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# Antioxidant Capacity of Tomato Seed Oil in Solution and Its Redox Properties in Cultured Macrophages

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**ABSTRACT:** The health benefits of tomato seed oil (TSO) have been suggested to be related to its antioxidant activity, although at the moment not much information is available on the antioxidant effects of TSO in biological systems. In this paper, we evaluated the antioxidant capacity of TSO using different spectrophotometrical antioxidant assays (LPSC, FRAP, $\alpha$TEAC, DPPH). Moreover, we determined the ability of TSO in inhibiting oxidative stress in human cultured macrophages. The peroxyl radical scavenging LPSC assay was the most sensitive assay to detect the antioxidant capacity of the TSO, followed by the DPPH, FRAP, and $\alpha$TEAC assay. TSO was able to counteract spontaneous and $H_2O_2$-induced oxidative stress in human macrophages, limiting intracellular ROS production and controlling oxidative stress signaling. In particular, TSO was able to decrease the phosphorylation of the MAPK ERK1/2, JNK, and p-38, activation of the redox-sensitive NF-kB, and expression of the heat shock proteins 70 and 90. When the antioxidant capacity of TSO was compared with that of purified lycopene, inhibition of ROS production by TSO was remarkably higher. This was due to the high content of other antioxidants in TSO, including (5*Z*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-lycopene isomers, $\beta$-carotene, lutein, $\gamma$-tocopherol, and $\alpha$-tocopherol.

**KEYWORDS:** *tomato seed oil, antioxidant capacity, ROS production, redox signaling, lycopene, lycopene isomers, vitamin E*

## INTRODUCTION

The expanding tomato processing industry has been facing severe economical and technical environmental problems with the handling and disposal of resulting wastes.[1] About one-third of the total tomato production of 2.8 million tons goes to the industry for processing. The major component of such wastes is seed. For example, in Greece, over a million tons of tomatoes are processed each year, and the resulting quantity of seeds might be used to produce up to 2000 tons of oil.[2]

The oil, whose composition has been studied by several researchers,[3,4] could be used in cooking.

Studies have documented that tomato seeds are highly rich in lycopene.[5] Such a carotenoid has been reported to play an important role in prevention of some chronic diseases, including cancer of the digestive tract, prostate, and pancreas as well as cardiovascular diseases and inflammation.[6,7] Lycopene has been also used for prevention of cutaneous signs of skin damage, and tomato seed oil (TSO) has been suggest to protect from aging, eczema, psoriasis, and UV damage to the skin.[8] Such chemopreventive effects of lycopene are thought mainly to be due to its role as an antioxidant. In fact, tomato seeds have been reported to possess antioxidant properties and protect fats from oxidation.[9,10]

In addition to lycopene, TSO contains a wealth of fatty acids, vitamins, nutrients, carotenes, and phytosterols. In particular, TSO has been reported to possess a high unsaturated fatty acid content, with over 50% linoleic acid, followed by oleic acid.[11]

Moreover, it is rich of n-3, n-6, and n-9 polyunsaturated fatty acids. Besides, tomato seed cake was found to contain 23−39% protein with good functional and nutritional properties[12,13] since it is rich in lysine and when added to bread is able to improve loaf volume, texture, and crumb quality.

Though data on tomato seed oil composition are already available in the literature, more detailed information on its properties and on its possible applications in the clinical field is needed. The objective of the present work was to contribute to extend knowledge of the beneficial effects of tomato seed oil as an antioxidant. For this purpose, the antioxidant capacity of TSO in solution as well as the antioxidant ability of TSO in cultured macrophages were evaluated. THP-1 macrophages were used as a cell model to mimic human peripheral macrophages. In fact, they have been reported to possess properties similar to human macrophages and to be extremely sensitive to oxidative stress and changes induced by nutrient compounds, including lycopene.[14] Such data are potentially useful for studying the behavior of the oil during heating and processing and also for evaluating a possible use in prevention of chronic diseases.

## MATERIALS AND METHODS

**Chemicals.** Methanol (MeOH), dimethyl sulfoxide (DMSO), *tert*-butylmethyl ether (TBME), tetrahydrofuran (THF), and all others solvents used were of HPLC grade. HPLC-grade water (18 MΩ) was prepared using a Millipore Milli-Q purification system (Millipore GmbH, Schwalbach, Germany). Buffer salts and all other chemicals were of analytical grade. Specific chemicals were obtained of the highest quality available (95−99%) and used without purification. Concentrations of the carotenoid standard solutions as well as tocopherol and tocotrienols standards in solution were determined using the absorption coefficient at the specific wavelength in the

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specific solvent as published recently.[15,16] TSO was kindly provided by BioLycos.r.l., Rome, Italy.

**Analysis of Carotenoid Composition.** For quantification of the contents of carotenoids and lycopene isomers in TSO, 500 mg was diluted in 25 mL of MTBE (20 + 80, v/v) with $\beta$-apo-8-carotenal as an internal standard. Contents were analyzed using a gradient $C_{30}$-HPLC method using a Merck-Hitachi HPLC system (Darmstadt, Germany) and a Jetstream plus column oven (JASCO, Groß-Umstadt, Germany) as described recently.[15] A $C_{30}$-column (Trentec Stability 100 C30 PEEK, 250 × 4.6 mm, 5 $\mu$m, Trentec, Rutesheim, Germany) preceded by a $C_{18}$ProntoSil 120-5-C18 H (10 × 4.0 mm, 5 $\mu$m) column (Bischoff, Leonberg, Germany) was used. As mobile phase (1.0 mL/min) a gradient procedure consisting of MeOH (solvent A) and MTBE (solvent B) was used as published recently.[15] Column temperature was 10 ± 1 °C, and the detection wavelength was 470 nm. Carotenoid contents were quantified using external standards. Retention behavior was used to identify lycopene isomers. Carotenoids used were obtained from CaroteNature (Lupsingen, Switzerland) with a purity of 94−98% by HPLC.

**Analysis of Tocopherols and Tocotrienols.** For vitamin E quantification of the TSO using the HPLC procedure, 200 mg of the oil was dissolved in 10 mL of mobile phase (*n*-hexane:MTBE, 98:2, v/v) with $\alpha$-tocopherol acetate ($\alpha$-TA, Sigma-Aldrich, Taufkirchen, Germany) as the internal standard. After centrifugation (5 min, 14 000*g*), the isocratic HPLC procedures with fluorescence detection using a normal phase Eurospher DIOL-column (Knauer, Berlin, Germany) followed the descriptions of Franke et al.[17] Concentration series of all tocopherols ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T, Calbiochem Darmstadt, Germany) and tocotrienols ($\alpha$-, $\beta$-, $\gamma$-, and $\delta$-T3, Davos Life Sciences, Singapore) were used for quantification.

**Antioxidant Capacity.** In general, different dilutions of TSO in *n*-hexane [for ferric reducing antioxidant power (FRAP assay), $\alpha$-tocopherol equivalent antioxidant capacity ($\alpha$TEAC assay), and 2-diphenyl-1-picrylhydrazyl assay (DPPH assay) and MTBE:DMSO (1 + 4, v/v, for luminol-chemiluminescence-based peroxyl radical scavenging capacity (LPSC assay)] were used for analysis of antioxidant capacities of TSO in various chemical in vitro assays. $\alpha$-T standards in the specific solvent (5−250 $\mu$M) were used for calibration in each assay. *n*-Hexane was used as blank. On the basis of the $\alpha$-T calibration, antioxidant capacities were calculated as millimoles of $\alpha$-tocopherol equivalents ($\alpha$-TE) per liter.

**FRAP Assay.** FRAP of TSO was analyzed by mixing TSO (100 $\mu$L, dissolved in *n*-hexane) with 1.0 mL of FRAP reagent on a thermal shaker (6 min, 25 ± 1 °C, 1400 rpm) following the FRAP procedure used for oil samples by Müller et al.[15] FRAP reagent contained 300 mM acetate buffer pH 3.6, 10 mM TPTZ (Sigma-Aldrich, Taufkirchen, Germany) in 40 mM hydrochloric acid and 20 mM aqueous $FeCl_3$ solution. After shaking the solution was transferred completely into half-microcuvettes (1.5 mL, polystyrene) and centrifuged for 30 s at 1000*g*. Exactly 8 min after starting shaking, the absorbance at 595 nm of the aqueous layer was detected in a JASCO V-530 spectrophotometer (Groß-Umstadt, Germany).

**$\alpha$TEAC Assay.** To analysize the capacity of TSO to reduce the free $ABTS^{\bullet+}$ radical cation (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Sigma-Aldrich, Taufkirchen, Germany), the $\alpha$TEAC assay was used, as published by Müller et al.[16] TSO solution (100 $\mu$L, in *n*-hexane) was shaken with 1.0 mL of freshly prepared $ABTS^{\bullet+}$ solution (in 75 mM PBS, pH 7.4, $Abs_{734\ nm}$ = 0.70 ± 0.02). After 30 s of shaking on a Vortex mixer Genie 2 (Scientific Industries Inc., Bohemia, NY) at the highest speed, the mixture was transferred into cuvettes and centrifuged as described for the FRAP assay above. Exactly 2 min after starting shaking, the absorbance at 734 nm of the ABTS layer was measured on the spectrophotometer.

**DPPH Assay.** To determine the capacity of TSO to scavenge the chromogenic free nitrogen $DPPH^{\bullet}$ radical, 0.5 mL of fresh prepared 0.3 mM $DPPH^{\bullet}$ (Sigma-Aldrich, Taufkirchen, Germany) solution in ethanol was mixed with TSO solution (1.0 mL, in *n*-hexane). The mixture was shaken on a thermal shaker (15 min, 25 ± 1 °C, 1000 rpm). Afterward the solution was transferred into half-microcuvettes. The absorbance of the mixture was detected at 540 nm using a spectrophotometer to avoid interferences with the absorbance of TSO carotenoids, as recently mentioned.[16]

**LPSC Assay.** The LPSC assay was carried out in white 96-well microplates (Greiner, Frickenhausen, Germany) and measured using a FluoStar Optima plate reader (BMG Labtech, Offenburg, Germany) at 37 ± 0.2 °C, as described recently.[16] Luminol (Fluka, Buchs, Switzerland) was used as the luminescent dye and AAPH (2,2′-Azobis(2-amidinopropane) hydrochloride (Acros Organics, Schwerte, Germany) as the thermal-sensitive peroxyl radical generator. The chemiluminescence signal was measured every second minute for up to 1.5 h. Final LPSC values were calculated using the area under the curve (AUC) technique, with $\alpha$-T standards for calibration.

**Cell Culture.** THP-1 (American Type Culture Collection, Rockville, MD, USA) were grown in RPMI Dutch Modified (Sigma, Milan, Italy) without antibiotics and supplemented with 10% fetal calf serum, nonessential amino acids, 2 mM glutamine, and 1 mM sodium pyruvate. Cells were maintained in log phase by seeding twice a week at a density of $3 \times 10^8$ cells/L at 37 °C under 5% $CO_2$/air atmosphere. TSO and purified lycopene (provided as an extract of 99.9% purity from LycoRed Natural Products Industries Ltd., Beer Sheva) were delivered to the cells using THF as a solvent. To avoid formation of peroxides, the solvent used contained 0.025% butylated hydroxytoluene (BHT). Stock solutions of TSO (10 mg/mL) or lycopene (2 mM) in THF were prepared immediately before each experiment. From the stock solutions, dilutions were made and aliquots of TSO or lycopene were rapidly added to the culture medium to give the final concentrations indicated. Incubation was performed for 24 h. Lycopene was incorporated in THP-1 macrophages, as described in ref 18. The amount of THF added to the cells was not greater than 0.5% (v/v). Control cultures received an amount of solvent (THF) equal to that present in TSO- or lycopene-treated ones. No differences were found between cells treated with THF and untreated cells in terms of cell number, viability, and reactive oxygen species (ROS) production. After addition of lycopene, the medium was not further replaced throughout the experiments. Experiments were routinely carried out on triplicate cultures. At the times indicated, cells were harvested and quadruplicate hemocytometer counts were performed. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

**Measurement of ROS.** Cells were harvested to evaluate reactive oxygen species (ROS) production using the di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR) as described in ref 14 . Before addition of the fluorescent probes, $2 \times 10^6$ cells were washed to eliminate the amount of lycopene not cell associated. Fluorescent units were measured in each well after 30 min incubation with DCF (10 $\mu$M) by use of a Cytofluor 2300/2350 Fluorescence Measurement System (Millipore Corp., Bedford, MA). Lycopene did not alter the basal fluorescence of DCF.

