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Antioxidant bioactive compounds in tomato fruits at different ripening stages and their effects on normal and cancer cells

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ABSTRACT

The changes in antioxidant content of tomato fruits at different ripening stages (mature green, breaker and mature red) were determined and hydrophilic extracts were tested on normal and cancer cells. Large differences existed in the content of bioactive compounds at different ripening stages, with breaker tomatoes containing a higher content of hydrophilic antioxidants. We demonstrated a high cytotoxic effect of α-tomatine in green, but not in breaker, tomato extracts on all cell lines analysed. Cell death was found to be an apoptotic independent mechanism, probably due to α-tomatine binding to cell membrane cholesterol, disruption of cell integrity and necrosis. These results help in understanding which harvesting stage corresponds to the highest functional power of tomato fruits and may lead to the development of tomato-based functional foods.

Abbreviations: FW, fresh weight; HAA, hydrophilic antioxidant activity; LAA, lipophilic antioxidant activity; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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1. Introduction

Tomato (*Solanum lycopersicum*) fruits at different developmental stages synthesize a wide array of phytochemicals that are reported to have health-promoting effects in humans, such as the carotenoid pigments lycopene and β-carotene, the glycoalkaloids dehydrotomatine, α-tomatine and esculeoside A and many phenolic compounds (Choi et al., 2010). Many of these compounds are potent antioxidants capable of scavenging free radicals, which are responsible for DNA, protein and membrane lipid damage (Raiola, Rigano, Calafiore, Frusciante, & Barone, 2014; Weaver et al., 2009). In humans, oxidative stress, due to the imbalance between reactive oxygen species (ROS) generation and the cellular capacity to detoxify these species, is an important causative factor in the development of several chronic diseases, such as cardiovascular disease and atherosclerosis (H. Li, Deng, Liu, Loewen, & Tsao, 2013). Several publications have reported that the antioxidative effect of tomato fruits is due to the presence of polyphenols (flavonoids and hydroxycinnamic acids), which are able to scavenge peroxyl radicals (García-Valverde, Navarro-Gonzalez, Garcia-Alsonso, & Periago, 2013). Moreover, polyphenols extracted from peel, pulp and seeds of selected fruits, including tomatoes, have an antiproliferative effect in several cancer cell lines (F. Li et al., 2013).

Tomato lipophilic compounds exert a beneficial effect on humans, as carotenoids possess an apoptotic inducing effect in cancer cells (reviewed in Niranjana et al., 2015), and lycopene has been demonstrated to reduce oxidized-LDL cholesterol levels (Abete et al., 2013).

Tomato fruits at the green stage are rich in the glycoalkaloid α-tomatine which has been found to inhibit the growth of human cancer cells *in vitro* and to reduce serum LDL cholesterol through the formation of a tomatine–cholesterol complex (Choi et al., 2013). Tomatine-containing extracts of green tomatoes inhibited human lung, breast, colon, liver and stomach cancer cells (Sucha et al., 2013). However, literature is still contrasting on the cytotoxic effects of α-tomatine on normal cells, as reported by Choi et al. (2010). There are many factors that affect the nature and concentration of synthesized compounds in tomato fruits, including agricultural practices, environmental factors, variety and ripening stage (García-Valverde et al., 2013). During ripening, the level of carotenoids increases between 10 and 14 fold, due to the increased expression of genes involved in isoprenoid biosynthesis, such as 1-deoxy D-xylulose 5-phosphate (Tohge, Alseekh, & Fernie, 2014). Red colour, due to lycopene accumulation, starts to appear from the breaker stage (44 ± 1 day after anthesis) when ripening is triggered by ethylene. The content of total phenolics strongly depends on the ripening stage of tomato fruits, as they are more abundant in green and intermediate ripening stages, decreasing in full red tomatoes (Choi et al., 2010; García-Valverde et al., 2013; Kozukue & Friedman, 2003). Finally, steroidal glycoalkaloids change their composition through a ripening-dependent conversion of tomatines to esculeosides via hydroxylation and glycosylation reactions (Tohge et al., 2014).

The main objective of this study was to investigate how the composition and content of antioxidant bioactive compounds in tomato fruits are influenced by on-vine ripening. Here we analysed the content of ascorbic acid, individual phenolic compounds, chlorophylls, carotenoids and glycoalkaloids in the *S. lycopersicum* variety M82, harvested at three ripening stages (mature green-S1, breaker-S2 and mature red-S3). We evaluated the antioxidant activity using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and Ferric Reducing Ability of Plasma (FRAP) assays. Due to conflicting reports in the literature regarding the effects of tomato extracts from fruits at different ripening stages, we tested the activity of the hydrophilic fraction of the three tomato fruit extracts on two normal and two human cancer cell lines. The hydrophilic fraction was chosen since it is known that hydrophilic antioxidant activity has a far more significant impact on total antioxidant activity (83%) than lipophilic antioxidant activity (17%) (García-Valverde et al., 2013). The cytotoxicity experiments have been followed by studies investigating the possible mechanisms of action of the hydrophilic tomato extracts in several cell lines.

Data reported in this manuscript could be used by fresh and industrial tomato processors in order to obtain tomato products and tomato-based functional foods with added beneficial effects for the consumers.

2. Materials and methods

2.1. Chemicals and reagents

All standards and reagents were purchased from Sigma (St. Louis, MO, USA). Solvents were obtained from Fluka (Schweiz, Switzerland). Chromatographic solvents were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., Phoenix, AZ, USA).

2.2. Plant material and growth conditions

Seeds from cultivated line M82 (LA3475) were kindly provided by the Tomato Genetics Resource Centre (TGRC) (University of California, Davis, USA). They were grown according to a completely randomized design with three replicates (10 plants/replicate), in an experimental field located in Acerra (Naples, Italy) in the year 2014. Samples of about 20 full fruits per plot were collected at three different stages of ripening: mature green (S1, 37 ± 1 day after anthesis), breaker (S2, 44 ± 1 day after anthesis) and mature red (S3, 10 ± 1 day after breaker). Tomato fruits were chopped, ground in liquid nitrogen using a blender Fimar FRI150 (Rimini, