Journal of Functional Foods 42 (2018) 271–280

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Bioactive compounds of tomato: Cancer chemopreventive effects and influence on the transcriptome in hepatocytes

Inmaculada Navarro-González, Javier García-Alonso, María Jesús Periago*

Department of Food Technology, Food Science and Nutrition, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, Espinardo, 30071 Murcia, Spain
Instituto Murciano de Investigaciones Biomédicas-IMIB, Spain

ARTICLE INFO

Keywords:
HepG2 cells
Phenolic compounds
Lycopene
Tomato
Gene expression
Apoptosis
Cell cycle arrest

ABSTRACT

The mechanisms of the effects of tomato extracts (containing lycopene and phenolic compounds) and these pure bioactive compounds on the viability, apoptosis, cell cycle, and the scavenging of ROS of HepG2 cells were investigated. Simultaneously, a transcriptomic analysis in human hepatocytes from obese patients, after the intake of tomato juice, was carried out using Human Gene Expression Array (Affymetrix). The viability of HepG2 decreased after exposure to tomato extracts and pure compounds, but none showed apoptotic activity; however, lycopene was able to inhibit cell cycle progression in phase S. Tomato extracts and pure compounds were able to decrease the ROS generation, the phenolic compounds being more effective than lycopene. Transcriptomic analysis suggested a involvement in the regulation of the cell cycle and apoptosis pathways after the intake of tomato juice, through the lycopene accumulation. These results showed that tomato juice have potential as functional foods for maintenance of liver health.

1. Introduction

Liver cancer is one of the most common carcinomas, with poor prognosis and a low survival rate since it is a type of malignant tumor that is highly resistant to chemotherapeutic compounds (Rougier, Mitry, Barbare, & Taieb, 2007). Many factors are associated with the hepatocarcinoma (HCC) risk, obesity being one of the most important, as observed in several epidemiological and clinical studies (Schlesinger et al., 2013).

Human studies indicate that the consumption of vegetables and fruits can prevent HCC formation, associated with a low intake of saturated fats and the intake of antioxidant phytochemical compounds, such as phenolic compounds and carotenoids (Montella, Crispo, & Giudice, 2011). Different mechanisms have been described for the prevention of tumor development by phytochemicals, such as regulation of the cell cycle (Chen & Kong, 2005), improvement of the antioxidant status (Chen & Kong, 2005), anti-inflammatory activity (Ye et al., 2004), and reduction of cell proliferation and induction of apoptosis (Feng et al., 2016; Kalra et al., 2007; Kundu, Dey, Roy, Siddiqi, & Bhattacharya, 2005). Plant foods contain a wide range of antioxidant bioactive compounds (polyphenols, carotenoids, vitamin, glucosinolates, etc.), and it has been proposed that their beneficial effects may arise from additive and synergistic actions of the different phytochemicals present in the food matrix (García-Alonso, Navarro-González, Ros, & Periago, 2015). Synergism is, in general, the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced overall effect than that derived from a simple additivity concept (Laguerre, Lecomte, & Villenueve, 2007).

Tomato is one of the most consumed vegetables in the world and its consumption has been associated with the reduction of cardiovascular diseases (Mordente et al., 2011) and cancer (Sharoni et al., 2016). This effect is mainly due to its content of antioxidants of differing chemical nature, providing a wide variety of dietary lipophilic and hydrophilic antioxidants – such as carotenes (lycopene as well as β-carotene), ascorbic acid, tocopherol, and phenolic compounds (chlorogenic acid, caffeic acid, ferulic acid, and naringenin) (García-Valverde, Navarro-González, García-Alonso, & Periago, 2013; Periago et al., 2009). Lycopene is the main carotenoid of tomato, giving raw fruits and tomato products their red color, and shows a series of biological effects, including anti-inflammatory, antimutagenic, and anticarcinogenic activities (Sharoni et al., 2016). After consumption, lycopene accumulates in the liver, modulating the redox balance and lipid metabolism (Bernal et al., 2013; Martín-Pozuelo et al., 2015); hence, it is considered one of the most potent antioxidant compounds with regard to improving liver health (Ferramosca, Di Giacomo, & Zara, 2017; Vitaglione, Morisco,

* Corresponding author at: Departamento de Tecnología de los Alimentos, Nutrición y Bromatología, Facultad de Veterinaria, Campus Universitario de Espinardo, 30071 Murcia, Spain.
E-mail addresses: inmaculada.navarro@um.es (I. Navarro-González), mjperi@um.es (M.J. Periago).

https://doi.org/10.1016/j.jff.2018.01.003
Received 10 October 2017; Received in revised form 2 January 2018; Accepted 3 January 2018

Caporaso, & Fogliano, 2005). The mechanisms underlying the inhibitory/preventive effects of lycopene on carcinogenesis and mutagenesis could involve radical oxygen species (ROS), interference with cell proliferation, induction of gap-junctional communication, inhibition of cell cycle progression, and modulation of signal transduction pathways (Sharoni et al., 2016), showing chemopreventive effects against HCC (Ip, Lui, Ausman, Von Lintiq, & Wang, 2014; Wang, Ausman, Greenberg, Russell, & Wang, 2010). Moreover, the main phenolic compounds of tomatoes are dietary antioxidants, which can also exert chemopreventive effects against different cancers (Pan & Ho, 2008). However, unlike lycopene, either the content of phenolic compounds or their bioavailability is very low; for this reason, their accumulation in the liver is insignificant (Navarro-González, Pérez-Sánchez, Martín-Pozuelo, García-Alonso, & Periago, 2014).

Observational and clinical studies have shown that dietary intake of tomatoes is associated with a reduced risk of human cancers at numerous sites, as reviewed previously (Palomo, Moore-Carrasco, Carrasco, Villalobos, & Guzmán, 2010). This association remains to be explored with liver cancer, although higher intake of tomatoes or tomato-based products has been associated with a lower risk of HCC (Wang et al., 2010). However, with the results obtained from epidemiological studies it is often difficult to conclude whether the observed chemopreventive effects are tomato or lycopene-dependent, or are dependent on the other compounds naturally present in tomato, such as phenolic compounds.

Taking into consideration the beneficial effect of tomatoes associated with the content of different bioactive compounds, the aim of the present study was to determine the effects of tomato extracts (lipophilic, containing lycopene; hydrophilic, with chlorogenic acid, caffeic acid, ferulic acid, and naringenin) and pure compounds on the cell viability, apoptosis, cell cycle, and scavenging of ROS of human hepatoma cells (HepG2). Simultaneously, to ascertain the plausible mechanisms, we have investigated the changes in gene expression of human hepatocytes from liver biopsies of obese patients after the intake of tomato juice.

2. Material and methods

2.1. Preparation and characterization of organic tomato extract

Organic tomato extract was prepared using the method described by García-Alonso et al. (2015), with minor modifications. To obtain the lipophilic tomato (Lt) extract, 30 g of organic tomato juice were extracted in a mixture of 750 mL of hexane and 60 mL of water for 30 min, using a magnetic stirrer. The hexane upper layer was dried in a rotary evaporator and the resulting extract was re-dissolved in 40 mL of DMSO. Similarly, the hydrophilic tomato (Ht) extract was prepared by magnetic stirring of 30 g of organic tomato juice in 750 mL of 70% ethanol, for 30 min. Then, the 70% ethanol was removed using a rotary evaporator and the extract was re-dissolved in 40 mL of distilled water. The contents of lycopene and phenolic compounds in the tomato extracts were analyzed by HPLC as described by Böhm (2001) and García-Valverde et al. (2013), respectively. The compounds detected in the Ht extract were chlorogenic acid (13 μM), caffeic acid (5 μM), ferulic acid (2 μM), and naringenin (4.5 μM), so that the total concentration of phenolic compounds in the Ht extract was 25 μM (the sum of the individual compounds). Lycopene was only detected in the Lt extract, at a concentration of 46 μM, whilst no phenolic compounds were detected in the Lt extract.

2.2. Preparation of stock solutions of pure bioactive compounds of tomato

In order to resemble the composition of the lipo- and hydrophilic tomato extracts, stock solutions of pure lycopene (Lp) and pure phenolic compounds (Cp) were prepared. The Cp stock solution was prepared in DMSO by mixing 130 μM chlorogenic acid, 50 μM caffeic acid, 20 μM ferulic acid, and 45 μM naringenin. Similarly, the Lp stock solution contained 1 mM lycopene in DMSO. Lycopene, chlorogenic acid, caffeic acid, ferulic acid, and naringenin were purchased from Sigma (Madrid, Spain).

2.3. Cell lines and cultures

The HepG2 cell line was derived from a liver hepatocellular carcinoma and was acquired from the American Type Tissue Culture Collection (ATCC, USA). This cell line was cultured in EMEM (Eagle's Minimum Essential Medium), supplemented with fetal bovine serum (10%), non-essential amino acids (1%), 2 mM glutamine, penicillin (100 $U{\cdot}mL^{-1}$), and streptomycin (100 $\mu g{\cdot}mL^{-1}$). The HepG2 cells were subcultured twice a week and seeded in a new 75-cm^2 flask at a dilution of 1:4, at 37 °C, in a humidified atmosphere of 5% CO_2. Before and after the experiments, the HepG2 cell line was tested for Mycoplasma using the MycoSEQ™ *Mycoplasma* Real-Time PCR Detection Kit (Applied Biosystems, Foster City, USA). For the assays, tomato extracts or stock solutions, as well as their combinations, were diluted in the cell medium to reach the desired test concentrations. The final DMSO concentration in the culture medium never exceeded 1% (v/v), and control cultures were treated with the same amount of solvent.