**Western Blot Analysis of p38 and p-p38, ERK1/2, pERK1/2, JNK, p-JNK, hsp-70, and hsp-90 Expression.** Cells ($10 \times 10^6$) were harvested, washed once with ice-cold PBS, and gently lysed for 30 min in ice-cold lysis buffer (1 mM $MgCl_2$, 350 mM NaCl, 20 mM Hepes, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM $Na_4P_2O_7$, 1 mM PMSF, 1 mM aprotinin, 1.5 mM leupeptin, 1 mM $Na_3VO_4$, 20% glycerol, 1% NP40). Cell lysates were centrifuged for 10 min at 4 °C (10 000*g*) to obtain the supernatants, which were used for Western blot analysis. The anti-p38 (clone C-20, cat. no. SC-535), anti p-p38 (clone D-8, cat. no. 7973), anti-ERK1/2 (clone K-23, cat. no. SC-94), anti p-ERK1/2 (clone E-4, cat. no. SC-7383), anti-JNK (clone C-17, cat. no. SC-474), and anti-p-JNK (clone G-7, cat. no. SC-6254) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antihsp 70 (clone K-20, cat. no. sc-1060) and anti-hsp90$\alpha$ (clone C-20, cat. no. sc-8262) goat polyclonal antibodies were also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were washed and exposed to horseradish peroxidase-labeled secondary antibodies (Amersham Pharmacia Biotech, Arlington Heights, IL) for 45 min at room temperature.

Immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantified by densitometric scanning.

**Electrophoretic Mobility-Shift Assay.** Frozen cell pellets were processed to obtain nuclear extracts. The pellet was treated as indicated in ref 14 . Binding reactions containing 5 $\mu$g of nuclear extracts, 10 mMTris-HCl (pH 7.6), 5% glycerol, 1 mM EDTA, 1 mM DTT, 50 mM NaCl, and 3 mg of poly(dI-dC) were incubated for 30 min with 5000 cpm of $\alpha$-$^{32}$P-end-labeled double-stranded oligonucleotide in a total volume of 20 $\mu$L. The probe was 5′-AGTTGAGGGGACTTTCCCAGGC3′. Labeling of the probe was obtained by incubating 5 pmol of oligonucleotide with 10 pmol of [$\alpha$-$^{32}$P]ATP and 3 UT4 polynucleotide kinase for 30 min at 37 °C. The probe was then purified with MicroBIO-Spin P-30 columns. Complexes were separated on 60 g/L polyacrylamide gels with 45 mM Tris-borate, 1 mM EDTA, pH 8 buffer. After fixation and drying, gels were exposed on phosphor screens which were then analyzed by a phosphor/fluorescence imager STORM 840 (Molecular and Dynamics, Sunnyvale, CA). The intensity of the revealed bands was directly quantified by Image QuaNT software (Molecular Dynamics, Sunnyvale, CA).

**Analysis of p65 Protein.** Nuclear extracts, 25−30 $\mu$g of protein, were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis with 40−120 g/L Bis-Tris gels (NOVEX, San Diego, CA) and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) with the use of a semidry system. Immunoblots were blocked overnight at 4 °C in 50 g/L dried milk in PBS, pH 7.4 plus 0.05% Tween 20. Blots were incubated with polyclonal primary antibodies to p65 (Santa Cruz, Biotechnology, CA, clone 49.Ser 311, cat. no. SC-135769) in PBS plus 0.05% Tween 20 for 1−2 h at room temperature. Blots were visualized as described in the Western blotting assay.

**Statistical Analysis.** Values were presented as means ± SEM. One-way ANOVA was used to determine differences between different treatments in Figure 1A−F. When significant values were found ($P <$ 0.05), posthoc comparisons of means were made using Fisher's test. Multifactorial two-way analysis of variance (ANOVA) was adopted to assess any differences among the treatments and doses (Figure 4A). When significant values were found ($P < 0.05$), posthoc comparisons of means were made using the Tukey's Honestly Significant Differences test. Differences were analyzed using Minitab Software (Minitab, Inc., State College, PA).

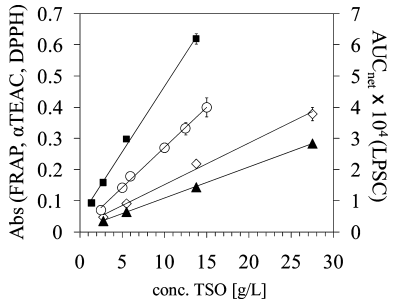


**Figure 1.** Blank-corrected absorbances (in FRAP, $\alpha$TEAC, and DPPH assay) and AUC$_{net}$ (in LPSC assay) after reaction with a different concentration of TSO: FRAP assay at 595 nm (▲), $\alpha$TEAC assay at 734 nm (◇), DPPH assay at 540 nm (■), and LPSC assay (○).

## RESULTS

**Antioxidant Capacities Chemically Measured.** Figure 1 shows the linearity between the used concentrations of TSO (1−30 g/L) and the absorbances of the oxidant solutions in the different spectrophotometrical antioxidant assays (FRAP, $\alpha$TEAC, DPPH). The blank-corrected absorbance of the FRAP solution containing $Fe^{II}TPTZ_2$ as oxidant as well as the absorbance of the ABTS$^{\bullet+}$ solution in the $\alpha$TEAC assay and the absorbance of the DPPH$^{\bullet}$ in the corresponding assay increased linearly after addition of various concentrations of TSO. The peroxyl radical scavenging LPSC assay was the most sensitive assay to detect the antioxidant capacity of the TSO, followed by the DPPH, FRAP, and $\alpha$TEAC assay. The antioxidant capacities of the TSO ranged between 3.2 mmol of $\alpha$-TE/kg in the FRAP assay and 15.1 mmol of $\alpha$-TE/kg in the LPSC assay (Figure 2). The higher antioxidant capacity of the TSO in the LPSC assay compared to FRAP, $\alpha$TEAC, and DPPH assay is due to the high activity of lycopene in this assay compared to tocopherols[16] combined with the high lycopene content in the TSO. In contrast, the antioxidant capacities in the four assays ranged more closely, if the calculation is based on lycopene as reference, between 0.53 and 0.85 mmol of lycopene equivalents (LYC-E) per kg TSO (Figure 3). The TSO was more potent as an antioxidant than pure lycopene. Figure 4A−D displays the lycopene content in the specific dilution of the TSO against the antioxidant capacities of the specific dilution measured in the four assays. For comparison, pure (all-*E*)-lycopene was measured at a similar concentration. In all assays used, the TSO was more potent as an antioxidant than a pure lycopene solution. The developing curves in both SET-based assays (FRAP, $\alpha$TEAC) were very similar (Figure 4A and 4B). With increasing concentration of pure lycopene or lycopene concentration in the diluted TSO, the blank-corrected absorbances of the oxidant solutions increased. However, the curves of the lycopene in TSO increased much faster compared to the curves of the pure lycopene solutions. In the DPPH assay, pure lycopene did not lead to a change in the absorbance

