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Supercritical CO_2-Extracted Tomato Oleoresins Enhance Gap Junction Intercellular Communications and Recover from Mercury Chloride Inhibition in Keratinocytes

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A nutritionally relevant phytochemical such as lycopene, found in tomatoes and other fruits, has been proposed to have health-promoting effects by modulating hormonal and immune systems, metabolic pathways, and gap junction intercellular communication (GJIC). This work analyzes lycopene extracts, obtained from tomato and tomato added with grape seeds by using a safe and environmentally friendly extraction process, based on supercritical carbon dioxide technology (S-CO_2). Analysis of the innovative S-CO_2-extracted oleoresins showed peculiar chemical composition with high lycopene concentration and the presence of other carotenoids, lipids, and phenol compounds. The oleoresins showed a higher in vitro antioxidant activity compared with pure lycopene and β-carotene and the remarkable ability to enhance the GJIC and to increase cx43 expression in keratinocytes. The oleoresins, (0.9 μM lycopene), were also able to overcome, completely, the GJIC inhibition induced by 10 nM $HgCl_2$, mercury(II) chloride, suggesting a possible action mechanism.

KEYWORDS: Antioxidants; *Solanum lycopersicum* L.; grape seeds; lycopene; GJIC; connexin 43; keratinocytes; tumor promoter

INTRODUCTION

Epidemiologic studies suggest statistically significant inverse association between the consumption of fruits and vegetables and reduced incidence of chronic degenerative illnesses such as certain types of cancer and cardiovascular diseases (*1*). The significance of this finding for cancer prevention is related to a large number of compounds, including the antioxidant vitamins C and E, minerals, phenolics, and carotenoids present in fresh fruits and vegetables (*2*−*4*). Carotenoids are natural fat-soluble pigments that are structurally quite diverse and widespread in all higher plants, along with some bacteria and algae (*5*,*6*). They are potent antioxidants and efficient free radical scavengers and can modulate the pathogenesis of cancers and coronary heart disease (*7*). Of the more than 50 dietary carotenoids, lycopene, a linear carotenoid found in tomatoes and in tomato products (the main component responsible for their characteristic deep-red color) has attracted attention in different fields of research (*8*,*9*). Several epidemiological studies have been published (*10*) showing inhibition of prostate cancer cell proliferation by lycopene at physiological levels and a synergistic effect of this compound when it was used in combination with other antioxidants such as vitamin E or other carotenoids (*11*). The action mechanism of lycopene in reducing cancer risk is thought to be due to its antioxidant and singlet oxygen-quenching properties and, thereby, its ability to trap peroxyl radicals. (*8*, *12*, *13*). The benefits associated with lycopene consumption for disease protection are amplified through both additive and synergistic interactions with various other tomato carotenoids (such as phytoene, phytofluene, lutein, and β-carotene) and with polyphenolic phytochemicals also abundant in the tomato fruit (*14*, *15*). The superior protection of mixtures might be related to the specific positioning of different carotenoids in the membranes (*16*).

Carotenoids and, in particular, lycopene are thought to enhance gap junction intercellular communication (GJIC) between cells, a property seemingly independent of their antioxidant ability, and that might be a basis for protection against cancer development (*17*, *18*). GJIC is essential to maintain tissue homeostasis by exchanging ions, signaling molecules, nucleotides, and other small molecules (<1 kDa) between adjacent cells through gap junctions, which consist of transmembrane proteins called connexins. Trosko and Chang (*19*) discussed the involvement of the GJIC during the tumor promotion phase of carcinogenesis and established the rational chemopreventive value to prevent the regular, chronic reversible down-regulation of GJIC during the tumor promotion phase; as a consequence, GJIC might be a suitable model for investigating the tumor-promoting and anti-tumor-promoting effects of phytochemicals.

Many phytochemicals, such as polyphenols, are able to modulate GJIC and to reverse or prevent the GJIC inhibition mediated

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by several tumor promoter chemicals (*17*, *20*−*24*). Carotenoids reversibly inhibited neoplastic transformation in the promotion phase of carcinogenesis, and this activity closely was correlated with carotenoids' ability to up-regulate expression of connexin 43 (Cx43), one of the most abundant GJIC proteins, ubiquitous in many tissues, thereby increasing gap junction communication (*17*). Lycopene and some conjugate isoforms were proposed to increase GJIC in different cell types, by increasing the levels of Cx43. Lycopene enhanced GJIC in human fetal skin fibroblasts (*25*), in KB-1 human oral cancer cells (*26*), in MCF-7 breast cancer cells (*27*), and in human keratinocytes (*28*). Human keratinocytes (the most common type of skin cells) exposed at noncytotoxic mercury(II) chloride ($HgCl_2$) concentrations showed an unbalancing of the redox cellular state and the activation of a redox signaling and the inhibition of GJIC (*29*). Indeed, GJIC has been proved to be specifically affected by various toxicants, such as heavy metals (*30*, *31*), including mercuric ions. In oral keratinocytes, a subcytotoxic concentration of $HgCl_2$ stimulated the release of low levels of tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) and inhibited the release of interleukin-1 α (*32*). In addition, lycopene-enriched oleoresin significantly increased the production of IL1-β and TNF-α pro-inflammatory cytokines in $HgCl_2$-treated human keratinocytes (*28*). Recently, several phytochemicals, including lycopene, alone or used in mixtures, were found to be protective agents against oxidant-induced DNA damage, to decrease proliferation of nontumorigenic and tumorigenic keratinocytes, and to show antitumor effects in an in vivo short-term skin carcinogenesis assay (*33*).

In cell culture systems and in tissues and tumors explanted from test animals and patients, the tumor-promoting agents, such as heavy metals, act as a carcinogen promoter by inducing aberrant expression and function of several connexin proteins (*34*). Thus, the ability of any select drug/natural product to repair/restore the altered GJIC might be a useful end point by which to assess the potential anticancer activities of that agent. Moving from these assumptions, the chemopreventive effects of new natural compounds could be tested by investigating whether they recover the toxicant-induced inhibition of GJIC. Our previous results indicated that tomato-derived oleoresins high in lycopene affected GJIC functionality and recovery in $HgCl_2$-treated keratinocytes (*28*).

Lycopene is considered to be a commercially important health-promoting compound for the emergent market of nutraceutical products. Nevertheless, it has become clear that the active natural chemicals seldom work independently. The prevalence of potentiating chemical interactions, especially for plant-derived extracts, has been increasingly documented in recent years (*6*). A complex interplay between diverse chemical components modifies the effects of functional foods, nature-derived supplements, or pharmaceuticals, as they modulate biological processes in human metabolism. The bioactive constituents in edible plants are always ingested in the form of natural mixtures, and the epidemiological evidence for health benefits may actually be accounted for by specific interactions between phytochemicals in the most common traditional foods (*35*). In addition, the processing involved in the formulation of some plant extracts into dietary supplements can disrupt the delicate associations between natural co-occurring phytochemicals, resulting in erratic or much-attenuated efficacy in the commercial product. In this respect, there is a great interest in the use of environmentally friendly processes, such as supercritical fluid extraction (SFE), for the industrial production of products containing lycopene. SFE is regarded as an environmentally safe technology and a promising process to obtain functional ingredients from natural sources compared with the traditional extraction methods, which have several drawbacks. More recently, the supercritical CO_2-extracted lycopene (S-CO_2) is highly demanded because it proposes a new safer and stabler product that has a composition rich in more bioactive compounds. There are few experimental data on the effects of S-CO_2-extracted oleoresins rich in lycopene obtained from tomato matrix on human keratinocytes and, to our knowledge, there are no data about lycopene coextracted by S-CO_2 from tomato and grape seeds.

