (Radzikow, Poland). Seeds were sown in an experimental field, and the material was collected during the second year of cultivation at the beginning of flowering. Aerial parts of harvested plants (150 g) were freeze-dried, finely powdered, defatted with chloroform, and extracted with 70% methanol overnight at room temperature. This procedure was repeated twice, the obtained mint extracts were combined and filtered, and the solvent was removed under reduced pressure. The crude extract was suspended in water and passed through short column (10 cm × 6 cm), 40−63 μm LiChroprep RP-18 (Merck) previously preconditioned with water. The column was washed first with water to remove sugars and then with 40% methanol to elute phenolics (9.5 g).

The composition and the concentration of individual phenolics in the corresponding fraction were determined by using a LC-ESI-TQ-MS/M method as already described (*13*).

Antioxidant Assays. ABTS assay (*14*) was performed using an antioxidant assay kit following the manufacturer's instructions (CS0790, Sigma). Results are expressed as Trolox equivalent antioxidant capacity per gram of dry powder (TEAC, μmol/g) and are the mean of three different experiments performed in triplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) (*15*) was dissolved in EtOH 70% to a final concentration of 0.25 mM. Increasing volumes of samples in a final volume of 1 mL of EtOH 70% were added to 0.5 mL of DPPH solution and incubated in the dark for 30 min. Samples were read against blank (EtOH 70%) at 519 nm. Results are expressed as the sample concentration that quenches 50% of DPPH radical (IC_{50}, μg/mL), calculated by linear regression, plotting together data obtained for at least three different experiments performed in duplicate.

Cell Culture. The human keratinocyte cell line (NCTC2544) was routinely cultured at 37 °C in a humidified atmosphere with 5% CO_2 in minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 20 mM tricine, 0.1 mM nonessential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin.

Cell Viability. To assess the toxicity of all the substances, cells were incubated for 24 h with antioxidant compounds and EtOH 70% as solvent, and viability was determined by MTT assay (*16*). Briefly, 20000 cells per well were plated in a 96-multiwell plate, and the day after were incubated with increasing concentrations of the different substances for a further 24 h. After medium change, MTT (3-(4,5-dimethylthiazolyl)diphenyl tetrazolium bromide, 5 mg/mL) was added to each well and incubated for an additional 4 h, and formazan crystals were solubilized overnight by 10% SDS, 0.01 M HCl, and read at 540 nm. Results are expressed as percentage with respect to untreated control cells.

We also evaluated cell survival after incubation with increasing concentrations and different incubation periods with H_2O_2 by the MTT assay using the same procedure.

Cell Treatments and Lysate Preparation. Cells (3.4×10^6) were plated in 10 cm plates and after 24 h were incubated with the highest nontoxic doses, determined by MTT, of the different antioxidants in complete medium for 1 h. Hydrogen peroxide 500 μM was then added, without changing medium, and incubated for further 2, 16, and 24 h. Cells were then collected by trypsinization, counted, washed with PBS, lysed with three cycles of freezing/thawing in 1 mM sodium vanadate, and stored at −20 °C until use. For glutathione analysis, fresh cell pellets corresponding to 5×10^5 cells were extracted with 10% metaphosphoric acid and centrifuged, and the supernatant was stored at −20 °C until use. Protein concentration was determined according to Lowry's method (*17*).

Western Blot Analyses. Fifty micrograms of total proteins were separated by 12% SDS-PAGE (*18*), electroblotted onto PVDF membrane, probed (*19*) with anti-HSP 70 (clone BRM-22) 1:5000, antiphospho-histone H2AX (ser139) (clone JBW301, Upstate Biotech, Lake Placid, NY) 1:2000, and anti-actin (C-11) (sc-1615, Tebu-bio, France) 1:1800 primary antibodies following the manufacturer's instructions. Primary antibodies were detected with 1:20000 HRP-conjugated goat anti-mouse IgG (sc-2005) secondary antibody and Western Lightning *Plus*-ECL Enhanced Chemoluminescence Reagent (Perkin-Elmer). The area quantification was made with digital scanning (CAMAG Reprostar3 and CAMAG Videostore program).