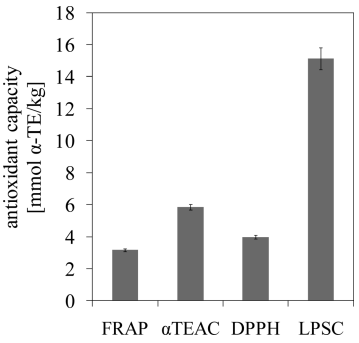


**Figure 2.** Evaluation of the antioxidant capacity of TSO in various assays with respect to $\alpha$-tocopherol (mmol $\alpha$-TE/kg TSO).

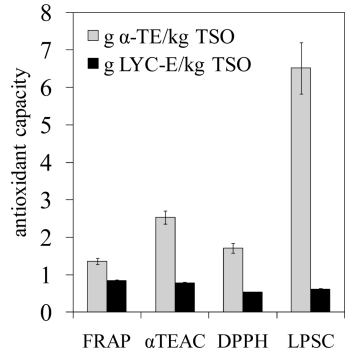


**Figure 3.** Evaluation of the antioxidant capacities of TSO in various assays with respect to $\alpha$-tocopherol (grams of $\alpha$-tocopherol equivalents [$\alpha$-TE] per kilogram of TSO) as well as to lycopene (grams of lycopene equivalents [LYC-E] per kilogram of TSO).


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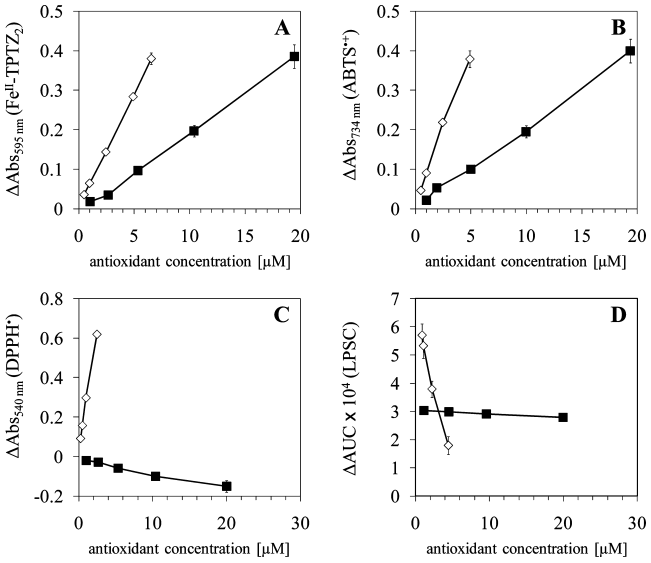


**Figure 4.** Evaluation of the antioxidant capacities of TSO in various assays ((A) FRAP assay, (B) $\alpha$TEAC assay, (C) DPPH assay, (D) LPSC assay) compared to pure lycopene, (■) pure lycopene, and (◇) lycopene concentration in TSO.

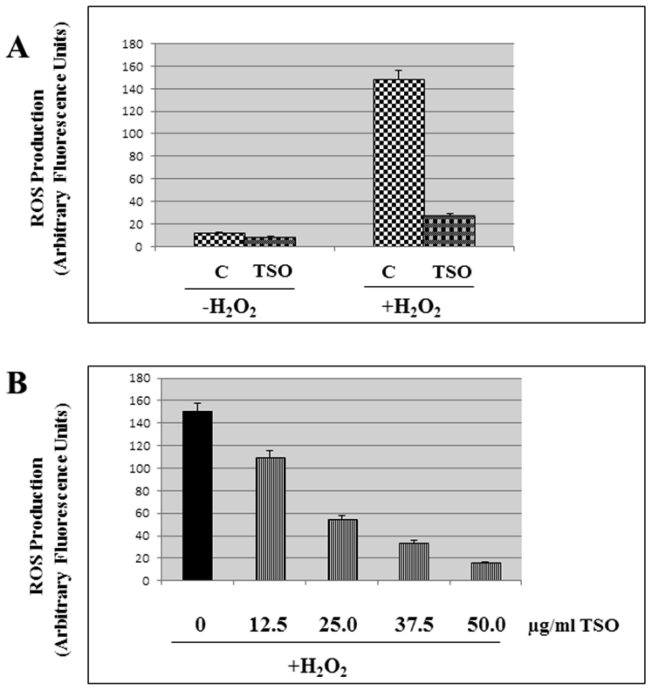


**Figure 5.** ROS production in THP-1 cells pretreated with tomato seed oil (TSO) for 24 h. (A) Spontaneous and $H_2O_2$-induced ROS production in control cells and in cells pretreated with 50 $\mu$g/mL TSO and exposed, when indicated, to 100 $\mu$M $H_2O_2$ for 30 min. (B) $H_2O_2$-induced ROS production in the presence of varying TSO concentrations in cells treated with 100 $\mu$M $H_2O_2$ for 30 min. Values were the means ± SEM of three experiments.

of the DPPH$^\bullet$ solution (Figure 4C), whereas the TSO dilution containing the same lycopene content bleached the DPPH$^\bullet$ solution very strong. Such a strong antioxidant effect of the TSO compared with pure lycopene was also observed in the LPSC assay (Figure 4D). The TSO dilutions containing a specific lycopene concentration scavenged peroxyl radicals, shown as decreasing AUC, on a greater extent than pure lycopene itself.