MATERIALS AND METHODS

Chemicals. Methanol, ethanol, hexane, ethyl acetate, and acetonitrile (HPLC grade) and acetic acid were purchased from Merck Darmstadt, Germany; caffeic acid, ferulic acid, coumaric acid, chlorogenic acid, rutin, naringenin, lutein, β-carotene, *trans*-lycopene, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate (dipotassium peroxdisulfate), $HgCl_2$, Folin−Ciocalteu's phenol reagent, Epilife Medium, keratinocyte supplement, penicillin, streptomycin, and dimethyl sulfoxide (DMSO) were purchased from Sigma, St. Louis, MO; trypsin−EDTA solution was from Sigma-Aldrich, Milan, Italy; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Hoffman-La Roche.

Supercritical CO_2-Extracted Oleoresins. Ripe fresh tomatoes (*Solanum lycopersicum* L.) grown in Apulia were purchased from a local market. Tomatoes were crushed and autoclaved for 20 min at 120 °C, and then the pulp was separated from the seeds and skins, placed into an Erlenmeyer flask, and centrifuged. The samples were dried at 40 °C under vacuum condition and powdered (about 18 mesh), and the obtained dried powder (DT) was stored under vacuum in the dark at −20 °C (unpublished method). Grape seed powder (GS) was obtained by washing, drying, and powdering grape seeds collected from a local winery. Extraction matrices were obtained from DT and by mixing tomato dried powder with dried grape seed powder at 3:2 (DT/GS 3:2) and 2:3 (DT/GS 2:3) (w/w) ratios. Pilot plant equipment for supercritical CO_2 extraction was used to obtain the oleoresins by using the same procedure reported as in ref 36. The lycopene-rich oleoresins obtained by S-CO_2 fluid extraction from tomato matrix and tomato−grape seed mixtures were used for carotenoid, phenol, and lipid compound determination.

Carotenoid Determination. Carotenoids were extracted from the S-CO_2-extracted oleoresins by stirring 0.1 g of the oleoresins in 10 mL of hexane/acetone/ethanol (2:1:1, v/v/v) solution (*37*), and then an equal volume of distilled water was added followed by 5 min of stirring. After separation of the aqueous phase from the hexane lipophilic, 20 μL of the hexane solution containing carotenoids was injected into the high-performance liquid chromatography (HPLC) system 126 solvent module equipped with a System Gold 168 diode array detector (Beckman-Coulter, Fullerton, CA). The analysis was carried out using a reverse phase (RP) C_{30} column (4.6 mm × 2.5 cm, YMC, Milford, MA). A methanol/methyl butyl ether/ethyl acetate (50:40:10) solution was used as the mobile phase, at a flow rate of 1 mL/min (*38*). Carotenoid detection was achieved at a 475 nm wavelength. Carotenoid quantification was achieved by comparison with the standard curves of lutein, β-carotene, and *trans*-lycopene authentic standards (Sigma). Moreover, the *cis*-β-carotene and *cis*-lycopene isomers were quantified on the *trans*-lycopene standard curve because of similarity in the extinction coefficient (*39*). All procedures were performed under dim light to reduce photo-oxidation. To measure the total carotenoid content, the hexane extracts were analyzed spectrophotometrically at 470 nm using a Beckman-Coulter DU-640 spectrophotometer, and the sample absorbance values were compared with those of the known amount of the standard β-apo-8′-carotenal. The total carotenoid concentration was expressed as milligrams of β-apo-8′-carotenal equivalents per gram of the oleoresin.

Phenol Compound Determination. Phenolic compounds were extracted by stirring 0.1 g of the oleoresins in 10 mL of 80% (v/v) methanol solution at room temperature for 30 min (*40*). After centrifugation (4000*g* for 10 min), the supernatant was recovered, filtered through a 0.45 μm membrane (Millipore), and analyzed. Total phenols were estimated by using the Folin−Ciocalteu method (*41*). Total phenol content was standardized against gallic acid and expressed as milligrams of gallic acid equivalents per gram of oleoresin. The individual phenols were identified

and quantified with HPLC by comparison with external standards of corresponding known phenols and expressed as milligrams per gram of oleoresin (*40*). HPLC analysis was carried out using an RP-C_{18} Beckman Ultrasphere column (4.6 mm × 2.5 cm, Beckman-Coulter) and methanol/water/acetic acid 34:65:1 (v/v/v) at a flow rate of 1 mL/min as mobile phase. Two different wavelengths at 280 and 320 nm were used for detection of the catechin and the other phenolic compounds, respectively.

Lipid Determination. Total lipids were extracted from S-CO_2-extracted oleoresins by stirring 0.05 g of the oleoresins in 5 mL of hexane/methanol solution (2:1, v/v), followed by the addition of an equal volume of water. The two phases were separated by centrifugation, and the upper lipid-containing phase was concentrated on a rotary evaporator under vacuum at 60 °C (*42*). Total lipids were saponified with 2 M potassium hydroxide in methanol and re-extracted by hexane. The hexane layer was separated and analyzed by a gas chromatograph coupled to a mass spectrometer (QP5050 GCMS, Shimadzu, Kyoto, Japan) equipped with a 30 m DB-5MS (J&W Scientific, Folsom, CA) capillary column (0.25 mm i.d., 0.25 μm film thickness) for the determination of fatty acid methyl esters (FAMEs). The oven temperature was as follows: 80 °C for 5 min raised to 100 at 10 °C/min and then to 250 at 5 °C/min and maintained at this temperature for 15 min. The injector and interface temperatures were 250 °C. Helium was used as carrier gas at a flow rate of 1.1 mL/min. The fatty acids were fragmented by electron impact at 70 eV, identified by a comparison of their retention time and mass spectrum with those of standard FAME, and quantified according to their percentage area by integration of the peak.

In Vitro Antioxidant Activity Analysis. Evaluation of antioxidant activity of S-CO_2-extracted oleoresins was determined by using the Trolox equivalent antioxidant capacity (TEAC) method based on the suppression of the absorbance of radical cations of 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants in the test sample. The method was modified for screening the antioxidant capacity of lipophilic, hydrophilic, and hydroalcoholic soluble components in the S-CO_2-extracted oleoresins (*43*). Briefly, S-CO_2-extracted oleoresins were diluted with tetrahydrofuran (THF), phosphate-buffered saline (PBS),or 80% methanol to give 20−80% inhibition of the blank absorbance with 10 μL of the sample. A Trolox calibration curve in a range of 2.5−30 μM was prepared under the same conditions of the samples. The antioxidant capacity of the samples was calculated, on the basis of the inhibition exerted by standard Trolox concentrations at 734 nm, inhibition time being fixed at 6 min. Results were expressed as micromoles of Trolox equivalents per gram of oleoresin or standards.