Enzymatic Assays. Analysis of total GSH content, GSH peroxidase and reductase, SOD, and catalase activities of 2 h stressed cell lysates were performed as previously described (*20*).

Lipid Peroxidation Assessment. The end products of lipid peroxidation (malondialdehyde, MDA) were measured in cell extracts as thiobarbituric acid reactive substances (TBARS) and quantified using a fluorometric assay (*20*).

Statistics. Results are presented as mean ± standard deviation (SD) or standard error (SE) for each treatment group, as indicated. Comparisons between groups were calculated by a two-tailed unpaired t test with two levels of significance: $p < 0.05$ and $p < 0.01$.

RESULTS AND DISCUSSION

Mint Purification and Extract Composition. The extraction of *M. longifolia* aerial parts with 70% MeOH yielded an extract rich in phenolics. The extract after solid phase fractionation provided the phenolic fraction with an efficiency of 6.5% of plant dry matter. The chromatographic profile of this extract showed the presence of several phenolic compounds including two luteolin-glucuronides (sugars substituted at two different positions), methylated luteolin-glucuronide, luteolin-diglucuronide, luteolin-glycopyranosyl-rhamnopyranoside, eriodictyol-glycopyranosyl-rhamnopyranoside, rosmarinic acid, L-salvianolic acid, and dedihydrosalvianolic acid, as already reported (*13*). The total phenolic concentration in phenolic fraction was rather high, approaching 275 mg/g of dry weight (dw). The dominant compound was the eriodictyol-glycopyranosyl−-rhamnopyranoside; its content was 120 mg/g of dw. Also, the flavone luteolin-glycopyranosyl-rhamnopyranoside was present in high amounts, 56 mg/g of dw. Luteolin-glucuronide and luteolin-diglucuronide were found in amounts of 40 and 22.5 mg/g of dw, respectively. Among the other dominant secondary metabolites, rosmarinic acid was also identified, and its concentration was 30.5 mg/g of dw.

Antioxidant Capacity Determination. To evaluate the antioxidant power of mint PE as well as that of its corresponding fresh extract (MFE), and the two reference synthetic compounds, rosmarinic acid and resveratrol, we performed two common and reliable in vitro assays, ABTS and DPPH assays.

Results are reported in **Table 1**; with both assays the mint extracts showed a lower antioxidant activity with respect to the reference compounds, even if the differences are in the ranges of approximately 250-fold using ABTS assay and 10-fold using DPPH assay. On the other hand, mint PE displayed approximately a double antioxidant activity with respect to its corresponding MFE, and 5−13-fold higher antioxidant activity with respect to fresh extracts obtained by other mint species (*21*−*25*), indicating that the applied purification procedure improves the antioxidant capacity of the raw material, maintaining the potential of the polyphenolic components.

Table 1. Antioxidant Capacity of the Different Compounds[a]

sample	TEAC (mmol/g)	IC_{50} (μg/mL)
mint purified extract	3.08 ± 0.34 *§	16.38 ± 2.25
mint fresh extract	1.55 ± 0.25	22.11 ± 1.48
rosmarinic acid	842.13 ± 35.08	2.65 ± 0.25
resveratrol	886.46 ± 9.40	3.31 ± 0.43

[a] Data are reported as mean value ± SD. *, $p < 0.05$ vs mint fresh extract; §, $p < 0.01$ vs reveratrol and rosmarinic acid.