2.4. Cell viability

Cytotoxicity was determined using the MTT (methylthiazoletetrazolium) colorimetric assay. HepG2 cells, in 200 μL of medium, were seeded at 20,000 cells/well in a 96-well plate. After 24 h, the medium was replaced with medium containing the tomato extracts or pure bioactive compounds at different concentrations, alone or in combination, to evaluate possible synergistic effects. The cells were incubated for 6 and 24 h at 37 °C in a humidified atmosphere of 5% CO_2. After treatment (6 and 24 h), the medium was removed and the cells were carefully washed, twice, with phosphate-buffered saline (PBS) to eliminate the bioactive compounds. Serum-free medium containing 0.2 mg/mL MTT was added to each well and incubation took place for 4 h at 37 °C. After that, the incubation solution was removed and the water-insoluble formazan was dissolved in DMSO, measuring the absorbance at 550 nm with a FLUOstar Omega microplate reader (BMG labtech, Offenburg, Germany). The percentage of viable cells was expressed as a percentage compared with the control.

2.5. Apoptosis assays

For the apoptosis assays, 1×10^5 cells were typically seeded in a 6-well plate. Hepatocarcinoma cancer cells were exposed to Lt, Ht, Lp, Pp, and their combinations. After that, the cells were collected and washed twice with PBS as described above (no PBS/ethanol mix was used in this case). After removing the PBS, 40 μL of a solution containing Annexin V (Annexin-V-Fluos from Roche) and propidium iodide (PI) and 160 μL of incubation buffer (10 mM Hepes, 140 mM NaCl, 5 mM $CaCl_2$, pH = 7.4) were added to the cell pellet. The cells were resuspended in this solution and left, in the dark, at room temperature for 15 min. Two hundred microliters of PBS were added prior to the measurements in a Becton-Dickinson FACScalibur flow cytometer, registering the emission at wavelengths of λ = 620 and 525 nm for PI and Annexin V, respectively. In each case, 10,000 events were acquired. Staurosporine was used as a positive control.

2.6. Mitochondrial membrane potential assay

Briefly, cells were seeded at 1×10^5 cells per well in 6-well plates and left to incubate overnight. The Ht and Lt extracts were added at different concentrations and the cells were left to incubate for 24 h. The cells were collected, washed twice with PBS, and then 1 μL of a solution of Rho123 (1 μM, Sigma Aldrich) was added. Samples were

immediately analyzed on a Becton-Dickinson FACScalibur flow cytometer.

2.7. Detection of intracellular ROS

The intracellular level of ROS in the HepG2 cells was monitored with H_2DCFDA. In this method, the probe DCFHDA is taken up by cells and deacetylated to DCFH by intracellular esterases. Free radicals generated from tBOOH lead to the oxidation of DCFH to fluorescent DCF, and the level of fluorescence measured upon excitation is proportional to the level of oxidation. Antioxidant compounds present in the samples quench free radicals and inhibit the generation of DCF. HepG2 cells were seeded at a cell density of 62,500 cells/cm^2 and incubated at 37 °C in their optimal growth conditions for 24 h. After this, the cells were incubated with DCFHDA for 15 min at 37 °C. Then, two different methodologies were followed: (1) cells were washed and incubated with the tomato extracts and pure compounds for 10 min and then 250 μM tBOOH was added, and (2) cells were washed and incubated with 250 μM tBOOH for 30 min to induce oxidation. After this, the tomato extracts and pure compounds were added prior to a 10-min incubation. The intensity of the fluorescence was registered with an excitation and emission wavelength at 482 nm and 520 nm, respectively, using a FLUOstar galaxy microplate reader (BMG Labtech, Offenburg, Germany).

2.8. Cell cycle analysis

For the study of cell cycle arrest, 1×10^5 cells were seeded in 6-well plates and left at 37 °C in a 95% H^a, 5% CO_2 atmosphere for 24 h. After that, the corresponding concentrations of Ht and Lt extracts, their combination, alone or combined with pure compounds, were added to the wells, using one as a control (without tomato extract or pure compounds) and other with 1% DMSO. The cells were left with the extracts and pure compounds for 24 h and then removed from the wells with trypsin, collected, and centrifuged (200*g*, 10 min). The supernatant was discarded and the pellets were washed with EMEM medium and centrifuged again under the same conditions. The supernatant was discarded and the cells treated with 70% ethanol in PBS for 45 min at 4 °C. After that, the ethanol was removed by centrifugation and the cells were resuspended in PBS, adding RNase solution and PI (at final concentrations of 0.1 mg/mL and 40 mg/mL, respectively). The cells were incubated in this medium for 30–60 min. The PI fluorescence was measured for each cell in a Becton-Dickinson FACScalibur flow cytometer. In each case, 30,000 events were acquired. The data were processed with the software ModFit LT, Version 4.1.7 (Verity Software House).

2.9. In vivo experiment, study design

Thirty-six obese patients (12 men and 24 women), who presented the indications for bariatric surgery, were operated on at "Santa Lucía" University Hospital of Cartagena (Murcia, Spain). All patients showed the inclusion criterion: body mass index (BMI) greater than 40 kg/m^2 or greater than 35 kg/m^2 with at least one comorbidity (type 2 diabetes, dyslipemia, hypertension, apnoea, or weight-induced rheumatological disease). All patients signed the informed consent form after being informed appropriately about the study protocol and surgery. The local Ethical Committee approved the experimental protocol and the study adhered to the "Tenets of the Declaration of Helsinki".

In general, the plasmatic analytical parameters of the patients measured before surgery were not diagnostic of steatosis or steatohepatitis, nor of diabetes, for any patient. The patients were classified in two groups: a control group with 19 subjects and an experimental group with 16 subjects, who drank a 200-mL bottle of commercially available tomato juice every day during the month prior to the surgical intervention. For all participants, the bariatric surgery was programmed according to the medical criteria and a sample of liver, of each patient, was taken with a biopsy needle kit. Samples were taken for carrying out this study, since lycopene is accumulated in this organ. The total RNA was extracted and isolated from each liver sample, using the RNeasy Lipid Tissue Mini Kit (Quiagen, Hilden, Germany). Once validated the quantity and quality of the RNA, 100 ng of RNA were processed to study the transcriptome using a Microarray- GeneChipPrimeView Human Gene Expression Array PN/901837 (Affymetrix Incorporated, Santa Clara, CA, USA). The biotin-labeled target synthesis reactions, as well as the arrays hybridization, staining, and scanning, were performed in compliance with MIAME guidelines.

2.10. Statistical analysis

All experiments with cells were carried out at least three times. The results for the different parameters analyzed were expressed as mean ± standard deviation (SD). To compare independent groups of numerical data, the ANOVA test was used in the *in vitro* study, using superscript letters to indicate significant differences among the means for the different assays. All statistical analyses were performed using the SPSS 19.0 package, with a significance level of $p \leq .05$. For the *in vivo* study, analysis of gene expression data was performed using Partek Genomics Suite 6.6 and Partek Pathway (Partek Incorporated, St. Louis, MO, USA) programme. Differentially expressed genes (DEGs) were identified using the paired Student's *T*-test ($p < .05$) and adjusted using the Benjamini-Hochberg false discovery rate (FDR) with 5% of false discovery. A *p-value* < .05 and a cutoff of 1.5-fold change were taken into account for the selection of DEGs. The Go Enrichment analysis of DEGs was applied (GSEA) to identify the groups of genes that share a common biological function, and analysis of the pathways was carried out with a KEGG data-base.

3. Results

3.1. Effect of bioactive compounds of tomato on viability in HepG2 cells

The tomato extracts diminished the HepG2 cell viability as a function of the time of treatment (Fig. 1), with a reduction of approximately 50% when the HepG2 cells were treated with different concentrations of the Lt extract for 24 h. By contrast, no effect on the HepG2 cells viability was observed after their incubation with the Ht extract for 6 h. The Lt extract showed greater inhibitory activity than the Ht extract, but, when the cells were incubated with both extracts simultaneously (Lt + Ht) the cell viability decreased more than when the extracts were assayed separately, showing a non-concentration-dependent combined effect.

Fig. 2 illustrates the effect of the pure compounds and their combinations on cell viability. In contrast to the tomato extracts, pure lycopene (Lp) did not significantly affect the viability of the HepG2 cells, and only small reduction was achieved with the concentration ≥ 5 μM (Fig. 2A). However, the pure phenolic compounds (Pp) significantly reduced cell viability in a dose- and time-dependent manner, reaching a decrease higher than 80% at 6 and 24 h of exposure (Fig. 2B). As shown in Fig. 2C, the combined effect of the pure compounds was weaker than those of the tomato extracts, and only a slight additive effect on cell viability was observed at 24 h under some conditions. Therefore, taking into consideration, these cell viability results and the physiological concentrations that the bioactive compounds reach in the human body, the test concentrations indicated in Table 1 were used for the remaining experiments, using tomato extracts and pure bioactive compounds.