**Antioxidant Activity of TSO in THP-1 Cells.** Furthermore, the antioxidant capacity of tomato seed oil extract (TSO) was analyzed in THP-1 cells in the absence and in the presence of the well- known prooxidant agent $H_2O_2$. Its efficiency was evaluated as inhibition of intracellular reactive oxygen species (ROS) production induced spontaneously or in the presence of the prooxidant (Figure 5A). TSO was added to the system at a concentration of 50 $\mu$g/mL for 24 h, and ROS production was measured after 30 min of incubation in the absence or in the presence of 100 $\mu$M $H_2O_2$. Intracellular radical species were detected by measuring the fluorescence intensity values due to oxidation of DCF. In the absence of the prooxidant, a very small amount of ROS production was observed, but it increased significantly in the presence of $H_2O_2$. Treatment of THP-1 cells with TSO caused a decrease in ROS production, which was remarkably evidenced during incubation with $H_2O_2$. A weak but significant inhibition of ROS production by TSO in the absence of $H_2O_2$ was also observed. Such an effect was specific for TSO, since cells treated with $H_2O_2$ and THF alone as a vehicle did not differ in ROS expression from cells treated with $H_2O_2$ alone (data not shown).

The antioxidant effects of TSO were clearly dose dependent, as it can be observed by the progressive decrease of $H_2O_2$-induced ROS production after addition of increasing TSO concentrations to THP-1 cells (Figure 5B).

We next investigated intracellular redox signal transduction in THP-1 cells. For this purpose, possible modulation of the mitogen-activated protein kinase (MAPK) cascade was analyzed in macrophages pretreated with TSO and then exposed to $H_2O_2$ (Figure 6). These kinases have been reported to be activated by various stress stimuli, including an overproduction of ROS, and they have been also implicated in the modulation of several intracellular redox functions. Treatment with $H_2O_2$ induced a remarkable increase in the level of the phosphorylated forms of extracellular signal-regulated kinase1/2 (p-ERK1/2) (Figure 6A and 6B), Jun N-terminal kinase (p-JNK) (Figure 6C and 6D), and p-38 (p-p38) (Figure 6E and 6F) after 3 h of incubation. Such increases were all prevented by addition of TSO. A reduction of MAPK phosphorylation by TSO was also observed in basal conditions (without $H_2O_2$ addition).

In view of recent studies suggesting that NF-kB plays an important role in regulating redox signaling, we investigated the involvement of this redox-sensitive transcription factor in THP-1 macrophages treated with $H_2O_2$ (Figure 7A). Following THP-1 cell incubation in the presence of $H_2O_2$, net enhancement of nuclear translocation was evident at 3 h treatment. However, the up-regulation of NF-kB nuclear binding was completely prevented by addition of TSO. Similar results were found when nuclear extracts were prepared from macrophages treated with $H_2O_2$ alone and in combination with TSO and nuclear translocation of the NF-kB p65 subunit was detected by Western blotting (Figure 7B). The prooxidant induced the nuclear translocation of the NF-kB subunit p65 within 3 h. Such an effect was inhibited by addition of TSO.

Since it has been reported that the heat shock proteins hsp70 and hsp90 are nuclear binding proteins involved in oxidative stress, we measured the expression of hsp70 and hsp90 proteins in THP-1 cells exposed to 0.1 $\mu$M $H_2O_2$ for 3 h in the absence and in the presence of TSO (Figure 7C and 7D). Addition of the prooxidant increased the expression of both hsp70 and


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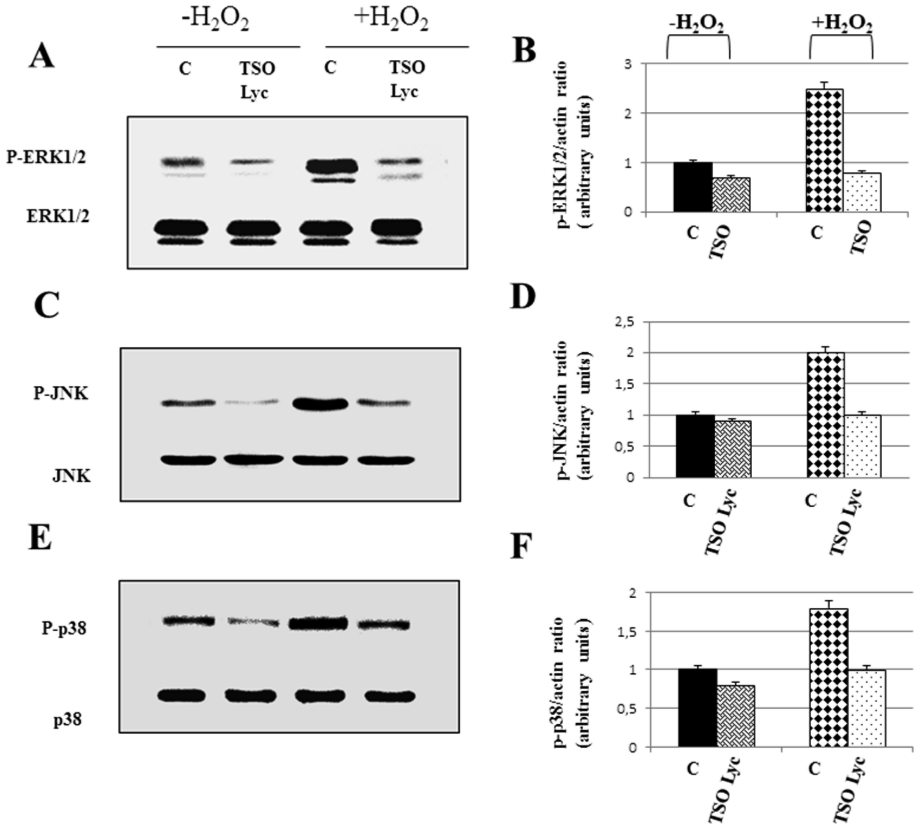


**Figure 6.** MAPK expression in THP-1 cells pretreated with tomato seed oil (TSO). Cells were pretreated with 50 $\mu$g/mL TSO and exposed, when indicated, to 100 $\mu$M $H_2O_2$ for 3 h. (A, C, and E) Representative Western blotting analyses. (B, D, and F) Densitometric analyses. Values were the means ± SEM of three experiments. Values not sharing the same letter were significantly different ($P < 0.05$, Fisher's test).