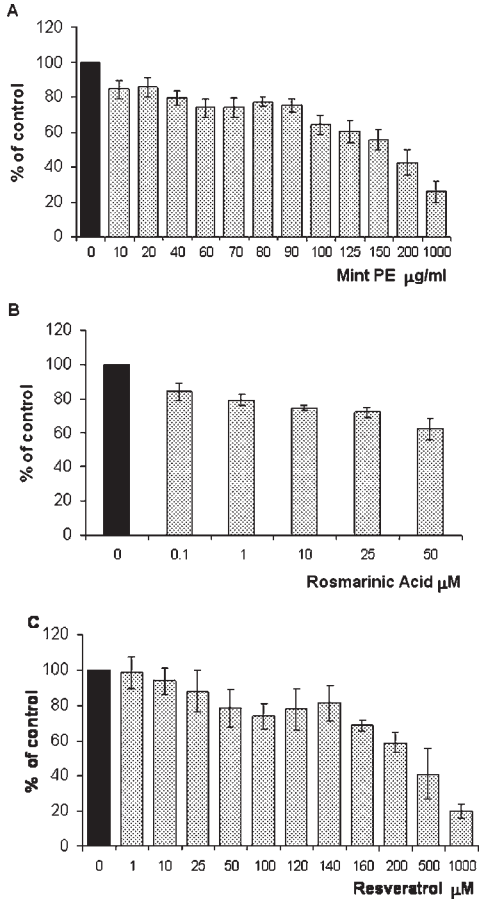

Figure 1. Viability of NCTC human keratynocytes, determined by MTT assay, after 24 h of treatment with (**A**) mint purified extract (range of 10 μg/mL−1 mg/mL), (**B**) rosmarinic acid (range of 0.1−50 μM), or (**C**) resveratrol (range of 1 μM−1 mM). Data are presented as mean ± SD percentage values with respect to untreated control cells by at least two independent experiments performed in eight replicates.

Viability of NCTC Treated with Mint Purified Extract and Antioxidant Reference Compounds. Human normal NCTC2544 keratinocytes, which express high activities of ROS detoxifying enzymes, are frequently used as a model to simulate ROS-induced skin injury including hydrogen peroxide and UV-caused damages (*26*, *27*). The toxicity profile of each antioxidant was evaluated, incubating for 24 h cells with increasing concentrations of mint PE, range, 10 μg/mL−1 mg/mL; rosmarinic acid, range, 0.1−50 μM; resveratrol, range, 1 μM−1 mM (**Figure 1**). For successive experiments we decided to employ, for each compound, the highest nontoxic dose that leads to 80% survival.

Table 2, column 2, reports, for each antioxidant, the experimental concentration suitable for further cell treatments; with these doses, the corresponding supplied antioxidant equivalents (**Table 2**, column 3) were very similar between mint PE and rosmarinic acid and extremely high for resveratrol (approximately 100-fold higher). Solvent influence was assessed, and excluded, because the ethanol final concentration never exceeded 0.027 M (data not shown).

Table 2. Highest NCTC2544 Nontoxic Doses for the Different Compounds, Implied by MTT Assay, and Corresponding Trolox Equivalents Provided to Cells in Further Treatments

compound	80% survival concentration	corresponding TEAC (mM)
mint purified extract	80 μg/mL	0.246
rosmarinic acid	1 μM−360.31 μg/mL	0.303
resveratrol	140 μM−31.95 mg/mL	28.33

Oxidative Stress Induced by Hydrogen Peroxide. Hydrogen peroxide, which can freely permeate cell membrane, is generated and released by a wide variety of normal and malignant cell types in response to a variety of stimuli. Increasing evidence suggests that at low concentrations hydrogen peroxide is effective in stimulating cell proliferation, whereas it leads to cell death as concentration increases (*28*).

We evaluated the H_2O_2 cell toxicity profile coupled with the HSP70 expression at increasing H_2O_2 concentrations (range, 50−500 μM) incubated for 2 h. HSP70 are molecular chaperones expressed under both unstressed and stressed conditions (*29*) that prevent inappropriate protein aggregation and mediate transport of immature or damaged proteins to the target organelles for final packaging, degradation, or repair.

As can be clearly seen by data shown in **Figure 2A**, both markers slightly changed up to 250 μM H_2O_2, whereas there was a sensible oxidative stress induction with 500 μM H_2O_2, which corresponded to approximately 60% cell viability and 50% HSP70 induction. These results are consistent with the keratinocytes' capability to be extremely resistant to the effects of fairly high concentrations of peroxides (*30*).