3.2. Apoptotic and mitochondrial membrane potential assay

Fig. 3A shows the percentages of living, apoptotic, and necrotic HepG2 cells after incubation with tomato extracts, pure compounds, or their combinations, whereas Fig. 3B shows the differences in the

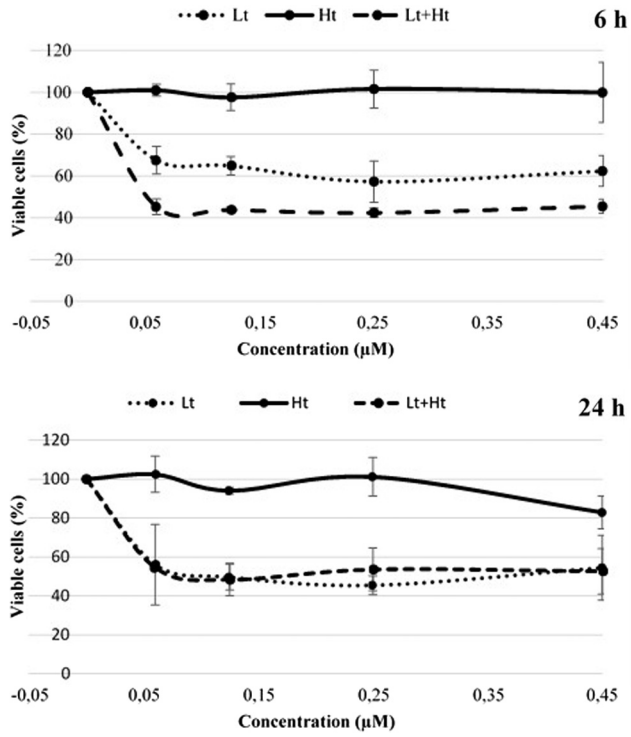


Fig. 1. Effect of extracts of tomato on cell lines viability after 6 h and 24 h exposure using MTT assays. Lt (lipophilic tomato extract), Ht (hydrophilic tomato extract), and Lt + Ht (both extracts combined). All analyses were performed in triplicate. Data are expressed as means ± SD.

percentages of living and apoptotic cells according to the assayed compounds. In general, cells exposed to tomato extracts showed a high proportion of living cells and a low proportion of apoptotic cells, whereas pure compounds induced HepG2 cell apoptosis (Fig. 3 A-B) almost at the same intensity as staurosporine. It is of note that the tomato extracts, alone or combined, did not show apoptotic activity; there was a high percentage of live cells after application of Lt, Ht, or their mixture (Lt + Ht) for 24 h. In addition, a low number of necrotic cells were detected in each assayed treatment (Fig. 3A). Rhodamine 123 staining was applied to test the changes in the mitochondrial membrane potential of HepG2 cells, providing information on a plausible mechanism by which the tomato extracts or pure compounds induced apoptotic cell death. There was a reduction in the mitochondrial membrane potential of between 50% and 56% in the HepG2 cells, for all assay conditions, compared with the control group, with significantly lower values in the assays with pure compounds (data not shown).

3.3. ROS

Both Ht and Pp reduced the generation of ROS: by between 20% and 40% when coincubation was performed (Fig. 4A), and between 50% and 60% when applied as a pre-treatment to reverse the oxidation provoked by tBOOH (Fig. 4B). However, lycopene – either pure (Lp) or from tomato (Lt) – showed lower effectiveness in the inhibition/prevention of ROS generation than the pure phenolic compounds. In addition, no synergistic effect was observed in the combination of Lt + Ht extracts and Lp + Pp (Fig. 4B).

3.4. Cell cycle analysis

To elucidate the mechanisms responsible for the reduction of cell viability after the exposure to different compounds, we examined the effect of the bioactive compounds in tomato on HepG2 cell cycle progression (Fig. 5). Treatment with Lp led to the highest decrease of the proportion of cells in the G2/M phase, without significant differences with the application of tomato extracts, alone or combined. In addition, the pure phenolic compounds did not affect the cell cycle progression (Fig. 5), compared with the control cells.

3.5. Transcriptomic analysis with obese patients

To determine the changes in hepatic gene expression induced by the intake of tomato juice, the expression of transcripts represented on the Affymetrix GeneChip PrimeView Human Gene Expression Array PN/901837 was analyzed. To detect changes in gene expression a the Student's *T*-test was applied. Fig. S1 (supplementary material) depicts all the genes analyzed, showing in red the down-regulated genes and in green the up-regulated genes, whereas the grey shadow indicates the 16,412 DEGs for $p < .05$. The DEGs were reduced to 10,334 after applying the FDR < 5%, and to 910 when the two following criteria were applied: fold change ≥ or ≤ 1.5 and $p < .05$ (715 were up-regulated and 195 down-regulated). The GSEA illustrates one of the strengths of this analysis, as it was able to indicate a strong involvement of genes related to cellular process, more concretely in the negative regulation of the cell cycle (GO: 0045786 $p \leq 7.7.10–5$, Fig. 6). In addition, the following pathways related to cellular processes were selected according to the objective of this research: "Apoptosis" and "Cell cycle" (Figs. S2 and S3, supplementary material), the up-regulated genes being shown in red and the down-regulated genes in green. To obtain the figures of both pathways (Figs. S2 and S3, supplementary material), the DEGs with a fold change > or < 1 were selected.

With respect to apoptosis pathway, 116 genes showed a significant differential expression, 41 being up-regulated and 75 down-regulated, but only the most relevant, biologically, are discussed below (Fig. 7). The microarray data reveal the activation of tumor suppressor p53 (TP53), which causes cell cycle arrest or apoptosis, in the obese patients that drank tomato juice. There was significant up-regulation of caspases 8 and 10 and anti-apoptotic protein (Bcl-2, Bcl-xL), and significant down-regulation of caspases 3 and 7 and pro-apoptotic protein (Bak). There were also changes in the expression of other genes that may be important in the regulation of apoptosis – such as Bid, Noxa1, TP53AIP1, and TRAILR2, which were down-regulated. With respect to the cell cycle pathway, 64 genes were up-regulated and 83 genes down-regulated. Of particular interest are the changes in the expression of cyclin-dependent kinase (Cdks)/Cyclin complexes. The expression of kinase 4, 6/Cyclin D1 was significantly elevated, while kinases Cdk2/Cyclin E, Cdk2/Cyclin A, Cdk7/Cyclin H, and Cdk1/Cyclin A were down-regulated.

4. Discussion

Cancer chemopreventive effects elicited by natural dietary compounds are due, at least in part, to the inhibition of cell growth signaling pathways, culminating in apoptosis and/or cycle arrest (Pan & Ho, 2008). Specifically, bioactive compounds of tomato (lycopene and phenolic compounds) have been associated with the prevention of different types of cancer, showing different mechanisms of action - including inhibition of cell viability and induction of cell death by activation of apoptosis (Sharoni et al., 2016). Although the MTT assay is often erroneously described as measuring cell proliferation, it is suitable for ascertaining cell viability, since it measures changes in the mitochondrial activity of cells. However, reducing compounds are known to interfere with this assay; for example, antioxidant vitamins and phenolic compounds can reduce tetrazolium salts and lead to increased absorbance values in the assay well (Riss et al., 2017). In this study, to avoid the interference with the reducing substances of the tomato extracts or with the pure compounds, after incubation the cells were washed twice with PBS to remove the free compounds in the assay well; the MTT reagent was then added. The cell viability study shows