Cell Culture and Cell Treatments. Primary human keratinocytes, provided by Istituto Zooprofilattico of Brescia (Italy), were cultured in Epilife medium supplemented with keratinocyte supplement, penicillin, and streptomycin (Sigma, St. Louis, MO) as described in ref*28*. A stock solution of 0.1 mM mercuric chloride ($HgCl_2$) was added to the culture medium to reach the final concentration of 10 nM. Cell viability was routinely assayed using the Neutral Red viability assay method.

Lycopene, β-carotene, and S-CO_2-extracted oleoresins were dissolved in DMSO (Sigma) and stored at −70 °C under a N_2 atmosphere. All procedures including cell treatment were carried out under dim light. Prior to the various assays outlined below, the keratinocyte cultures received either medium or medium containing 10 nM $HgCl_2$ for 24 h to establish baseline levels of effects on GJIC. Immediately before the experiment, the S-CO_2-extracted oleoresins and the standards, lycopene and β-carotene, dissolved in DMSO, were added to the cell culture medium and vigorously stirred, and the obtained medium was filtered through 0.45 μm Millex-HV (Millipore) and provided to the cells at the following final concentrations: 0.04, 0.09, 0.19, 0.37, 0.93, 4.65, 9.31, and 18.62 μM β-carotene or lycopene equivalent. The maximum amount of DMSO in the cell cultures was about 4% (v/v). The keratinocyte cultures were treated with the different concentrations of pure β-carotene or pure lycopene or lycopene contained in S-CO_2 oleoresins or only DMSO, for 4 h, to investigate its effect on GJIC. Recovery experiments were performed by incubating the cells in 10 nM mercury chloride for 24 h, followed by lycopene or β-carotene or S-CO_2-extracted oleoresins for 4 h, to evaluate their effect against any mercury-induced toxicities. Controls were not treated cells, or cells incubated in DMSO for 4 h, or cells incubated in 10 nM mercury chloride for 24 h followed by 4 h of treatment of DMSO (see the scheme in **Figure 5**).

GJIC Functionality. GJIC was measured using the dye transfer method, as described in ref *33*. At least 30 individual cells were microinjected for each sample, and the extent of intercellular communication (LY spreading into neighboring cells) was quantified by counting the number of dye-coupled cells with a fluorescence microscope fitted with a modified fluorescein filter block (435 nm). The values are referred to the means of the three independent experiments.

RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The total RNA was extracted from keratinocytes treated with both S-CO_2-extracetd oleoresin alone (DT oleoresin, 4 h) and mercury (10 nM $HgCl_2$ per 24 h), followed by 4 h of DT oleoresin treatment. Keratinocyte cultures treated for 24 h with 10 nM $HgCl_2$, for 4 h with DMSO, and with medium only were used as controls. Total RNA was isolated as described in ref *32*. cDNA was synthesized from the total RNA using a SuperScript II Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions. RT-PCR was performed using the primers of the human connexin 43 gene, forward 5′-ACATCAGGTGGACTGTTTCCT-3′ and reverse 5′-ACGACTGCTGGCTCTGCTT-3′. The PCR cycle used was 94 °C, 5 min, 1 hold; 94 °C, 30 s, 50 °C, 1 min, 72 °C, 1 min (30 cycles) followed by a final extension step of 7 min, 72 °C. Human actin primers (forward 5′-CGTGACATCAAGGAGAAGCT-3′, reverse 5′-ATCCACATCTGCTGGAAGGT- 3′) were used as control. RT-PCR products were separated on 0.8% agarose gel stained using ethidium bromide.

Statistical Analysis. When indicated, comparisons between mean values from control and treated samples were carried out with the two-tailed unpaired Student's t test and a one-way analysis of variance (ANOVA). A p value of < 0.05 was considered to be significant.

RESULTS

Tomato Oleoresin Composition. Three types of oleoresins obtained by S-CO_2 extraction, from tomato dried powder (DT) and tomato added with grape seed powder in ratios of 3:2 by weight (DT/GS 3:2) and 2:3 (DT/GS 2:3) were considered. Data concerning the overall content of the three different S-CO_2-extracted oleoresins are given in **Table 1**. The quali-quantitative analysis showed that in all of the S-CO_2-extracted oleoresins the main detected components were lipids, carotenoids, and phenols. Lutein, β-carotene, and lycopene, with their respective trans isoforms were the most representative carotenoids in all of the three oleoresins. The total carotenoid content, measured as β-apo-8′-carotenal equivalents, was higher in the oleoresins obtained from the tomato matrix (36.14 mg/g) and lower in the oleoresins from tomato−grape seed matrices. The concentration of carotenoid decreased as the coextracting grape seed components increased (15.25 and 4.81 mg/g in DT/GS 3:2 and DT/GS 2:3 oleoresins, respectively). The lycopene (as cis and trans isomers) was 28.45 mg/g of oleoresin extracted from DT oleoresin and 11.49 and 3.72 mg/g in DT/GS 3:2 and DT/GS 2:3 oleoresins, respectively. β-Carotene (as cis and trans isomers) was 2.29, 0.92, and 0.33 mg/g in DT, DT/GS 3:2, and DT/GS 2:3 oleoresins, respectively. In all oleoresins, however, the ratio between lycopene and β-carotene was not substantially changed. The concentration of *all-trans*-lutein was detectable at 0.38, 0.236, and 0.036 mg/g of DT, DT/GS 3:2, and DT/GS 2:3 oleoresins, respectively. The relative percentages of different carotenoid isomers were changed in the S-CO_2-extracted oleoresins compared with the raw tomato as both fresh tomato and a dried powder matrix. In particular, the ratio of *trans*-/*cis*-β-carotene ranged from 1.4 to about 3.3 in the oleoresins and was 3.7 and roughly 1 in the fresh and dried tomato, respectively. The trans/cis ratio of lycopene isomers ranged from 2.3 to 3.7 in the three different oleoresins, notably lower than in fresh tomatoes (ratio 6.3) and in the dried tomato matrix (ratio > 8) (data not shown).

Although S-CO_2 extraction is more specific for lipid compounds, also some phenols were identified in the tomato and tomato−grape oleoresins. Probably these compounds were