We evaluated the effects of longer exposures to 500 μM H_2O_2, incubating cells also for 16 and 24 h. As easily predictable, cell viability further decreased to approximately 50% in both cases, but there was no or very little change in HSP70 expression with respect to control cells (**Figure 2B**). This could be due to the fact that the increase of HSP70 (inducible form) is usually transient and its degree depends on many factors such as cell line, cell cycle, stress type, and the level and duration of the stress (*31*). Another hypothesis is related to the effects of extended stress on protein synthesis that might alter also HSP70 expression (*32*).

DNA damage is one of the most dangerous effects exerted by oxidative stress and could generate double-strand breaks (DSBs). Induction of DBSs in live cells triggers the phosphorylation of histone H2AX, one of the several variants of the nucleosome core histone H2A family, leading to γ-H2AX (*33*). This mechanism acts shortly after DNA damage and is considered to be part of the genome integrity guardians (*34*). Studying the profile of γ-H2AX histone phosphorylation induction with 500 μM H_2O_2 after 2, 16, and 24 h (**Figure 2C**), we detected a strong and significant induction at any time analyzed, with a maximum after 2 h (approximately 3.5-fold induction).

Protection from Oxidative Stress by Mint Purified Extract and Reference Compounds. *Cell Viability Defense*. To investigate the influence of the three antioxidants on cellular viability upon stress induction, NCTC2544 keratinocytes were pretreated for 1 h with their corresponding highest nontoxic doses, and then 500 μM H_2O_2 was added to the medium for a further 2, 16, or 24 h (**Figure 3**). Negative or positive control cells were incubated with routine culture medium or with H_2O_2 only, respectively. Viability was significantly improved by the preincubation with mint PE after 2 and 16 h, whereas there was apparently no effect at 24 h. Rosmarinic acid followed the same trend of mint, although it performed relatively better, also after 24 h of incubation. The increase