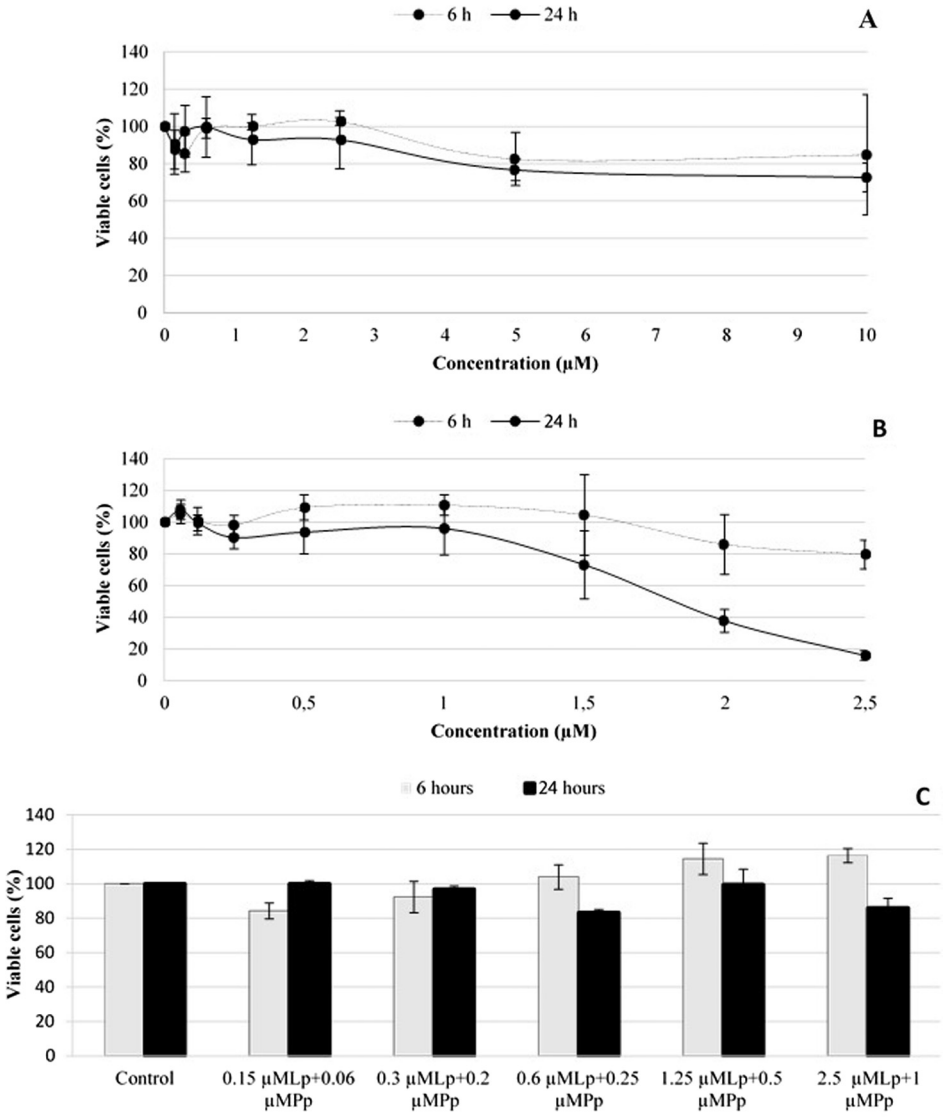


Fig. 2. Effect of pure lycopene (A), pure phenolic compounds (B), and their combination (C) on cell viability after 6 h and 24 h of exposure using MTT assays. All analyses were performed in triplicate. Data are expressed as means ± SD. Lp (Lycopene); Pp (Phenolic compounds).

that the Lt extract, containing lycopene, gave a greater reduction of cell viability than Ht, containing phenolic compounds. The lycopene concentrations used in these experiments were based on the physiological plasma concentrations of this carotenoid reported for European citizens by Jenab et al. (2005) and Stuetz et al. (2016). We observed very few changes in cellular viability when HepG2 cells were exposed to low concentrations of pure lycopene, concentrations ≥5 µM being more effective, similar to the results described by other authors (Arathi et al., 2016; Burgess et al., 2008). Other researchers have described an antiproliferative activity of lycopene, for higher lycopene concentrations (5 µM, 10 µM, and even higher) that cannot be achieved in human blood or tissues (Kim et al., 2015; Rafi, Kanakasabai, Reyes, & Bright, 2013). In relation to phenolic compounds, only the mix of pure compounds (chlorogenic, ferulic, and caffeic acids and naringenin), at concentrations higher than 1.5 µM, reduced significantly the growth of HepG2 cells. Interestingly, the effect on cell viability was greater when the two extracts – Ht and Lt – were combined, which indicates that the tomato extract led to cell death, reducing the viability by around 50%. This effect could be explained by mitochondrial damage, since the MMP is a sensitive indicator of mitochondrial function injury and the MTT

Table 1
Test concentrations (µM) of tomato extracts, pure compounds, and their combinations used in apoptosis, mitochondrial membrane potential assay, detection of intracellular ROS, and cell cycle experiments.

	Tomato extract			Pure compounds		
Compound	Lipophilic (Lt)	Hydrophilic (Ht)	Combined extract (Lt + Ht)	Lycopene (Lp)	Phenolic compounds (Pp)	Combined compounds (Lp + Pp)
Lycopene	0.22	–	0.22	0.22	–	0.22
Total phenolics	–	0.24	0.24	–	0.24	0.24
Chlorogenic acid	–	0.13	0.13	–	0.13	0.13
Caffeic acid	–	0.05	0.05	–	0.05	0.05
Ferulic acid	–	0.02	0.02	–	0.02	0.02
Naringenin	–	0.04	0.04	–	0.04	0.04

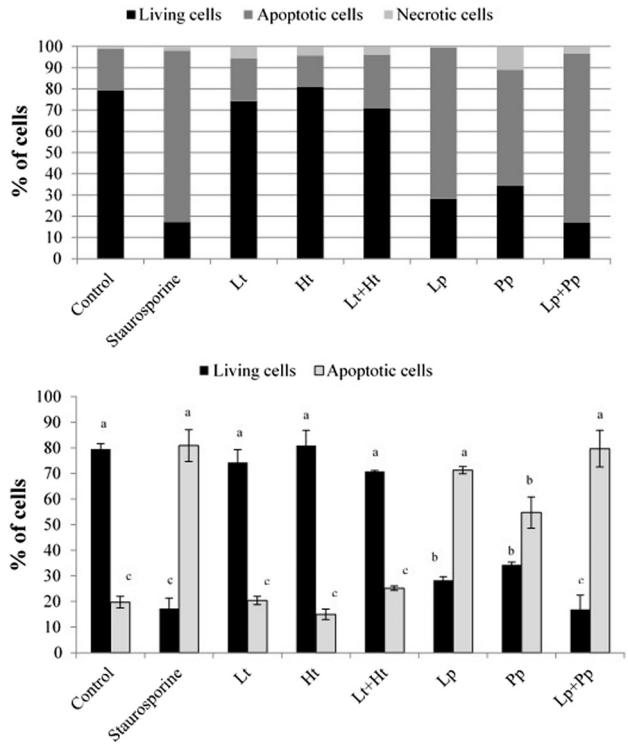


Fig. 3. Percentages of living, apoptotic, and necrotic cells after incubation with tomato extracts, pure compounds, and their combinations (A). Apoptotic effect of tomato extracts, pure compounds, and their combinations (B). Lipophilic tomato extract (Lt), hydrophilic tomato extract (Ht), combined tomato extracts (Lt + Ht), pure lycopene (Lp), pure phenolic compounds (Pp), and combination of pure compounds (Lp + Pp). Columns of living and apoptotic cells, bearing different superscript letters, indicate statistical significance ($p < .05$). All analyses were performed in triplicate. Data are expressed as means ± SD.

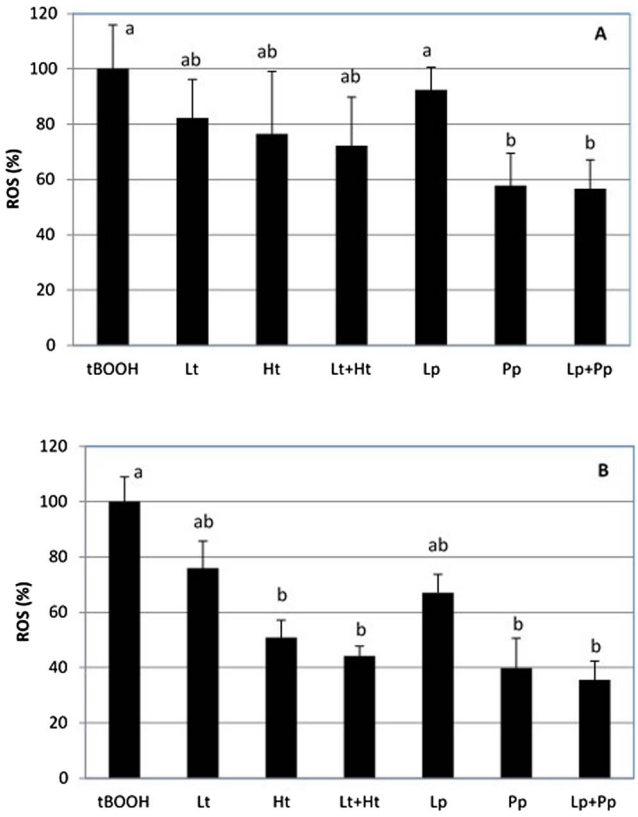


Fig. 4. Effect of pre-incubation (A) or co-incubation (B) of cells with lipophilic (Lt) and hydrophilic (Ht) tomato extracts, pure lycopene (Lp), pure phenolic compounds (Pp), and their combinations on tBOOH-induced intracellular ROS generation. Columns with different superscript letters indicate statistical significance ($p < .05$). All analyses were performed in triplicate. Data are expressed as means ± SD.