Table 1. Chemical Composition (Milligrams per Gram of Oleoresin)[a] of the Oleoresins Extracted by S-CO_2 from Processed Tomato Dry Powder (DT) and Processed Tomato Dry Powder and Grape Seed Powder in the Ratio 2:3 (DT/GS 2:3) and in the Ratio 3:2 (DT/GS 3:2)

	supercritical CO_2 extracted oleoresins		
compound	DT	DT/GS 3:2	DT/GS 2:3
carotenoids			
all-trans-lutein	0.380 ± 0.04	0.236 ± 0.04	0.036 ± 0.01
di-*cis*-β-carotene	0.421 ± 0.03	0.295 ± 0.04	0.062 ± 0.02
cis-β-carotene	0.116 ± 0.01	0.089 ± 0.01	0.016 ± 0.01
all-trans-β-carotene	1.754 ± 0.31	0.536 ± 0.04	0.256 ± 0.04
9,13-di-*cis*-lycopene	0.915 ± 0.07	0.553 ± 0.07	0.060 ± 0.01
13-*cis*-lycopene	4.518 ± 0.61	1.994 ± 0.34	0.282 ± 0.02
9-*cis*-lycopene	0.968 ± 0.03	0.904 ± 0.04	0.339 ± 0.06
cis-lycopene	0.104 ± 0.01	0.080 ± 0.03	0.092 ± 0.01
all-trans-lycopene	21.942 ± 2.01	7.957 ± 0.85	2.863 ± 0.47
other	0.324 ± 0.03	1.550 ± 0.01	0.184 ± 0.05
total (as mg of β-apo-8′-carotenal equiv)	**36.14 ± 2.87**	**15.25 ± 1.03**	**4.81 ± 0.68**
phenol compounds			
chlorogenic acid	0.110 ± 0.025	0.111 ± 0.012	0.024 ± 0.006
caffeic acid	0.314 ± 0.046	0.172 ± 0.068	0.190 ± 0.066
coumaric acid + ferulic acid	0.206 ± 0.041	0.104 ± 0.003	0.044 ± 0.008
rutin	2.127 ± 0.140	1.044 ± 0.054	0.599 ± 0.056
naringenin	0.123 ± 0.066	0.074 ± 0.008	0.062 ± 0.060
ND[b] (room temp, 24 min)	0.967 ± 0.218	0.438 ± 0.124	0.213 ± 0.095
total (as mg of gallic acid equivalents)	**3.85 ± 0.22**	**1.94 ± 0.09**	**1.13 ± 0.02**
lipids			
triacylglycerols	561.61 ± 32.0	953.43 ± 44.0	972.36 ± 22.0
diacylglycerols	250.52 ± 9.0	13.42 ± 0.1	12.52 ± 0.2
monoacylglycerols	108.49 ± 8.0	11.78 ± 0.1	3.93 ± 0.02
free fatty acids	44.09 ± 0.9	6.97 ± 0.02	5.04 ± 0.01
total	**964.7 ± 35.9**	**985.60 ± 32.2**	**993.87 ± 52.5**

[a] Data are means ± SD of three values from three independent experiments. [b] Unidentified peak.

mainly derived from tomato berries, because the phenol compounds were found to be higher in the DT oleoresin than in the DT/GS ones: the flavonoid rutin and caffeic acid were the most representative phenols. The rutin concentration in the DT, DT/GS 3:2, and DT/GS 2:3 extracts was 2.127, 1.044, and 0.599 mg/g of oleoresin, respectively, and the caffeic acid concentration was 0.314, 0.172, and 0.190 mg/g of oleoresin in DT, DT/GS 3:2, and DT/GS 2:3, respectively. Coumaric and ferulic acids and the flavonoid naringenin were detected in smaller, but significant, amounts in all three examined oleoresins. Chlorogenic acid was detectable at 0.111 mg/g of oleoresin in DT and DT/GS 3:2 and at only 0.024 mg/g of oleoresin in the DT/GS 2:3 extract.

Lipids were the major component of the S-CO_2 tomato and tomato−grape seed extracts, consisting in 96−99% (w/w) of the S-CO_2-extracted oleoresins. In DT oleoresin most of the total lipids were tri-, di-, and monoacylglycerols with a small amount of free fatty acids. In the oleoresins obtained from both tomato−grape seeds, the lipid fraction was mainly made up of triacylglycerols reflecting the lipid composition of grape seed, where they represent roughly 97% of the total lipids (**Table 2**). The fatty acid composition of lipids in the S-CO_2-extracted oleoresins is shown in **Table 3**. Unsaturated C_{18} fatty acids, such as oleic, linoleic, and linolenic acids, constitute about 72.3, 79.8, and 70.2% of the weight in DT, DT/GS 3:2, and DT/GS2:3 oleoresins, respectively. It is interesting to observe the shift of ratio between monounsaturated fatty acids (oleic, $C_{18:1}$) and polyunsaturated fatty acids ($C_{18:2}$, $C_{18:3}$) from a ratio of 1.7 in the oleoresin from tomato powder to a ratio of 0.3 in the two tomato−grape seed matrices. In particular, oleic acid decreased from 45 to about 17−18%, linolenic acid declined from 2.98 to 0.8−0.9%, and linoleic acid increased from 24.2 to about 52−61%. Saturated fatty acids, such as palmitic, stearic, and arachidic acids, represented 17−20% of the total lipids. The fatty acid composition of the oleoresins reflects the fatty acid content of both tomatoes and grape seeds, as shown in the **Table 3**. In these experimental conditions, lipids present in the grape seeds were extracted in bulk by S-CO_2, thus facilitating the coextraction of tomato lipids and highly lipophilic carotenoids. Apart from slight changes in the percentage of palmitic acid, stearic acid was 3.6% in DT oleoresin and increased to 6.6 and 5.2% in DT/GS 3:2 and DT/GS 2:3 oleoresins, respectively. Arachidic acid, detected in DT oleoresin, was 1.2%, and only 0.4% was present in oleoresins from tomato−grape seed mixed matrices. The most notable effect of grape seed−tomato coextraction by S-CO_2 was the increased percentage of linoleic acid, which was up to 50% of the total fatty acids, compared with the extraction from the matrix containing only tomato.

Table 2. Composition of the Lipids Present in Grape Seeds

lipid class	grape seed oil composition[a] (mg/g of seeds)
triacylglycerols	973.5
diacylglycerols	14.6
monoacylglycerols	4.6
free fatty acids	5.2
other[b]	2.1

[a] Data are means of three values from three independent extractions. [b] Not identified compounds.

In Vitro Antioxidant Activity of Supercritical CO_2 Extracted Oleoresins. Because the S-CO_2 oleoresins are to be regarded as a mixture of molecules with different polarities and antioxidant capacities, three different fractions, organic solvent extract (lipid fraction), hydroalcoholic extractable compounds (hydroalcoholic fraction), and hydrosoluble compounds (hydrosoluble fraction), were considered for antioxidant ability. As shown in **Figure 1**, the highest antioxidant activity, expressed as micromoles of Trolox equivalents (TE) per gram of oleoresin, was found in the lipid fraction (containing carotenoids) of each oleoresin. Minor activity

Table 3. Fatty Acid Composition of the Total Lipids Present in the S-CO_2-Extracted Oleoresins in Fresh Tomatoes, in Dried Powder of Processed Tomato, and in Oleoresins Extracted by S-CO_2 from Processed Tomato Dry Powder (DT) and from Processed Tomato Dry Powder and Grape Seed Powder in the Ratio 2:3 (DT/GS2:3) and in the Ratio 3:2 (DT/GS3:2)

	supercritical CO_2 extracted oleoresins (%)[a]				
fatty acid	DT	DT/GS 3:2	DT/GS 2:3	fresh tomatoes	grape seeds
$C_{16:0}$ (palmitic acid)	14.7	12.6	11.3	29.6	13.7
$C_{18:0}$ (stearic acid)	3.6	6.6	5.2	6.5	8.7
$C_{20:0}$ (arachidic acid)	1.2	0.4	0.4	1.8	0.42
$C_{18:1}$ (oleic acid)	45.1	17.4	18.3	6.3	19.9
$C_{18:2}$ (linoleic acid)	24.2	51.9	60.7	41.0	52.2
$C_{18:3}$ (linolenic acid)	3.0	0.9	0.8	7.5	0.58
others	8.2	10.2	3.3	7.3	4.46

[a] Data are means of three values from three independent extractions. Values are given as percentage of the total extracted lipids.