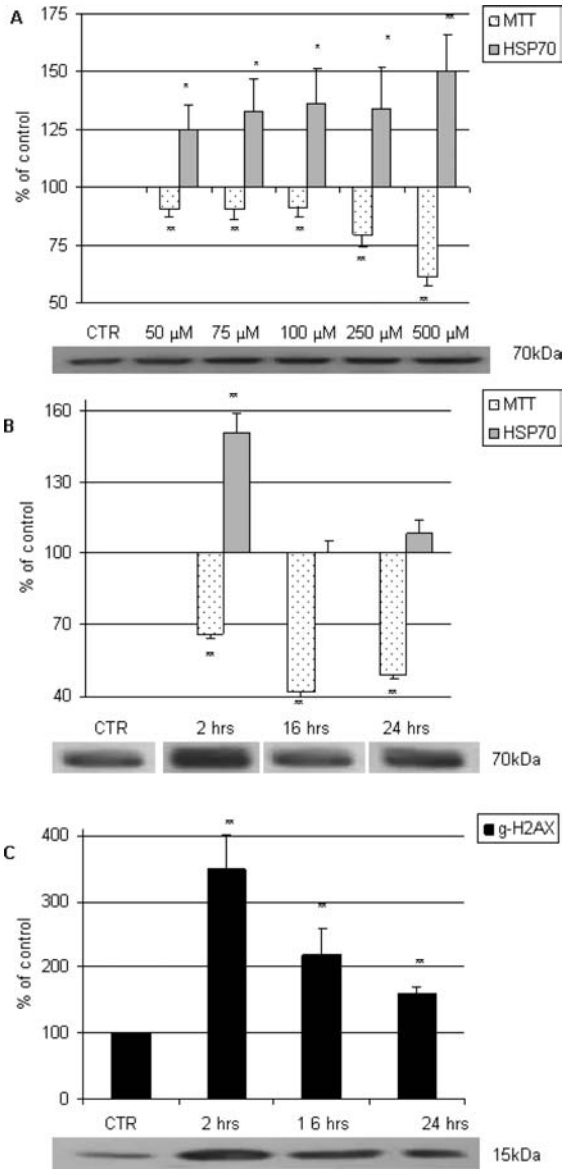

Figure 2. (**A**) Oxidative stress induced in NCTC human keratinocytes by increasing H_2O_2 concentrations (0−500 μM) for 2 h evaluated by cell viability (MTT, dotted bars) and HSP70 induction (gray bars). (**B**) Oxidative stress induced in NCTC human keratinocytes with 500 μM H_2O_2 for 2, 16, and 24 h evaluated by cell viability (MTT, dotted bars) and HSP70 induction (gray bars). (**C**) Histone H2AX phosphorylation induction by 500 μM H_2O_2 for 2, 16, and 24 h. Data are presented as mean ± SE percentage values with respect to untreated control cells. *, $p < 0.05$; **, $p < 0.01$. Bands at the bottom of each panel are representative of at least two independent Western blot analyses performed in duplicate; quantitative data were normalized by actin expression.

of viability by antioxidants, although statistically significant, indicates these molecules do not completely protect cells against H_2O_2 cytotoxicity; this may be an indication of a low bioavailability of the compounds related to solubility, cell membrane permeation, and time of preincubation.

Surprisingly, resveratrol not only did not protect cells from the induced injury after 2 and 16 h, but also it seemed to worsen it after 24 h. Taken together, these data are the first indication that this mint PE was able to counteract the cell toxicity induced by oxidative stress similarly to purified rosmarinic acid, providing cells with comparable antioxidant equivalents, and even better than resveratrol, despite a considerably lower antioxidant activity.

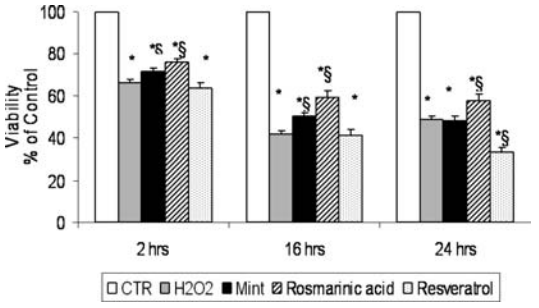

Figure 3. Cell viability of NCTC2544 keratinocytes preincubated for 1 h with the corresponding antioxidant and then treated with 500 μM H_2O_2 for 2, 16, and 24 h. Values are represented as mean ± SD of eight replicates of two independent experiments. *, $p < 0.05$ from negative control cells (CTR); §, $p < 0.05$ from positive control cells (H_2O_2). Cells were treated only with 500 μM H_2O_2).

Protein and DNA Damage Protection. To investigate the mechanisms responsible for the cell viability protection exerted by mint PE, we examined HSP70 and H2AX histone phosphorylation (γ-H2AX) induction in cells preincubated for 1 h with mint PE and then treated with H_2O_2 for 2, 16, and 24 h (**Figure 4**, panels **A**−**C** and **D**−**F**, respectively). We analyzed in parallel also the effects of the two synthetic antioxidants.

Mint PE was able to reduce HSP70 expression of approximately 20% in the shorter treatment, when HSP70 were sensitively induced in positive control cells. Therefore, one of the possible targets of mint PE action is the protection of proteins from early oxidative damages. After longer H_2O_2 treatments, actually no HSP70 induction was detectable in positive control cells (**Figure 2**) and no changes were measured also with mint extract, indicating that probably the extract influences mainly the inducible, rather than the constitutive, HSP70 expression. Rosmarinic acid and resveratrol showed different patterns, reducing HSP70 expression also after longer stress exposure.

It is worth noting that mint PE was the best performing antioxidant in reducing DNA damage, detected as γ-H2AX phosphorylated histone, confirming its highest activity after 2 h but showing a slight protection also after 16 h of H_2O_2-induced stress. This result is extremely important because it is the first indication of a protective effect at nuclear level of an extract obtained by a mint species.

However, after 24 h of stress induction, these compounds not only could not counteract DSB formation, but even seemed to increase it. This could probably be due to the combination of the long-term toxicities of both hydrogen peroxide and antioxidant treatments.

Glutathione Content and Antioxidant Enzyme Activities. Glutathione (GSH) is a tripeptide and is the most abundant natural antioxidant in the human body. Through its significant reducing power, GSH also makes major contributions to the recycling of other antioxidants that have become oxidized. There are a variety of mechanisms that could modulate cellular levels of GSH, including differences in GSH synthetase activity or changes in the cysteine transport (*28*). A loss of GSH is associated with the impairment of the electron transport chain and the breakdown of ATP synthesis that lead to further decrease in GSH content in cells.

Moreover, recent studies indicate that GSH depletion, via GSH oxidized form efflux from cells, is not only a common feature of apoptotic cell death triggered by oxidative stress but also a critical regulator of apoptosis itself (*35*). GR enzyme restores GSH from the oxidized form, oxidizing in turn NADPH coenzyme. In cells, GSH is mainly present in the reduced form, because GR is

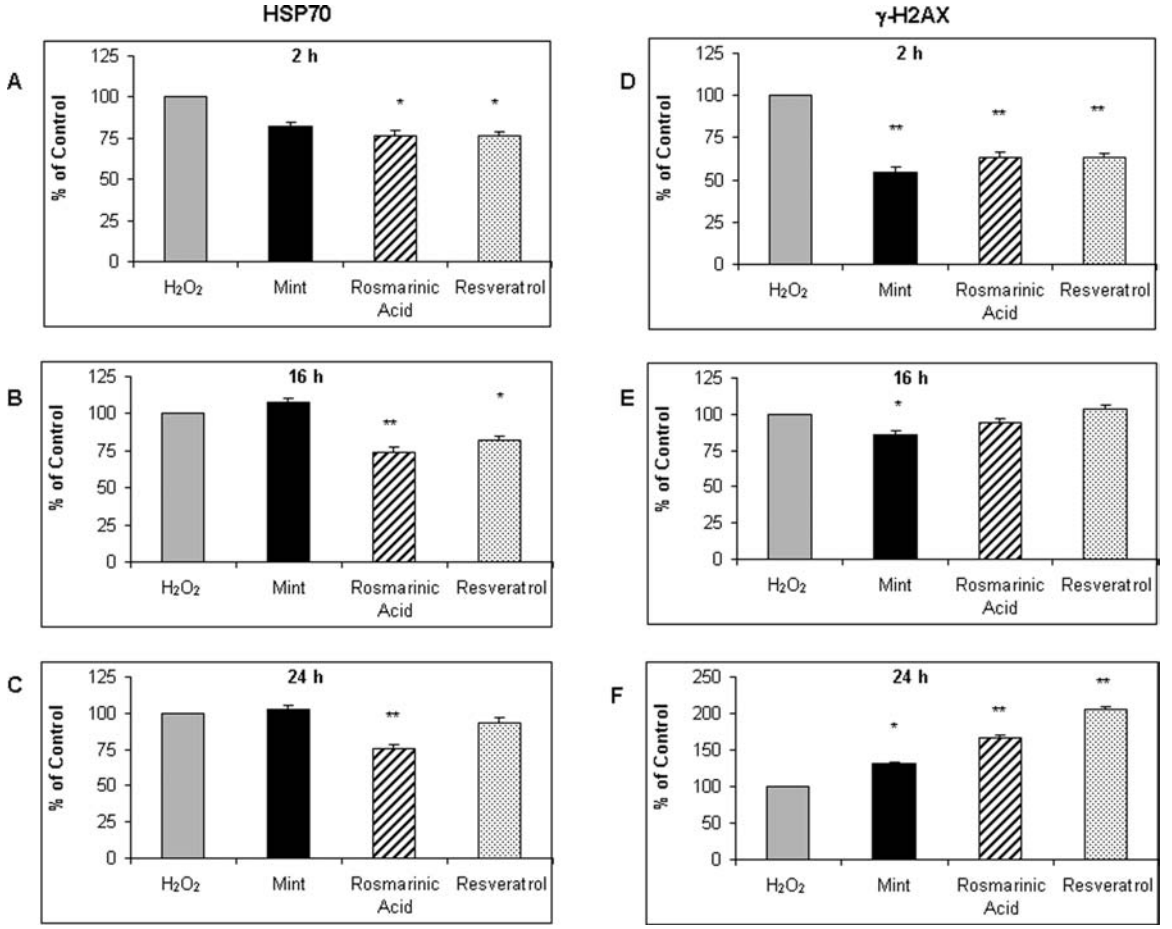

Figure 4. HSP70 and γ-H2AX expression detected by Western blot analysis of NCTC2544 cells pretreated for 1 h with the different antioxidants and then incubated with H_2O_2 for 2, 16, and 24 h (**A**−**C** and **D**−**F**, respectively). Results are normalized with actin expression and expressed as percentage respect to positive control cells (H_2O_2). Bars represent the mean ± SD values of two replicates of three independent experiments. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

constitutively active and also inducible by oxidative stress. Moreover, when intracellular H_2O_2 increases beyond a certain level, CAT is induced and helps GPx in detoxifying it. SOD enzymes act on an upstream reaction, producing H_2O_2 plus oxygen by the dismutation of superoxide anion, one of the more toxic ROS.

We investigated what happens to this complex and an integrated antioxidant system in the first phases of oxidative stress, that is, after 2 h of incubation with hydrogen peroxide. Moreover, this is the incubation time that showed the highest mint PE activity for all markers so far analyzed, that is, cell viability, HSP70 expression, and H2AX phosphorylation.