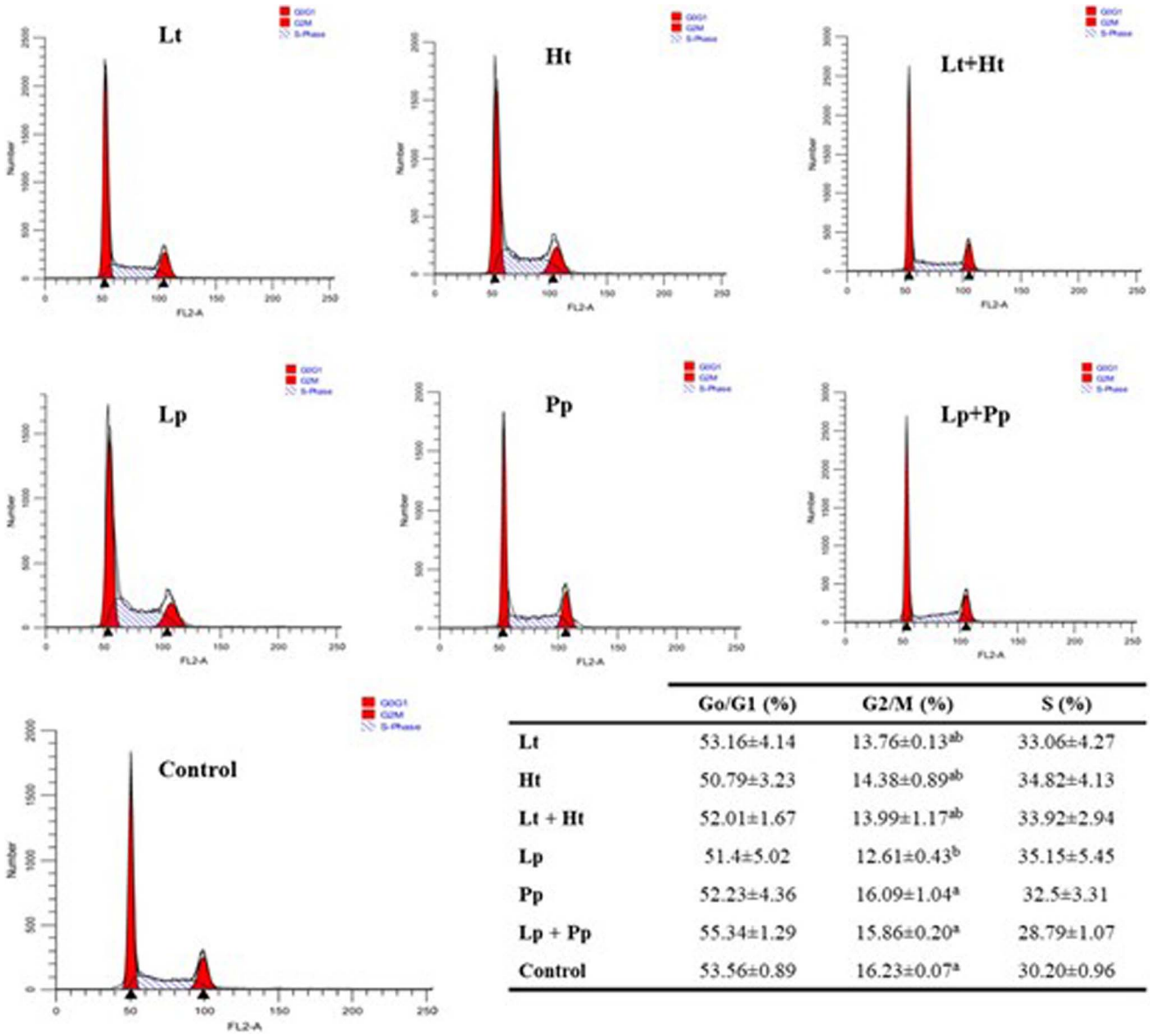
data agree with those obtained for MMP. In general, the inhibition on cell viability was greater with the food extracts than with pure compounds, showing the functional effect of tomatoes and tomato-based products.

To investigate the mechanisms, pro-apoptotic properties was assessed by flow cytometry, quantifying the apoptotic, live, and necrotic cells. However, the loss of cell viability in HepG2 cells treated with tomato extracts was not due to apoptosis, because only pure lycopene and its mixture with pure phenolic compounds induced apoptosis, in comparison with staurosporine. Rao (2006) described that the inhibitory effects of lycopene on mammary and prostate cancer cell growth were not accompanied by apoptotic or necrotic death – which commonly occurs in response to drugs, but not to dietary micronutrients or components. In this study, a decrease in the MMP and the percentage viability was only accompanied by apoptosis for pure lycopene. For this reason, we consider that pure lycopene, alone or in combination with pure phenolic compounds, can cause apoptosis.

The balance between the production and the clearance of ROS is very important to maintain normal biochemical functions; however the unbalance can induce oxidative damage to lipids, proteins, and DNA, activate the intrinsic apoptosis pathway due to mitochondrial dysfunction, and finally lead to apoptosis (Brunelle & Chandel, 2002). Generally, cells are capable of producing ROS immediately upon exogenous tBOOH, which can be scavenged by antioxidant molecules. The combination of both tomato extracts (Lt and Ht) was more effective in the prevention of ROS generation than each one alone, showing a slight additive effect. It is known that, in multiphase media, the interaction of antioxidants with different polarities, which are thus distributed in different phases or solvents, could induce synergy. In line with this, the additive effects observed in cell-based assays could rely on the presence of a multiphase medium (cell membrane and extra- and intracellular media) that might facilitate the distribution and interaction of lycopene and phenolics in the prevention of oxidation by means of different antioxidant mechanisms (e.g. radical scavenging, metal chelation) (García-Alonso et al., 2015). Tomato extracts reduce ROS generation, which prevents the lipid oxidation of cells and reduces mitochondrial injury, a plausible mechanism for the prevention of cancer in hepatic cells (Wang et al., 2010; Zhong et al., 2017). So, the peroxidation of cardiolipin (a mitochondria-specific inner membrane phospholipid) occurred in HCC progression; therefore, modulation of cardiolipin oxidation may have potential therapeutic use in cancer prevention and treatment (Zhong et al., 2017).

To further explore the mechanism by which the tomato bioactive compounds inhibited the cell growth, we investigated their effects on the cell cycle distribution. The addition of tomato extracts or pure bioactive compounds to HepG2 cells resulted in a significant decrease in the number of cells in the G2/M phase, especially after addition of pure lycopene. This effect was not significant for the tomato extracts or pure phenolic compounds, suggesting that lycopene can inhibit the cell cycle progression in phase S. Teodoro et al. (2012) reported that lycopene promoted cell cycle arrest, followed by decreased cell viability, in different cell lines. These observations suggest that lycopene may alter cell cycle-regulatory proteins and lead to apoptosis.

Although the human hepatoma HepG2 cell line has been widely used as an experimental model to ascertain the effect of bioactive compounds on hepatocytes, the aim of our *in vivo* approach was to determine the effect of tomato intake on the target genes associated with the mechanism of the cancer chemopreventive effects of tomato compounds. Taking into consideration that obesity has been considered one of the major risk factors for HCC, our study of gene expression, in



	Go/G1 (%)	G2/M (%)	S (%)
Lt	53.16±4.14	13.76±0.13^{ab}	33.06±4.27
Ht	50.79±3.23	14.38±0.89^{ab}	34.82±4.13
Lt + Ht	52.01±1.67	13.99±1.17^{ab}	33.92±2.94
Lp	51.4±5.02	12.61±0.43^{b}	35.15±5.45
Pp	52.23±4.36	16.09±1.04^{a}	32.5±3.31
Lp + Pp	55.34±1.29	15.86±0.20^{a}	28.79±1.07
Control	53.56±0.89	16.23±0.07^{a}	30.20±0.96

Fig. 5. Effect of tomato extracts, pure compounds, and their combinations on cell cycle progression in HepG2 after 24 h exposure: lipophilic tomato extract (Lt), hydrophilic tomato extract (Ht), combined tomato extracts (Lt + Ht), pure lycopene (Lp), pure phenolic compounds (Pp), and combination of pure compounds (Lp + Pp). Different letters, in the same column of the table, indicate statistical significance ($p < .05$). All analyses were performed in triplicate. Data are expressed as means ± SD.