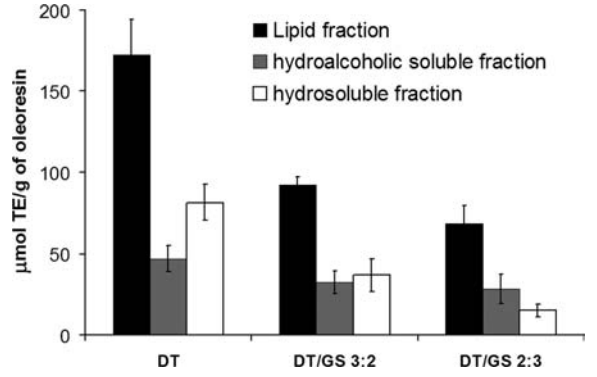

Figure 1. Antioxidant activity of the S-CO_2-extracted oleoresins from processed tomato dry powder (DT) and processed tomato dry powder and grape seed powder in the ratio 2:3 (DT/GS 2:3) and in the ratio 3:2 (DT/GS 3:2).

was measured in both hydroalcoholic and hydrosoluble fractions. The antioxidant activities of the three fractions obtained from the DT oleoresin were significantly higher compared to the fractions obtained from DT/GS oleoresins when measured per gram of oleoresin. Fewer differences in the total antioxidant activity of the three fractions between DT/GS 2:3 and DT/GS 3:2 oleoresins were observed.

A relationship between the percentage of tomato matrix utilized during the oleoresin extraction and antioxidant activity in the lipid fractions was also observed. A linear positive correlation (R^2 = 0.9868) between tomato powder amounts and the antioxidant activity of the lipid fractions obtained from the three oleoresins was calculated (**Figure 2A**). A similar positive correlation (R^2 = 0.9967) was established when the carotenoid contents and tomato powder concentrations were correlated (**Figure 2B**). These observations indicate that the antioxidant capability of the lipid fraction in the oleoresins is mostly due to the carotenoid components.

On the basis of the last observation and to reduce the effect of the differences in antioxidant phytochemical concentrations among the oleoresins, the detected antioxidant activities were referred to the amount of contained lycopene, as main antioxidant carotenoid in the lipid-soluble fraction, and compared with the contained total phenols in the hydroalcoholic fraction. The data presented in **Figure 3** show that the antioxidant activity, when it was expressed per milligram of contained lycopene, was higher in the DT/GS oleoresins than in DT oleoresin, with a significant difference between the DT and DT/GS 2:3 oleoresins. In addition, the antioxidant ability of lycopene present in all three S-CO_2-extracted oleoresins was significantly greater (from 2 to 3 times higher) when compared to the antioxidant activity of pure standard lycopene, measured in the same experimental conditions. When the antioxidant ability of hydroalcoholic soluble compounds was expressed as TE per total of the phenols contained (white columns in **Figure 3**), slight differences among the three types of oleoresins were detected. Probably the differences in antioxidant polyphenol composition of the three oleoresins have little or no influence on the overall antioxidant ability of this oleoresin fraction.

Effect of Supercritical CO_2-Extracted Oleoresins on Keratinocyte Viability. The S-CO_2-extracted oleoresins containing lycopene were assayed for their biological effects on keratinocytes and compared with the effect of standard lycopene and β-carotene. Different amounts of lycopene were evaluated (for oleoresins the concentration is referred to the actual lycopene contained assayed by HPLC). As shown in **Figure 4**, the standard β-carotene did not significantly affect the cell viability in all the range of concentrations considered (from 0.04 to 18.8 μM), whereas standard lycopene induced a decrease of keratinocyte viability at concentrations above 0.9 μM. The highest assayed lycopene concentration reduced the cell viability to 42% compared with the untreated control. The compounds present in the DT/GS oleoresins did not affect the cell viability in the range of concentration from 0.04 to 0.9 μM of lycopene equivalents. On the contrary, the DT oleoresin slightly reduced the cell viability even at concentrations between 0.04 and 0.9 μM, with a cell viability decrement of about 20% compared with the control. Treatments with all three S-CO_2-extracted oleoresins, at the higher lycopene concentration, induced a similar or slightly lower cell viability, compared to pure lycopene.

Oleoresins, Mercury Chloride, and GJIC in Keratinocyte Cell Culture. The CO_2-extracted oleoresins were evaluated for their ability to influence the GJIC in keratinocyte cultures and to release the $HgCl_2$-dependent GJIC inhibition. The experimental treatment of keratinocyte cells with $HgCl_2$, β-carotene, and lycopene or S-CO_2-extracted lycopene and the exposure time are summarized in the scheme reported in **Figure 5**. In all experiments with 0.9 μM lycopene, the higher concentration not affecting cell vitality was used. As shown in **Figure 6**, pure lycopene and β-carotene (both 0.9 μM) were ineffective or had very little effect on GJIC functionality compared with control treated with DMSO (vehicle), and they were unable to restore GJIC after $HgCl_2$ treatment. All three S-CO_2-extracted oleoresins (applied at an equivalent 0.9 μM lycopene concentration) were able to enhance remarkably the GJIC functionality in keratinocytes after 4 h of treatment (**Figure 6**, black columns). In fact, the extent of GJIC reached almost 12−13-fold the control value. Treatment with 10 nM $HgCl_2$ for 24 h decreased the gap junction-mediated intercellular communications by about 67% (±3.5) compared with the control (**Figure 6**, gray columns). When the keratinocytes were treated with tomato oleoresins for 4 h, after 24 h of mercury chloride treatment, the GJIC was completely

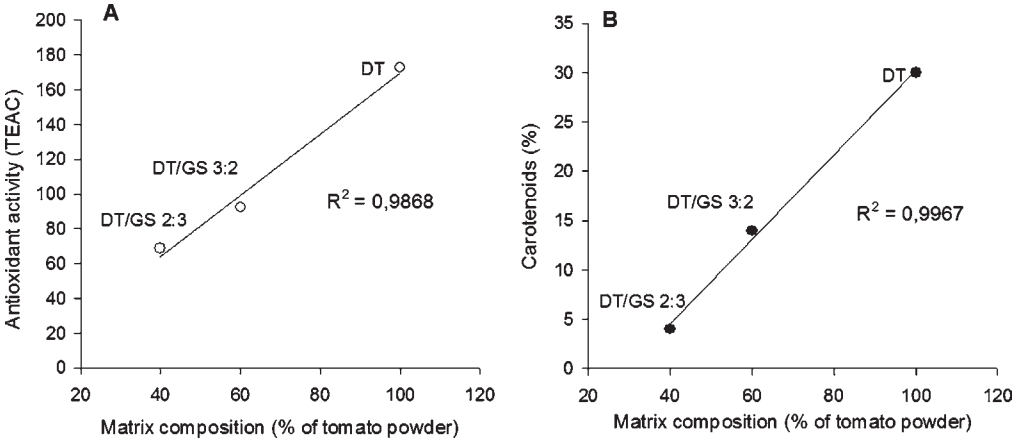

Figure 2. Correlation between matrix composition and the antioxidant activity (**A**) or the carotenoid content (**B**) in the correspondent S-CO_2-extracted oleoresins.