If, on the one hand, total GSH content was significantly reduced after hydrogen peroxide treatment (**Figure 5A**), as expected, on the other hand, all of the antioxidants increased its levels back, but only mint PE did so to a significant extent. Curiously, in positive control cells both GPx and GR activities are significantly reduced, but in these cases no antioxidant was able to restore them (**Figure 5**, panels **B** and **C**, respectively). These results could be probably explained by GSH depletion through a nonenzymatic GSH oxidation caused by electrophilic substances, such as free radicals and ROS (*26*).

CAT activity (**Figure 5D**) showed no alteration following hydrogen peroxide burst with or without antioxidant pretreatment; we conclude that 2 h of stress induction are not sufficient to induce CAT in these cells that already possess a relatively high activity. On the other hand, SOD activity, significantly reduced by H_2O_2 treatment, was restored only when cells had been preincubated with mint PE (**Figure 5E**).

Lipid Peroxidation. Lipid peroxidation is a useful marker to evaluate oxidative stress induction, because the phospholipid component of membranes is highly vulnerable and represents one of the first targets of oxidative damage due to susceptibility of its polyunsaturated fatty acid side chains to peroxidation (*36*). Damage to polyunsaturated fatty acids tends to reduce membrane fluidity, which is known to be essential for the proper biological function of membranes. Moreover, most of the proteins that play key roles in proliferative signal transduction or that are critical for ionic homeostasis and cell viability, for example, protein kinase C, ion channels, and Na^+/K^+-ATPase, actually are modulated by the lipids in the bilayer and are sensitive to lipid peroxidation (*28*). Our results show that NCTC2544 cell treatment with hydrogen peroxide induced a statistically significant increase in TBARS values (**Figure 5F**). Paradoxically, rosmarinic acid seemed to worsen lipid peroxidation, whereas mint PE and resveratrol significantly reduced it, indicating another possible mechanism of action of these antioxidants. This aspect well correlates with the observed GSH maintenance by mint PE, because the disruption in the GSH function is tightly bound with susceptibility to ROS-caused lipid peroxidation of cell membranes. Moreover, there are many observations (*28*) that indicate an apparent inverse relationship between levels of cellular lipid peroxidation and rates of cell proliferation, as clearly seen in our results. Therefore, the mint effect of decreasing lipid peroxidation has to be taken into consideration, because it involves many crucial aspects of cell survival under oxidative stress.