liver, was performed with the purpose of identifying the DEGs associated with apoptosis or the cell cycle pattern, when comparing obese patients with obese patients who consume tomato juice. Although we did not analyze the content of lycopene in plasma, the drinking of a daily bottle of tomato juice for 30 days allowed the assessment of an intake of more than 20 mg/day. The consumption of tomato juice increases significantly the plasmatic level of lycopene in humans (Colmán-Martínez, Martínez-Huélamo, Miralles, Estruch, & Lamuela-Raventós, 2016; Jacob, Periago, Böhm, & Berruezo, 2008), plasma lycopene being a robust biomarker of tomato intake. In a previous study, we showed that the intake of two doses of 250 mL of the same tomato juice, for a period of two weeks, was sufficient to increase significantly, by more than 150%, the plasma lycopene concentrations (Jacob et al., 2008). Therefore, for the current study, an increase of lycopene in plasma and its parallel accumulation in the liver were expected, as described in different animal studies (Martín-Pozuelo et al., 2015), suggesting that lycopene-rich foods could be proposed in therapeutic treatment of some liver pathologies (Vitaglione et al., 2005). Moreover, we have to point out that, although the phenolic compounds decreased the cell viability, induced apoptosis, and reduced significantly the ROS generation in HepG2 cells, their role in the *in vivo* study was limited by their low content in the tomato juice (chlorogenic acid 21.9 mg/kg, rutin 21.2 mg/kg, and naringenin 6.4 mg/kg being the main compounds), their low bioavailability, and the fact that they are quickly metabolized (Navarro-González et al., 2014). The DEGs revealed the activation of tumor suppressor p53 (TP53) in obese patients after the intake of tomato juice, which regulates cellular responses as apoptosis, cell cycle arrest and tumor suppression and could be triggered when cells are under stress to induce apoptosis or cell cycle arrest (Fischer, Quaas, Steiner, & Engeland, 2016; Lin et al., 2016). Most of the changes observed in cell apoptosis are caused by activation of the family of caspases, which are divided in two groups: initiator caspases (caspase-8, caspase-9, and caspase-10) and effector caspases (caspase-3, caspase-6, and caspase-7). Initiator caspases activate effector caspases (Kondratskyi, Kondratska, Skryma, & Prevarskaya, 2015). The dietary supplementation of tomato juice up-regulated the expression of initiator caspases (8 and 10) but led to a down-expression of effector caspases (3 and 7). Sayer (2011) reported that, in some cell lines, levels

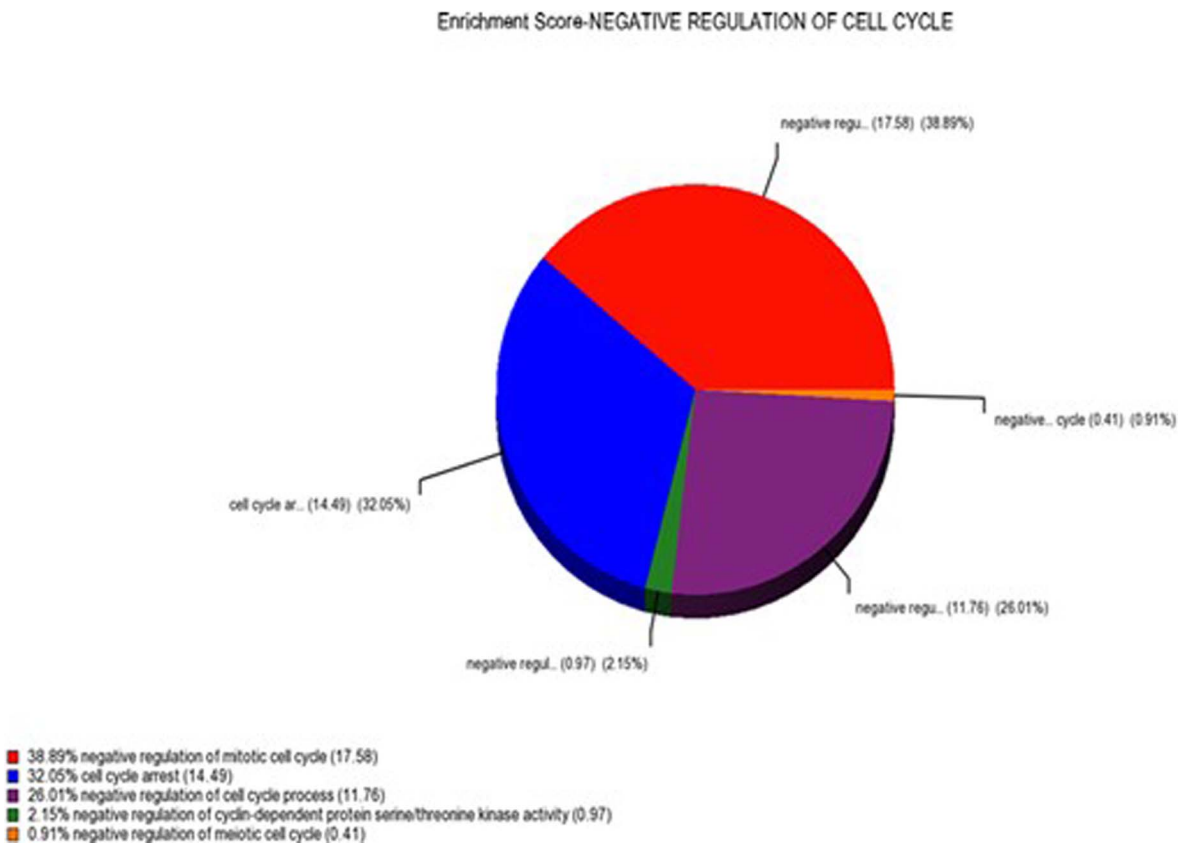


Fig. 6. Enrichment score of the DEGs related to the negative regulation of the cell cycle. A high value for each sector indicates that the functional group is over-represented in the gene-list, compared to the genes in the ontology database.

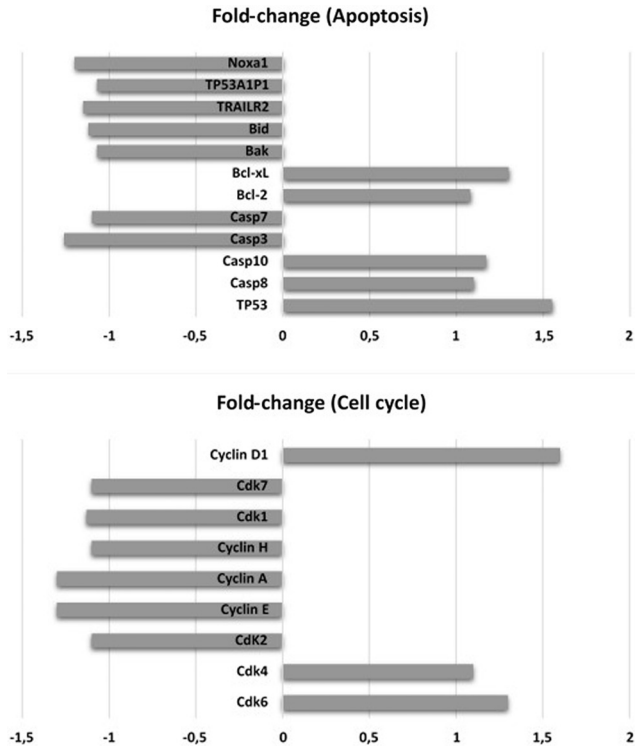


Fig. 7. Genes involved in the apoptosis and cell cycle pathways with a significant fold change (up or down 1.0, $p < .05$) in human hepatocytes, analyzed with the Affymetrix GeneChip prime View Human Gene Expression Array.

of caspase-8 activation are insufficient to directly promote apoptosis, with other proteins being implicated - such as the Bcl-2 family, including pro-apoptotic (Bax, Bak) and anti-apoptotic (Bcl-2, Bcl-xL) members (Sorrentino, Comel, Mantovani, & Sal, 2014). We observed an increase in the expression of mRNA for anti-apoptotic protein (Bcl-2, Bcl-xL) and down-regulation for pro-apoptotic protein (Bak). These results are in disagreement with those reported by Talvas et al. (2010), who did not observe changes in the expression of these genes in prostatic cells after exposure to the serum of humans obtained after supplementation of the diet with yellow and red tomatoes and a capsule of lycopene (16 mg/day during 1 week). However, up-regulation of the Bak:Bcl-2 ratio was observed after the supplementation of tomatoes, but it was unaffected by pure lycopene. In addition, other pro-apoptotic genes of the TP53 signaling pathway – such as Bid, NOXA, P53AIP1, and TRAILR2 – were down-regulated, suggesting that bioactive compounds of tomatoes do not inhibit cell growth by affecting apoptosis, as mentioned above for the *in vitro* assay. TP53 was up-regulated and acts as an important tumor suppressor gene, which regulates cancer cell progression through multiple mechanisms, including cell cycle arrest (Fischer et al., 2016; Lin et al., 2016). Cyclin-dependent kinase (Cdks)/cyclin complexes are the first proteins implicated in cell cycle control. The Cdks are perceived as the engine that drives cell cycle progression, whereas cyclins are considered the gears that are changed to aid the transition between cycle phases. Transcriptomic analysis showed the up-regulation of Cdk 4 and 6/cyclin D1, and the down-regulation of checkpoint kinases Cdk2/cyclin E, Cdk2/cyclin A, Cdk7/cyclin H, and Cdk1/cyclin A. Hence, the Cdk-cycline complex of the G1 phase was over-expressed and the expression of the complexes involved in the G1/S and S phases was decreased. Cyclin D1 is involved in the G1 phase of the cell cycle and regulates transcriptional pathways involved in the metabolism of carbohydrates, lipids, amino acids, and other substrates. Together with Cdk4 and Cdk6, it allows the progression of the cell cycle

through this phase (Mullany et al., 2008). Cdk2 exerts control of the G1/S phase of the cell cycle, in a complex with cyclin E and cyclin A, whereas cyclin A intervenes in the control of the S phase of the cell cycle in a complex with Cdk2 (Lim & Kaldis, 2013). These results are in concordance with those obtained in the *in vitro* assay with HepG2 cells, where there was a decrease in the proportion of cells in the G2/M phase – which could be due to the down-regulation of cyclins and cyclin-dependent kinases implicated in the transition between the G1/S and S phases. Previous reports also evidence that lycopene or lycopene extracts induce a G1/S cell cycle arrest (Palozza et al., 2010) and this has been corroborated by the down-regulation of cyclin in different cell lines, including Hep3B cell (Park, Hwang, & Moon, 2005).

The microarray data for the target genes are in concordance with those obtained in cell culture assays with HepG2, suggesting that tomato juice does not promote apoptosis, but does cause cell cycle arrest, probably through modulation of the tumour protein TP53. Talvas et al. (2010), in prostatic cells, observed that tomato extracts had a greater effect, as modulators of the genes associated with the apoptosis of cell cycle arrest, than lycopene alone, since other nutrients can also act synergistically. Our study have demonstrated that the combination of both tomato extracts did not cause apoptosis in hepatocytes, but reduced cell viability, preventing the ROS generation against the induced oxidation. This effect is greater in mixtures (extracts and pure compounds) than when evaluated independently, suggesting that mixtures of pure compounds exert anti-apoptotic effects due to the antioxidant effects. Therefore, tomato consumption may be preferable to the intake of pure compounds – lycopene or phenolic compounds – since the food matrix provides other compounds that can act synergistically in the prevention of cancer. These results are in agreements with those reported by Wang et al. (2010), who showed that tomato extract supplementation was more protective against HCC than lycopene, due to the synergistic effect of the different phytochemicals.

5. Conclusion

The results of this study show that bioactive compounds of tomato inhibit cell viability of HepG2 cells, through a decrease in ROS and the regulation of cell cycle progression. These findings suggest that the intake of tomato juice and other tomato-based products can be considered in the dietary recommendations for the maintenance of liver health. The bioactive compounds of tomato can prevent oxidative stress in hepatocytes and modulate the transcriptome response of genes related to apoptosis and regulation of the cell cycle, particularly through over-expression of the tumor-suppressor protein (TP53). Further studies are being carried out to verify the effect of the consumption of tomato juice on the transcriptome and its relationship with several disease pathways, to understand further the beneficial effects of its bioactive compounds on human health.

Acknowledgments

This research was supported by the project of “Secretaría de Estado de I+D+i, Ministerio de Econonomía y Competitividad” (Spanish Government) MINECO/FEDER-EU BFU2012-38103. We thank the General Surgery Service of Hospital “Santa Lucía” (Cartagena, Murcia, Spain) for the collaboration in the human intervention study, for the recruitment of patients, and for performing liver biopsies. We also thank Dra. M.J. López Andreo (Research General Service of Murcia University) for her assistance in the analysis of microarrays and Zumos Hesperia Filab (Cabezo de Torres, Murcia, Spain) for providing the tomato juice samples.Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2018.01.003.

References

Arathi, B. P., Sowmya, P. R. P., Kuriakose, G. C., Vijay, K., Baskaran, V., Jayabaskaran, C., & Lakshminarayana, R. (2016). Enhanced cytotoxic and apoptosis inducing activity of lycopene oxidation products in different cancer cell lines. *Food and Chemical Toxicology, 97*, 265–276. http://dx.doi.org/10.1016/j.fct.2016.09.016.

Bernal, C., Martín-Pozuelo, G., Lozano, A. B., Sevilla, A., García-Alonso, J., Cánovas, M., & Periago, M. J. (2013). Lipid biomarkers and metabolic effects of lycopene from tomato juice on liver of rats with induced hepatic steatosis. *The Journal of Nutritional Biochemistry, 24*(11), 1870–1881. http://dx.doi.org/10.1016/j.jnutbio.2013.05.003.

Böhm, V. (2001). Use of column temperature to optimize carotenoid isomer separation by C30 high performance liquid chromatography. *Journal of Separation Science, 24*, 955–959. http://dx.doi.org/10.1002/1615-9314(20011201)24:12<955::AID-JSSC955>3.0.CO;2-B.

Brunelle, J. K., & Chandel, N. S. (2002). Oxygen deprivation induced cell death: An update. *Apoptosis, 7*, 475–482. http://dx.doi.org/10.1023/A:1020668923852.

Burgess, L. C., Rice, E., Fischer, R., Seekins, J. R., Burgess, T. P., Sticka, S. J., & Klatt, K. (2008). Lycopene has limited effect on cell proliferation in only two of seven human cell lines (both cancerous and noncancerous) in an in vitro system with doses across the physiological range. *Toxicology in vitro, 22*, 1297–1300. http://dx.doi.org/10.1016/j.tiv.2008.03.001.

Chen, C., & Kong, A. N. (2005). Dietary cancer-chemopreventive compounds: From signaling and gene expression to pharmacological effects. *Trends in Pharmacological Science, 26*, 318–326. http://dx.doi.org/10.1016/j.tips.2005.04.004.

Colmán-Martínez, M., Martínez-Huélamo, M., Miralles, E., Estruch, R., & Lamuela-Raventós, R. M. (2016). A new method to simultaneously quantify the antioxidants: Carotenes, xanthophylls, and vitamin A in human plasma. *Oxidative Medicine and Cellular Longevity*9268531. http://dx.doi.org/10.1155/2016/9268531.

Feng, C. P., Tang, H. M., Huang, S., Hou, S. Z., Liang, J., Huang, W., & Lai, X. P. (2016). Evaluation of the effects of the water-soluble total flavonoids from *isidon lophanthoides* var. *gerardianus* (Benth.) H: Hara on apoptosis in HepG2 cell: Investigation of the most relevant mechanisms. *Journal of Ethnopharmacology, 188*, 70–79. http://dx.doi.org/10.1016/j.jep.2016.04.042.

Ferramosca, A., Di Giacomo, M., & Zara, V. (2017). Antioxidant dietary approach in treatment of fatty liver: New insights and updates. *World Journal Gastroenterology, 23*(23), 4146–4157. https://doi.10.3748/wjg.v23.i23.4146.

Fischer, M., Quaas, M., Steiner, L., & Engeland, K. (2016). The p53–p21-DREAM-CDE/CHR pathway regulates G2/M cell cycle genes. *Nucleic Acids Research, 44*(1), 164–174. http://dx.doi.org/10.1093/nar/gkv927.

García-Alonso, F. J., Navarro-González, I., Ros, G., & Periago, M. J. (2015). Assessment of the antioxidant properties or tomato extracts: A synergistic approach using in vitro chemical tests and cell-bases assays. *Acta Alimentaria, 44*(2), 297–303. http://dx.doi.org/10.1556/066.2015.44.0006.

García-Valverde, V., Navarro-González, I., García-Alonso, J., & Periago, M. J. (2013). Antioxidant bioactive compounds in selected industrial processing and fresh consumption tomato cultivars. *Food and Bioprocess Technology, 6*(2), 391–402. http://dx.doi.org/10.1007/s11947-011-0687-3.

Ip, B. C., Lui, C., Ausman, L. M., Von Lintiq, J., & Wang, X. D. (2014). Lycopene attenuated hepatic tumorigenesis via differential mechanisms depending on carotenoid cleavage enzyme in mice. *Cancer Prevention Research (Philadelphia, Pa)*, 7(12): 1219–1227. http://dx.doi.org/10.1158/1940-6207.CAPR-14-0154.

Jacob, K., Periago, M. J., Böhm, V., & Berruezo, G. R. (2008). Influence of lycopene and vitamin C from tomato juice on biomarkers of oxidative stress and inflammation. *British Journal of Nutrition, 99*(1), 137–146. http://dx.doi.org/10.1017/S0007114507791894.

Jenab, M., Ferrari, P., Mazuir, M., Tjonneland, A., Clavel-Chapelon, F., Linseisen, J., ... Ribol, E. (2005). Variations in lycopene blood levels and tomato consumption across European countries based on the European Prospective Investigation into Cancer and Nutrition (EPIC) Study. *Journal of Nutrition, 135*, 2032S–2036S.

Kalra, N., Seth, K., Prasad, S., Singh, M., Pant, A. B., & Shukla, Y. (2007). Theaflavins induced apoptosis of LNCaP cells is mediated through induction of p53, dow-regulation of NF-Kappa B and mitogen-activated protein kinases pathways. *Life Science, 80*(23), 2137–2146. http://dx.doi.org/10.1016/j.lfs.2007.04.009.

Kim, J. Y. H., Lee, J. S., Han, Y. S., Lee, H., Bae, I., Yoon, Y. M., ... Lee, S. H. (2015). Pretreatment with lycopene attenuates oxidative stress-induced apoptosis in human mesenchymal stem cells. *Biomolecules & Therapeutic, 23*(6), 517–524. http://dx.doi.org/10.4062/biomolther.2015.085.

Kondratskyi, A., Kondratska, K., Skryma, R., & Prevarskaya, N. (2015). Ion channels in the regulation of apoptosis. *Biochimica et Biophysica Acta, 1848*, 2532–2546. http://dx.doi.org/10.1016/j.bbamem.2014.10.030.