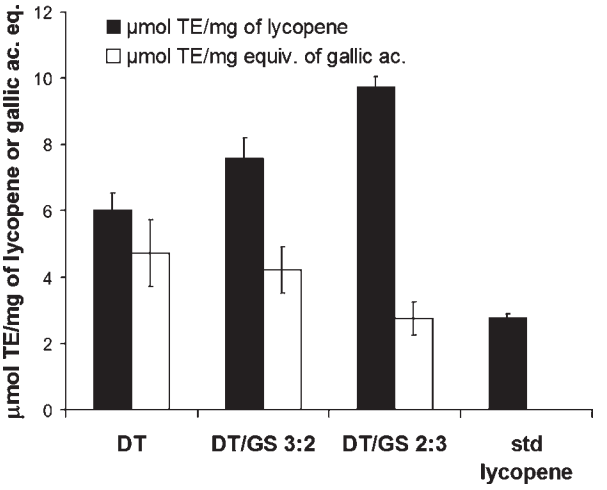

Figure 3. Antioxidant activity, detected in the lipid and hydroalcoholic fractions of S-CO_2-extracted oleoresins, related to the amount of lycopene or phenol content. Oleoresins were S-CO_2-extracted from processed tomato dry powder (DT) and processed tomato dry powder and grape seed powder in the ratio 2:3 (DT/GS 2:3) and in the ratio 3:2 (DT/GS 3:2). std lycopene, standard lycopene.

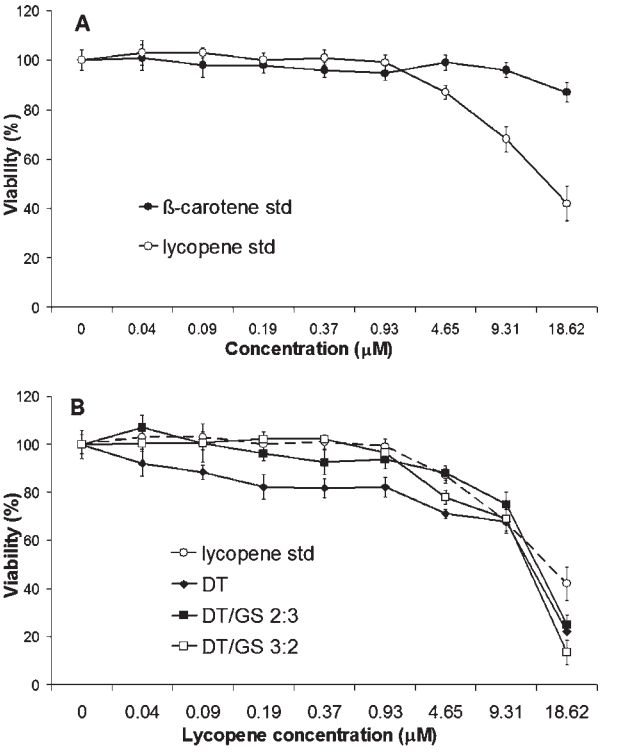

Figure 4. Cell viability of keratinocytes in culture treated with different doses of pure lycopene (lycopene std), pure β-carotene (β-carotene std) (**A**), and S-CO_2-extracted oleoresins from processed tomato dry powder (DT), processed tomato dry powder and grape seed powder in the ratio 2:3 (DT/GS2:3) and in the ratio 3:2 (DT/GS3:2), and pure lycopene (lycopene std, dotted line as reference) (**B**).

restored. In addition, all of the oleoresins were able not only to overcome the $HgCl_2$-mediated GJIC inhibition but to enhance the gap junction functionality approximately to the same extent reached when keratinocytes were merely treated with tomato oleoresins only. In particular, DT and DT/GS 3:2 CO_2-extracted oleoresins (the samples containing the higher amount of dry tomato powder) were able to reproduce the increase of GJIC obtained without $HgCl_2$ inibition as much as 1138 $\pm$ 56.9 and 1114 $\pm$ 55.7%, respectively. The DT/GS 2:3 oleoresin (containing a minor amount of dry tomato powder) notably increased the GJIC function (1349% of the control) in not $HgCl_2$-treated keratinocytes, but was able to overcome the mercury inhibition less efficiently, reaching only 586% of the control in $HgCl_2$-treated keratinocytes.

Effect of Lycopene-Containing Oleoresins on Connexin 43 Expression. To verify if the increase of the function of GJIC was dependent on an enhanced expression of connexin 43, the expression of the *cx43* gene was assayed by RT-PCR. The obtained result of the RT-PCR performed using specific primers for human connexin 43 and human actin, as control, is shown in **Figure 7**. Mercury chloride treatment has no influence on *cx43* expression. On the contrary, S-CO_2-extracted oleoresin clearly induces an increased expression of *cx43* on the mercury chloride treated keratinocytes. The S-CO_2-extracted oleoresins showed a capability to induce *cx43* expression to roughly at the same extent also in keratinocytes treated first with mercury chloride followed by oleoresin treatment.

DISCUSSION

The current study was designed to investigate the biological activity of tomato extracts, that is, S-CO_2-extracted oleoresins containing highly concentrated antioxidant compounds, particularly lycopene, on gap junction intercellular communication in

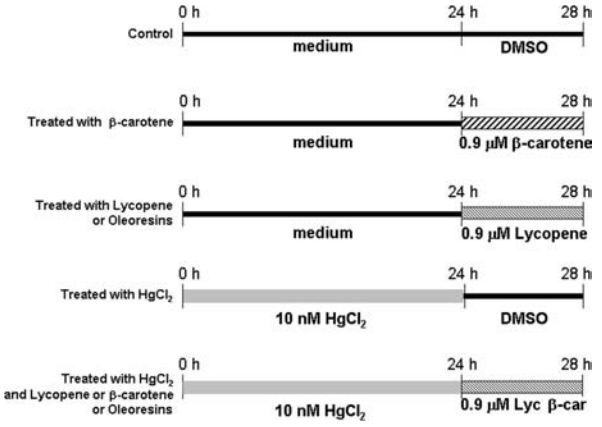

Figure 5. Experimental scheme of the treatments and timing on keratinocyte cultures.