In conclusion, this mint purified extract, rich in phenolics including rosmarinic acid, even if with an in vitro antioxidant power

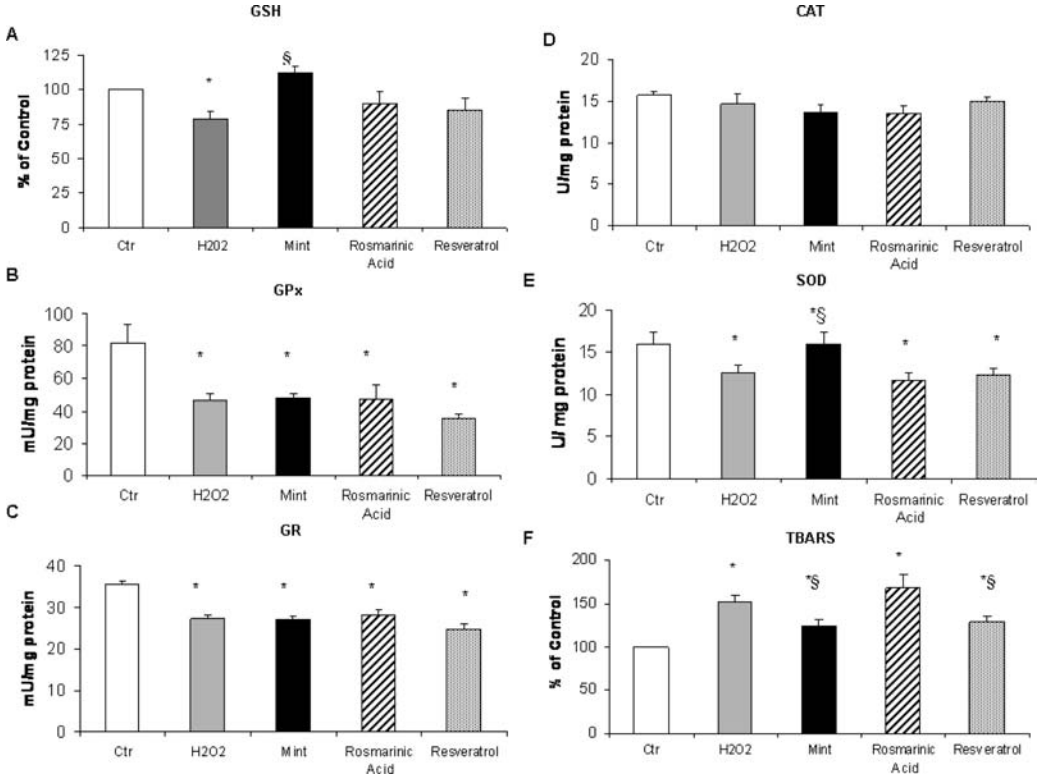

Figure 5. GSH content (**A**, expressed as percentage respect to control cells), GPx, GR activities (**B** and **C**, respectively, expressed as mU/mg protein); CAT and SOD activities (**D** and **E**, respectively, expressed as U/mg proteins), and TBARS (**F**, expressed as percentage respect to control cells) in NCTC2544 cell lysates. CTR, negative control cells; H_2O_2, positive control cells (treated for 2 h only with 500 μM H_2O_2); mint PE, cells pretreated with mint purified extract. Data are the mean values of two replicates of three independent experiments ± SE. ∗, $p < 0.05$ from CTR cells; §, $p < 0.05$ from H_2O_2 cells.

lower than those of the reference antioxidant compounds, showed a comparable, or even better, oxidative stress counteracting capacity on many different markers, finally resulting in a partial protection of cell viability. Moreover, these results corroborate the limitations of the employment of only in vitro antioxidant assays as screening methods for assessing the protecting capacity of a substance. Mint purified extract was apparently more efficient in the first phases of oxidative stress damage, and this should be more deeply investigated to verify if this could be due to preincubation or concentration-dependent factors. However, on the basis of these data, mint is confirmed to be more than a simple flavoring, and its regular intake in multifunctional foods or its employment for skin treatment in cosmetics has to be reevaluated and may be a useful strategy for the prevention of oxidative stress damage.

ABBREVIATIONS USED

ROS, reactive oxygen species; RNS, reactive nitrogen species; mint PE, mint purified extract; mint FE, mint fresh extract; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; DSBs, double-strand breaks.

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