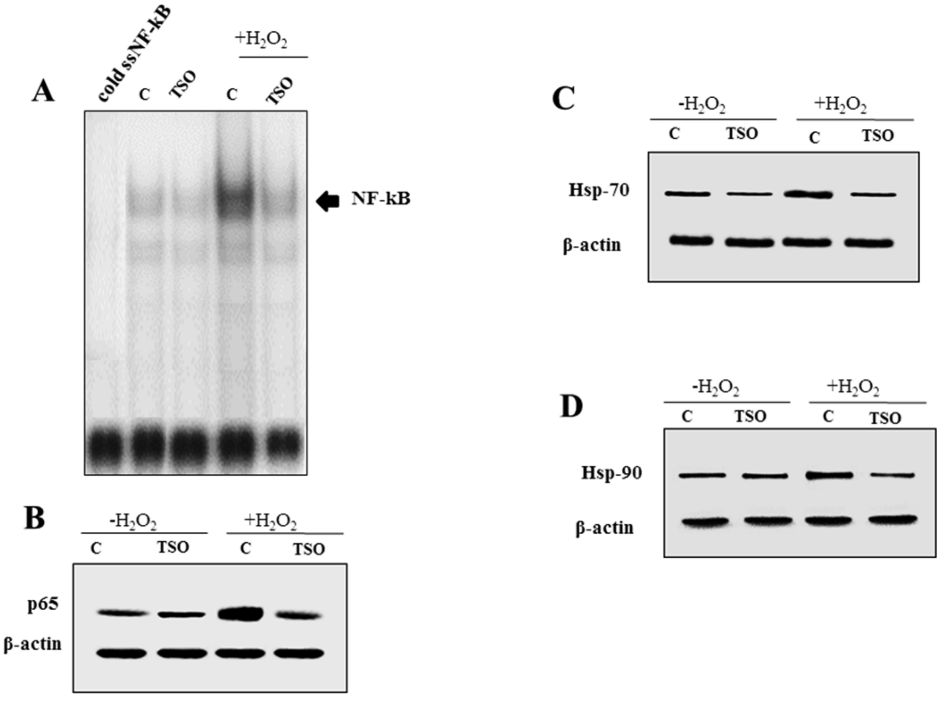


**Figure 7.** NF-kB activation in THP-1 cells pretreated with tomato seed oil (TSO). Cells were pretreated with 50 $\mu$g/mL TSO and exposed, when indicated, to 100 $\mu$M $H_2O_2$ for 3 h. (A) DNA binding activities of NF-kB subunits complexes. After treatment, binding activities of NF-kB subunits to DNA were determined using EMSA. Specifity was demonstrated using excess unlabeled NF-kB oligonucleotides (=cold ssNF-kB) which competed away binding. (B) Representative Western blotting analyses of p65 subunits. (C and D) Representative Western blotting analyses of hsp70 and hsp90, respectively.


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hsp90 with respect to untreated control cells. Both hsp70 and hsp90 expressions were inhibited by addition of TSO.

When the antioxidant capacity of TSO was compared with that of purified lycopene at concentrations ranging from 0.5 to 3.0 $\mu$M (Figure 8), inhibition of ROS production by TSO was

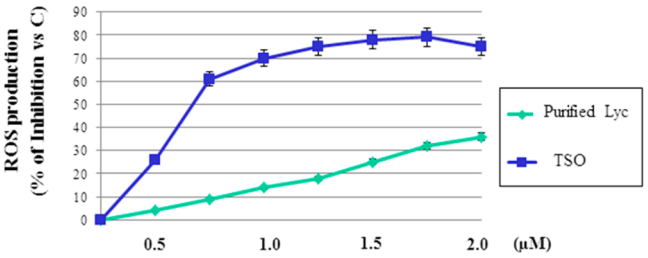


**Figure 8.** Inhibition of $H_2O_2$-induced ROS production in THP-1 cells treated with TSO or purified lycopene. Lycopene in TSO and purified lycopene were incubated at the concentrations indicated for 24 h, before 100 $\mu$M $H_2O_2$ for 30 min addition. Values were the means ± SEM of three experiments. Values not sharing the same letter were significantly different ($P < 0.05$, Tukey's test).

remarkably higher than that of the purified lycopene, as highlighted by the different percentages of inhibition. These data clearly support the notion that TSO contains several bioactive compounds, mainly lycopene but also other carotenoids, their metabolites and oxidative products, as well as vitamin E, vitamin C, and polyphenols, which could cooperate in increasing the antioxidant capacity of the extract with respect to purified lycopene.

According with this hypothesis, we measured carotenoid composition of TSO (Figure 9 and Table 1). Lycopene was the

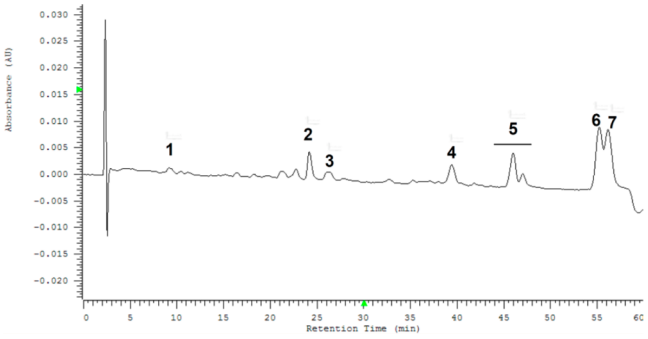


**Figure 9.** HPLC chromatogram (at 470 nm) of carotenoid composition of the tomato seed oil sample: (1) lutein, (2) (all-*E*)-$\beta$-carotene, (3) (9*Z*)-$\beta$-carotene, (4) (13*Z*)- + (15*Z*)-lycopene, (5) (5*Z*,9′*Z*)- + (9*Z*)- + (5*Z*,9*Z*)-lycopene, (6) (all-*E*)-lycopene, (7) (5*Z*)-lycopene. HPLC system by Merck-Hitachi; C30-column, preceded by a C18-precolumn. Mobile phase: gradient of MeOH (A) and MTBE (B). $T = 10 \pm 1$ °C.