Kundu, T., Dey, S., Roy, M., Siddiqi, M., & Bhattacharya, R. (2005). Induction of apoptosis in Human Leukemia cells by black tea and its polyphenol theaflavin. *Cancer Letters, 230*(1), 111–121. http://dx.doi.org/10.1016/j.canlet.2004.12.035.

Laguerre, M., Lecomte, J., & Villenueve, P. (2007). Evaluation of the ability of antioxidants to counteract lipid oxidation: Existing methods, new trends and challenges. *Progress Lipid Research, 46*(5), 244–282. http://dx.doi.org/10.1016/j.plipres.2007.05.002.

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKls: Roles beyond cell cycle regulation. *Development, 140*(15), 3079–3093. http://dx.doi.org/10.1242/dev.091744.

Lin, X., Wei, J., Chen, Y., He, P., Lin, J., Tan, S., ... Huang, Q. (2016). Isoorientin from *Gypsophila elegans* induces apoptosis in liver cancer cells via mitochondrial-mediated pathway. *Journal of Ethnopharmacology, 187*(2016), 187–194. http://dx.doi.org/10.1016/j.jep.2016.04.050.

Martín-Pozuelo, G., Navarro-González, I., González-Barrio, R., Santaella, M., García-Alonso, J., Hidalgo, N., ... Periago, M. J. (2015). The effect of tomato juice supplementation on biomarkers and gene expression related to lipid metabolism in rats with induced hepatic steatosis. *European Journal of Nutrition, 54*, 933–944. http://dx.doi.org/10.1007/s00394-014-0770-4.

Montella, M., Crispo, A., & Giudice, A. (2011). HCC, diet and metabolic factors: Diet and HCC. *Hepatitis Monthly*, 11(3), 159–162. PMCID: PMC3206690.

Mordente, A., Guantario, B., Meucci, E., Silvestrini, A., Lombardi, E., Martorana, G., & Bohm, V. (2011). Lycopene and cardiovascular diseases: An update. *Current Medicinal Chemistry, 18*(8), 1146–1163. http://dx.doi.org/10.2174/092986711795029717.

Mullany, L. K., White, P., Hanse, E. A., Nelsen, C. J., Goggin, M. M., Mullany, J. E., ... Albrecht, H. H. (2008). Distinct proliferative and transcriptional effects of the D-type cyclins in vivo. *Cell Cycle, 15*(14), 2215–2224. http://dx.doi.org/10.4161/cc.7.14.6274.

Navarro-González, I., Pérez-Sánchez, H., Martín-Pozuelo, G., García-Alonso, J., & Periago, M. J. (2014). The inhibitory effects of bioactive compounds of tomato juice binding to hepatic HMGCR: In vivo study and molecular modelling. *PLoS ONE, 9*(1), e83968. http://dx.doi.org/10.1371/journal.pone.0083968.

Palomo, I., Moore-Carrasco, R., Carrasco, G., Villalobos, P., & Guzmán, L. (2010). Tomato consumption prevents the development of cardiovascular events and cancer: Epidemiological antecedents and action mechanisms. *IDESIA (Chile), 28*(3), 121–129. http://dx.doi.org/10.4067/S0718-34292010000300016.

Palozza, P., Colanggelo, M., Simone, R., Catalano, A., Boninsegna, A., Lanza, P., ... Ranelletti, F. (2010). Lycopene induces cell growth inhibition by altering mevalonate pathway and ressignaling in cancer cell lines. *Carcinogenesis, 31*(10), 1813–1821. http://dx.doi.org/10.1093/carcin/bgq157.

Pan, M. H., & Ho, C. T. (2008). Chemopreventive effects of natural dietary compounds on cancer development. *Chemical Society Reviews, 37*(11), 2558–2574. http://dx.doi.org/10.1039/b801558a.

Park, Y. O., Hwang, E. S., & Moon, T. W. (2005). The effect of lycopene on cell growth and oxidative DNA damage of Hep3B human hepatoma cells. *Biofactors*, 23(3), 129–39. http://dx.doi.org/10.1002/biof.5520230302.

Periago, M. J., García-Alonso, J., Jakob, K., Olivares, A. B., Bernal, M. J., Iniesta, M. D., ... Ros, G. (2009). Bioactive compounds, folates and antioxidant properties of tomato (*Lycopersicum esculentum*) during vine ripening. *International Journal of Food Sciences and Nutrition, 60*(8), 694–708. http://dx.doi.org/10.3109/09637480701833457.

Rafi, M. M., Kanakasabai, S., Reyes, M. D., & Bright, J. J. (2013). Lycopene modulates grown and survival associated genes in prostate cancer. *The Journal of Nutritional Biochemistry, 24*(10), 1724–1734. http://dx.doi.org/10.1016/j.jnutbio.2013.03.001.

Rao, A. V. (2006). *Tomatoes, lycopene & human health. Preventing chronic diseases.* Scotland: Caledonian Science Press Ltd111–125.

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J., & Minor, L. (2017). Cell viability assays. In: G. S. Sittampalam, N. P. Coussens, K. Brimacombe, et al. (Eds.), Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences. https://www.ncbi.nlm.nih.gov/books/NBK144065/ Download 21st December 2017.

Rougier, P., Mitry, F., Barbare, J. C., & Taieb, J. (2007). Hepatocellular carcinoma (HCC): An update. *Seminars in Oncology,* S12–S20. http://dx.doi.org/10.1053/j.seminoncol.2007.01.007.

Sayer, T. J. (2011). Targeting the extrinsic apoptosis signaling pathway for cancer therapy. *Cancer Immunology Immunotherapy, 60*, 1173–1180. http://dx.doi.org/10.1007/s00262-011-1008-4.

Schlesinger, S., Alecksandrova, K., Pischon, T., Ferdiko, V., Jenab, M., Trepo, C., ... Nöthlings, U. (2013). Absominal obesity, weight gain during adulthood and risk of liver and biliary tract cancer in a European cohort. *International Journal of Cancer, 132*, 645–657. http://dx.doi.org/10.1002/ijc.27645.

Sharoni, Y., Linnewiel-Hermione, K., Khanin, M., Salman, H., Veprik, A., Danilenko, M., & Levy, J. (2016). Carotenoids and apocarotenoids in cellular signaling related to cancer: A review. *Molecular Nutrition & Food Research, 56*(2), 259–269. http://dx.doi.org/10.1002/mnfr.201100311.

Sorrentino, G., Comel, A., Mantovani, F., & Sal, G. D. (2014). Regulation of mitochondrial apoptosis by Pin1 in cancer and neurodegeneration. *Mitochondrion, 19*, 88–96. http://dx.doi.org/10.1016/j.mito.2014.08.003.

Stuetz, W., Weber, D., Dollé, M. E. T., Jansen, E., Grubeck-Loebenstein, B., Fiegl, S., ... Bürkle, A. (2016). Plasma carotenoids, tocopherols, and retinol in the age-stratified (35–74 Years) general population: A Cross-Sectional Study in Six European Countries. *Nutrients, 8*(10), 614–631. http://dx.doi.org/10.3390/nu8100614.

Talvas, J., Caris-Veyrat, C., Guy, L., Rambeau, M., Lyan, B., Minet-Quinard, R., ... Rock, E. (2010). Differential effects of lycopene consumed in tomato paste and lycopene in the form of a purified extract on target genes of cancer prostatic cells. *The American Journal of Clinical Nutrition, 91*(6), 1716–1724. http://dx.doi.org/10.3945/ajcn.2009.28666.

Teodoro, A. J., Oliveira, F. L., Martins, N. B., de Azevedo Maia, G., Martucci, R. B., & Borojevic, R. (2012). Effect of lycopene on cell viability and cell cycle progression in human cancer cell lines. *Cancer Cell International, 12*(1), 36. http://dx.doi.org/10.1186/1475-2867-12-36.

Vitaglione, P., Morisco, F., Caporaso, N., & Fogliano, V. (2005). Dietary antioxidant compounds and liver health. *Critical Reviews in Food Science and Nutrition, 44*(7–8), 575–586. http://dx.doi.org/10.1080/10408690490911701.

Wang, Y., Ausman, L. M., Greenberg, A. S., Russell, R. M., & Wang, X. D. (2010). Dietary lycopene and tomato extract supplementations inhibit nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats. *International Journal of Cancer, 126*, 1788–1796. http://dx.doi.org/10.1002/ijc.24689.

Ye, F., Wu, J., Dunn, T., Yi, J., Tong, X., & Zhang, D. (2004). Inhibition of cyclooxygenase-2 activity in head and neck cancer cells by genistein. *Cancer Letters, 211*, 39–46. http://dx.doi.org/10.1016/j.canlet.2004.03.043.

Zhong, H., Xiao, M., Zarkovic, K., Zhu, M., Sa, R., Lu, J., ... Waeg, G. (2017). Mitochondrial control of apoptosis through modulation of cardiolipin oxidation in hepatocellular carcinoma: A novel link between oxidative stress and cancer. *Free Radical Biology and Medicine, 102*, 67–76. http://dx.doi.org/10.1016/j.freeradbiomed.2016.10.494.