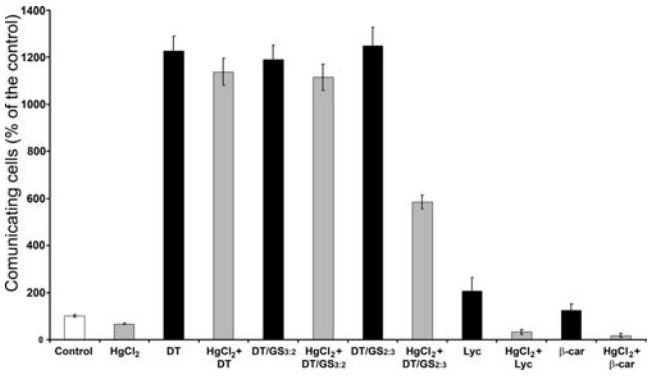

Figure 6. Functionality of gap junction intercellular communications (GJIC) in keratinocytes treated with 0.2% DMSO (control) or only with 10 nM $HgCl_2$ ($HgCl_2$), 0.9 μM lycopene, or 0.9 μM β-carotene or S-CO_2-extracted oleoresins containing 0.9 μM lycopene equivalent (Lyc, β-car, DT, DT/GS3:2 and DT/GS2:3) and with both $HgCl_2$ and lycopene or β-carotene or S-CO_2-extracted oleoresins ($HgCl_2$ + Lyc, $HgCl_2$ + β-car, $HgCl_2$ + DT, $HgCl_2$ + DT/GS 3:2 and $HgCl_2$ + DT/GS 2:3) (see scheme in **Figure 5**).

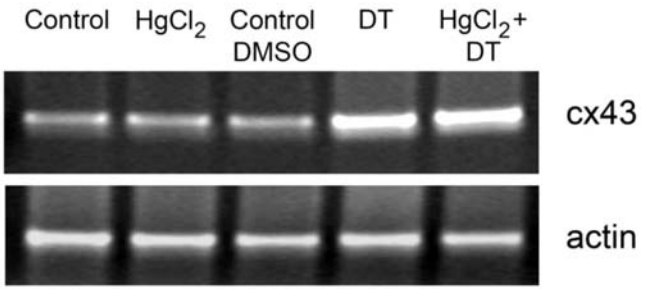

Figure 7. Expression of *cx43* mRNA, assayed by RT-PCR, in keratinocytes treated with 10 nM $HgCl_2$ or S-CO_2-extracted oleoresins from processed tomato dry powder (DT, containing 0.9 μM lycopene equivalent) and in keratinocytes treated only with 0.2% DMSO (control vehicle).

keratinocyte culture cells treated and not treated with the tumor-promoting agent mercury chloride. Here we examined the chemical composition and some biological aspects of the extracted oleoresins; the data on S-CO_2 extraction of tomato and tomato−coextracting matrices will be the object of a different publication. The results here presented indicate that lycopene-enriched oleoresins can be successfully coextracted by S-CO_2 from mixed tomato−grape seed powder. The grape seed matrix had the positive effect of improving the lipid and lipophilic antioxidant extraction and of significantly increasing the oleoresin amounts at the end of the extracting process (data not shown). This depends on the nonpolar nature of seed components (lipids), which are highly soluble in S-CO_2 fluid, and probably facilitated the extraction of highly hydrophobic components present in the plant matrices. This allowed not only a better solubilization of the unaltered lipid component but also an enhanced stabilization of carotenoids and other components such as phenols. Indeed, oleoresin from dry tomato alone contained triacylglycerols, diacylglycerols, and monocylglycerols at the rate of 4:2:1, respectively, whereas the two oleoresins obtained from dried tomato and grape seed mixed matrices consist mainly of triacylglycerols (corresponding to grape seed oil composition). First, an oleoresin with a high content of carotenoids (enriched in lycopene) was obtained, estimated at about 33% in weight in the oleoresin obtained from only tomato (DT). The supercritical fluid extraction of tomato−grape seed matrices produced a higher recovery of carotenoids from matrices (data not shown), entirely due to the major solubility of constituents present in tomatoes, because grape seed powder did not contain a significant amount of carotenoids. The carotenoid percentage was reduced depending on the grape seed oil dilution effect. Compared to S-CO_2 extraction of tomato matrix alone, the extraction of tomato−grape seeds allowed milder extraction conditions because of the synergetic effect of both tomato and grape seed oil components on antioxidant compound solubility. Specifically, supercritical fluid extraction of tomato matrix in combination with grape seeds offers the possibility of high selective extraction process and a reduced potential for oxidation of the extracted materials, making this technique especially suitable for extractive isolation of natural molecule such as lipids, carotenoids, and phenols.

The importance of the lipid environment is crucial for the carotenoid bioavailability and, maybe, for antioxidants and their biological activity. The source (*44*) and the isomeric configuration of carotenoids released from the matrix are thought to be an important determinant of their bioavailability (*45*). It is worth pointing out that the degree of carotenoid isomerization by S-CO_2 extraction in tomato matrix alone and in tomato−grape seeds was different. *all-trans*-Lycopene, representing about 60% of the total lycopene in DT oleoresin, decreased to about 50% in DT/GS oleoresins, whereas a slight increment of the four major cis isomers in the DT/GS 3:2 extract was observed. Similar observations about the isomerization of lycopene in tomato samples during supercritical fluid extraction were reported (*46*), in which it was stated that this is due to differences in solubility in supercritical CO_2 of cis and trans forms, rather than their trend to undergo transformation from all-trans to cis isomers. On the other hand, in vitro studies indicate that cis isomers are more easily taken up by mixed micelles in the intestine and hence are more bioavailable; indeed, *all-trans*-lycopene is a bulky, linear molecule that appears to be less soluble in bile acid micelles. In addition, cis isomers of lycopene might have less difficulty moving across plasma membranes and appear to be preferentially incorporated into chylomicrons (*47*). A significant increase in lycopene absorption was observed when tomato sauce was enriched in cis isomers by heat treatment (*48*). This led to the hypothesis that *cis*-lycopene isomers are preferentially absorbed in man, although an uptake of extracellular *all-trans*-lycopene and intracellular isomerization into at least two cis isoforms were recently shown in hepatic cells (*49*). From our data, it is reasonable to believe that the variation within the extracts produced by S-CO_2 was due to the diverse solubility of different isoforms of antioxidant molecules, in particular *cis*-lycopene, and an

enhanced lycopene bioavailability in the oleoresins could be expected. Finally, the matrix in which lycopene is found in foods appears to be an important determinant of its bioavailability, and the release of lycopene from this matrix is the first step in the absorptive process (*44*, *45*). In the S-CO_2-extracted oleoresins here studied, lycopene is ready-mixed in a lipid phase and could be a more bioavailable source of lycopene than both uncooked and cooked food sources.

The evidence that the oleoresins obtained from DT/GS by S-CO_2 technology have a high lycopene content with high in vitro antioxidant activity, compared with pure lycopene (**Figure 3**), suggests that the peculiar physical−chemical characteristics of the oleoresins also allowed a better antioxidant capacity. Therefore, considering that the lycopene obtained from the matrix with the high content of grape seeds (DT/GS 2:3, **Figure 3**) has a higher antioxidant activity, the idea that a difference in the relative amount of carotenoids/lycopene and lipophilic antioxidants could be responsible for the difference found in antioxidant ability is clearly sustainable. In addition, it can be postulated that the complex lipid phase (and lipophilic antioxidants) obtained from grape seed oil could preserve and/or enhance the antioxidant activity of the lycopene in the oleoresins.