predominant carotenoid with a contribution of approximately 82% to the total carotenoid content. As observed by HPLC analysis, TSO contains a large amount of (all-*E*)-lycopene but also a lot of lycopene isomers, including (5*Z*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-lycopene isomers. Moreover, it also contains remarkable amounts of $\beta$-carotene and lutein with portions of 14% and 4%, respectively. The TSO extract is also extremely rich of tocopherols and tocotrienols (approximately 1500 mg per kg), as shown in Table 1, whereas $\gamma$-tocopherol was the

**Table 1. Content of Carotenoids (in mg/kg) and Vitamin E in Tomato Seed Oil**

| compd | content |
|---|---|
| lutein | 4.8 ± 0.3 |
| $\beta$-carotene | 16.6 ± 0.4 |
| total lycopene | 95.6 ± 3.6 |
| thereof (all-*E*)-lycopene | 32.2 ± 0.9 |
| thereof (5*Z*)-lycopene | 33.5 ± 1.7 |
| thereof (13*Z*)- and (15*Z*)-lycopene | 9.9 ± 0.7 |
| thereof other lycopene (*Z*)-isomers | 20.0 ± 0.3 |
| vitamin E | 1451.6 ± 13.9 |
| thereof $\alpha$-tocopherol | 245.6 ± 3.0 |
| thereof $\beta$-tocopherol | 34.7 ± 0.5 |
| thereof $\gamma$-tocopherol | 1095.0 ± 9.3 |
| thereof $\beta$-tocotrienol | 76.3 ± 1.1 |

main vitamin E compound with a portion of about 75%, followed by $\alpha$-tocopherol with a portion of approximately 17%.

## DISCUSSION

Tomato pomace, a waste product from tomato processing plants, consists of skins, pulp, and seeds. The major component of tomato pomace is the seeds, which are considered to be a good source of edible oil.[19] It accounts for approximately 10% of the fruit and 60% of the total tomato industry waste.[20]

The oil has been studied extensively for its composition in fatty acids and chemical and physical properties.[2−4,20,21] Recently, its use in cooking as well as in skin creams has been suggested. Moreover, increasing evidence suggests that TSO may have beneficial effects in human health since it has been reported to possess antioxidant properties,[22] mainly due to its content of lycopene, lycopene isomers, and tocopherols. Administration of tomato seed oil can lower serum total cholesterol, triglyceride, and low-density lipoprotein cholesterol levels.[23] Moreover, an improvement of tomato seeds and vitamin C on potassium bromate-induced renal injury in rats has been reported.[24] Though data on tomato seed oil composition are already available in the literature, information is still missing on its antioxidant effects in biological models.

In this study, we reported that TSO exhibited a strong antioxidant capacity and acted as a potent antioxidant in human macrophages exposed to oxidative stress.

It has been suggested that the assessment of the antioxidant capacity of food samples requires the use of various methods.[16]

In the present study, we applied the LPSC, DPPH, FRAP, and $\alpha$TEAC to detect the antioxidant capacity of TSO. Therefore, we used assays based on a hydrogen-atom transfer, which measure the activity of the antioxidant to scavenge peroxyl radicals, such as the LPSC method, and assays based on electron transfer reactions, such as the FRAP, $\alpha$TEAC, and DPPH assays. In the presentstudy, the peroxyl radical scavenging LPSC assay was the most sensitive assay to detect the antioxidant capacity of the TSO, followed by the DPPH, FRAP, and $\alpha$TEAC assay. This is presumably due to the fact that LPSC assay is the only method, except for the oxygen radical absorbance capacity (ORAC) assay often used for determination of the antioxidant capacity of hydrophilic compounds and oil samples, combining in the detection both the inhibition time and the degree of inhibition.[16] In fact, carotenoids are highly concentrated in the TSO, and recent data demonstrated the high antioxidant activity of carotenoids in the LPSC assay compared to the other antioxidant assays.[16]


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On the other hand, in DPPH assay, tocopherols showed very high antioxidant capacity while carotenoids did not.[16] In addition, FRAP and αTEAC reacted similarly since both assays are based on the same mechanism in which the redox potential of the compounds analyzed is important.

In comparison to other plant oils, TSO was 40% more antioxidant than olive oil and 10 times more antioxidant than sunflower oil or walnut oil in the lipophilic FRAP assay. The relation between the antioxidant capacities of typical oils of human diet and the TSO was similar in the $ABTS^{\bullet+}$ bleaching method (αTEAC assay) however on a twice lower level. TSO was 4−5 times more potent as an antioxidant than sunflower and walnut oil and 45% more potent than olive oil. Moreover, in the $DPPH^{\bullet}$ bleaching assay, TSO presented a 2 times higher antioxidant capacity than sunflower and walnut oil and 10 times higher antioxidant capacity than olive oil. Olive oil was frequently found to be a weak $DPPH^{\bullet}$ scavenging plant oil due its low vitamin E content.[16,25] However, fish oil was in the used DPPH assay 2.5 times more antioxidant using this method. In the LPSC assay, TSO was 4−20 times more active in scavenging peroxyl radical than olive oil and sunflower oil due to the high content of carotenoids such as lycopene, which were observed to be multiple active in scavenging peroxyl radicals compared to vitamin E.[16]

TSO also acted as potent antioxidants in intact cells, reducing spontaneous and $H_2O_2$-induced intracellular ROS production, modulating the redox-sensitive MAPK-NF-kB pathway and inhibiting expression of oxidative stress-induced proteins, including hsp70 and hsp90. Such observations are not surprising in view of the fact that TSO contains a high content of lycopene, and this carotenoid has been reported to act as a redox agent in several cell models.[26] Recent studies from our laboratory demonstrated that lycopene is incorporated in THP-1 macrophages[18] and protects them against several oxidative stressors.[26] Moreover, the carotenoid has been reported to act as a quencher of singlet oxygen[27] and to function as inhibitor of several free radicals species,[28] including hydrogen peroxide, nitrogen dioxide ($NO_2^{\bullet}$), thiyl ($RS^{\bullet}$), and sulfonyl ($RSO_2^{\bullet}$) radicals.[29] Recently, it has been also reported that the carotenoid can also decrease hypochlorous acid levels.[30] Lycopene molecule is highly lipophilic and is most commonly located within cell membranes and other lipid components. It is therefore expected that in the lipophilic environment lycopene will have maximum ROS scavenging effects. Lycopene was shown to be the most effective antioxidant in protecting the 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN)-induced lipid peroxidation of the liposomal membranes[31] and inhibit ROS production in different cell models.[25−28] Moreover, increasing evidence shows that it may modulate expression of ROS-producing enzymes, including NADP(H) oxidase, cyclo-oxygenase, inducible nitric oxide synthase, and 5-lipooxygenase in isolated cells.[32] There is also growing evidence that lycopene, alone or in combination with other natural products, is able to regulate ROS-mediated cell signaling pathways, including MAPKs by affecting phosphorylation and NF-kB by inhibiting its activation.[33,34] The carotenoid was reported to attenuate the phenotypicand functional maturation of murine bone marrow (BM) dendritic cells (DC), mainly in lipopolysaccharide (LPS)-induced DC maturation, by down-regulating the expression of costimulatory molecules (CD80 and CD86) and major histocompatibility complex type II molecules and by inhibiting activation of MAPK and NF-kB.[35] It has been reported that lycopene is able to inhibit platelet-derived growth factor (PDGF)-BB-induced signaling and migration in human fibroblasts by inhibiting activation of ERK1/2, p38.[33] In recent studies from our laboratory, lycopene suppressed both redox-based MAPK phosporylation and NF-kB activation in oxysterol-stimulated macrophages as well as in prostatic cancer cells.[14,26]

Interestingly, TSO was more potent as an antioxidant than pure lycopene, as observed in solution and in intact cells, clearly underlying the presence of other antioxidants in TSO.

In fact, we determined several lycopene isomers in TSO, including (5*Z*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-lycopene isomers. The lycopene isomer pattern in TSO differs compared to the lycopene isomer pattern in fresh tomatoes or tomato products. In tomato products, such as catsup or tomato sauce, the portion of (all-*E*)-lycopene was determined with 60−96% of the total lycopene content. The presence of (3*Z*)-lycopene isomer has been also reported.[9] Moreover, TSO also contains remarkable amounts of β-carotene and lutein with portions of 14% and 4%, respectively. The TSO extract is also extremely rich of tocopherols and tocotrienols, as it can be observed in Table 1. While in fresh tomatoes as well as in tomato products α-T is the predominant vitamer,[36] in TSO γ-tocopherol was the predominant vitamin E active compound. High γ-tocopherol contents are typical for seed oils. Total vitamin E content in tomato products varied between 0.68 mg/100 g in fresh tomatoes and 4.98 mg/100 g in canned tomato paste. The vitamin E content in the TSO investigated was 30−300 times higher compared to these tomato products. Compared to typical used plant oils in the diet, the vitamin E content of the TSO was 5 times higher than the vitamin E content determined in olive oil and 4 times higher than in sunflower oil. Vitamin E is able to influence the lipophilic antioxidant activity of plant oils, as recently published by our research group.[16] It should be also considered that TSO contains vitamin C and phenolic compounds, whose antioxidant activity is well known.[37] The total content of phenolics in 100 g of dry matter ranged from 70 mg in tomato seeds. The skin and seed fractions of tomato, together, contributed 43% of the total ascorbic acid.

On the basis of the content of vitamin E compounds and carotenoids in the analyzed TSO (see Table 1) and the antioxidant activities of the compounds known from the literature,[15,16] we calculated the theoretical contribution of these compounds to the antioxidant activity of the TSO in the used assays. In all assays, γ-tocopherol was the major antioxidant with a contribution of 22−56% of the antioxidant activity of TSO. All lycopene isomers together account for 11% and 12% of the FRAP and αTEAC activity, respectively, whereas the total tocopherol content account for 80% and 54% in FRAP and αTEAC assay, respectively. In addition, vitamin E was the major active antioxidant of TSO in the DPPH assay. All vitamers together contribute for about 80% to the TSO activity in DPPH assay, whereas carotenoids did not.[16] In the peroxyl radical scavenging-based LPSC assay, lycopene and vitamin E, especially γ-tocopherol due to its high concentration, were the most antioxidant active components. These lipophilic compounds account for 20% (by lycopene) and 28% (by total vitamin E; with 22% by γ-tocopherol itself) of the peroxyl scavenging antioxidant activity of TSO.

However, in all assays, 8−45% of the antioxidant activity of TSO could not be clarified by the single antioxidant activity of carotenoids and vitamin E. The unknown part of the antioxidant activity of TSO is maybe due to unidentified minor compounds, such as lipophilic compounds as known

from olive plant[38] or by synergistic effects between carotenoids and vitamin E. Such synergistic effects of the antioxidant activity in in vitro assays are described by Edge et al.[39] They show that lycopene is able to reduce tocopheryl radicals as well as radicals of lutein and zeaxanthin. Several observations showing synergistic effects of carotenoids and tocopherols have also been recently reported by Palozza et al.[40]

From our data we can conclude that TSO is able to determine potent antioxidant effects in biological systems. It possesses a strong antioxidant capacity in solution, and it is able to counteract $H_2O_2$-induced oxidative stress in human macrophages, limiting ROS production and controlling oxidative stress signaling.

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## ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,29-azobis(2,4-dimethylvaleronitrile); ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); $\alpha$TEAC, $\alpha$-tocopherol equivalent antioxidant capacity; $\alpha$-TE, $\alpha$-tocopherol equivalents; AUC, area under the curve; BHT, butylated hydroxytoluene; DCF, di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ERK, extracellular signal-regulated kinase; FRAP, ferric reducing antioxidant power; HPLC, high-performance liquid chromatography; hsp, heat shock protein; JNK, Jun N-terminal kinase; LPSC, luminol-chemiluminescence-based peroxyl radical scavenging capacity; MAPK, mitogen-activated protein kinases; NF-kB, nuclear factor-k; $NO_2^{\bullet}$, nitrogen dioxide; ORAC, oxygen radical absorbance capacity; PDGR, platelet-derived growth factor; ROS, reactive oxygen species; $RS^{\bullet}$, thiyl radicals; $RSO_2^{\bullet}$, sulfonyl radical; THF, tetrahydrofuran; TSO, tomato seed oil

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