From literature data, a comparison between the effects of dietary lycopene and dietary tomato powder showed that lycopene was not as effective as whole tomato in preventing prostate cancer in animal models, suggesting there may be additional bioactive components in the whole food that function in concert with lycopene. Indeed, significant flavonoid content is also found in tomato fruits. The oleoresins considered in this work contained phenol compounds ranging from 3.85 to 1.13 mg/g of oleoresin, depending on the matrix; the phenol content was higher in the oleoresin from only tomato powder and lower in the oleoresins from tomato−grape seed matrices, quite following the antioxidant activity in the hydroalcoholic soluble fraction. The percentage composition of assayed phenols was not changed, with respect to the berry, the presence of rutin, caffeic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, and naringenin being notable; a wide range of biological effects (*50*) has been attributed to these compounds. Individual compounds, as well as combination of compounds commonly present in tomato fruits, such as flavonoids, were seen to have significant synergistic interactions in both acellular and cell culture experimental systems (*14*, *15*). Although the main antioxidant activity of the oleoresins was in the lipid fraction (**Figure 1**), corresponding mainly to carotenoid fraction, it is however notable that oleoresins represent a balanced mix of lipid- and non-lipid-soluble antioxidants, with the double advantage of a better solubilization in aqueous media and an antioxidant protection in different cell compartments.

To further characterize the biological activity of S-CO_2-extracted tomato oleoresins beyond their in vitro antioxidant activity, we used the keratinocyte cell culture, which overcame most problems associated with bioavailability in the in vivo studies. We focused on the analysis of the effect of oleoresins on GJIC. Cancer-preventive activities of carotenoids have been associated with their antioxidant properties, but effects on cell signaling have also been discussed as a possible mechanism. Several carotenoids stimulate intercellular communication via gap junctions, which provides a direct pathway for the exchange of signaling molecules of low molecular weight between pairs of cells (*17*). However, the biochemical mechanism underlying the activation of GJIC by carotenoids is not yet fully understood. The importance of gap junctional intercellular communication and the biological functions of the complex of diverse connexin system, for normal development and differentiation of human epidermis, as well as their role in growth control and tumor genesis in skin is well-known (*51*). In this study we used keratinocyte cell culture, both not-treated and pretreated with the tumor promoting agent mercury chloride. Our results evidenced the ability of tomato and tomato−grape seed oleoresins to enhance, strongly, GJIC in normal human keratinocytes, compared to control. This result confirmed previous results obtained with a different type of S-CO_2-extracted lycopene (*28*). The presented results also show that the enhancement of GJIC functionality is associated with the increased level of *cx43* mRNA; this does not exclude the possibility of some posttranslational modifications of the connexin 43 protein, and it can represent a starting point to get insight into the mechanism underlying the activation of GJIC by carotenoids. In addition, the oleoresins were able to completely overcome the $HgCl_2$-mediated GJIC inhibition. The mercury chloride treatment induced on keratinocytes a significant inhibition of GJIC but no changes in *cx43* expression. In our experimental conditions, lycopene and β-carotene, supplied as pure compounds, were not active in overcoming the $HgCl_2$ effect. Application of lycopene-enriched oleoresins resulted not only in the complete recover of communications but also in an enhancement of GJIC functionality and in the up-regulation of *cx43* at the same level of keratinocytes not treated with $HgCl_2$. Connexin 43 is the most abundant connexin in human skin and is expressed in the human interfollicular epidermis, in the basal cell layer, in sebaceous glands, and in hair follicles. Changes in the proliferation and differentiation program of keratinocytes, such as those associated with skin diseases, coincide with changes of GJIC and with a dynamically regulated switch of the pattern of Cx expression in skin (*51*). These observations suggest that a higher level of GJIC is necessary to maintain epidermal homeostasis during periods of rapid epidermal growth and differentiation. On the contrary, the disruption of GJIC might be associated with abnormal cell growth and carcinogenesis. Lycopene was shown to up-regulate connexin 43 expression in human KB-1 cells (*26*), in human fibroblasts (*25*), and in MCF-7 cells (*27*) at the mRNA and protein level. The present results obtained with tomato oleoresins were interesting for the reasons that tomato compounds present in the oleoresins, and in such extent lycopene, were more efficient in increasing GJIC, compared with the pure lycopene and β-carotene. It can be postulated that (1) the physical−chemical properties of oleoresins give additional activity and/or stability to the contained carotenoids and (2) diverse compounds present in the oleoresins synergistically act to achieve the observed effect. Especially interaction with the fatty acids, which contributed to the effect on GJIC, although often they show an inhibitory effect on gap junction, could be considered.

Most tumor cells have dysfunctional GJIC and are down-regulated in the majority of skin human tumors. Consequently, it has been postulated that gap junctions serve as conduits for growth inhibitory signals. The application of a pulse of carcinogen applied to an actively growing cell population resulted in the initiation of a small percentage of exposed cells. During this time, cells seem to be reactive to tumor promoters and chemopreventive agents, such as retinoids and carotenoids (reviewed in ref *17*). This hypothesis seems to be substantiated also by the presented results obtained by using noncytotoxic (*52*) mercury chloride concentration (10 nM), able to inhibit GJIC in keratinocyte cell cultures. Recently, it was reported that lycopene in vivo inhibited epidermal hyperplasia in induced murine skin carcinogenesis model and that lycopene, in combination with acid ellagic, decreased proliferation of MT1/2 papilloma cell line in an in vitro assay (*33*). The present results make it possible to postulate that the up-regulation of *cx43* could be a possible mechanism behind the protective/preventive action of lycopene.

It has to be remarked that the level of lycopene used in this study is in the range of that commonly seen in the serum of adult humans, depending on the population being studied (*53*). Taking into account that significant differences in lycopene uptake among organs, tissues, and cell types (*11*) and that differences in measuring methods in skin (*54*) can occur, the concentration used in this study did not influence cell viability and is compatible with physiological levels. In conclusion, the obtained data confirm the potential value of S-CO_2-extracted lycopene also in combination with grape seed oil, as an excellent source of biocompounds with antioxidant and biological properties. These results established that a high-added-value product with potentially healthy properties can be obtained from natural sources (e.g., tomato and waste winery byproduct) by environmentally friendly methods such as S-CO_2 extraction. The oleoresins produced and here analyzed seem to have a phytochemical composition approximately corresponding to that found in the plant sources; thus, the observed effects could be derived from a balanced carotenoid mixture and/or the presence of other synergistically bioactive compounds. In the meantime, these results would be a better model for this type of study and provide a contribution to understanding the possible influence that carotenoids exert on cancer defense mechanisms. In addition, the effect on GJIC in keratinocyte cell cultures could suggest the value of lycopene-enriched oleoresins as an additive antioxidant and/or preservative in cosmetic formulations. Further work is necessary to determine other factors influencing the effect of bioactive compounds present in S-CO_2 extracts from tomato−grape seed on cancer cell lines.

ABBREVIATIONS USED

Cx43, connexin 43; DMSO, dimethyl sulfoxide; DT, oleoresin extracted by supercritical CO_2 from processed tomato dry powder; DT/GS 3:2, oleoresin extracted by supercritical CO_2 from processed tomato dry powder and grape seed powder in the ratio 3:2; DT/GS 2:3 oleoresin extracted by supercritical CO_2 from processed tomato dry powder and grape seed powder in the ratio 2:3; GJIC, gap junctional intercellular communication; HPLC, high-performance liquid chromatography; S-CO_2, supercritical carbon dioxide; TE, Trolox equivalents.

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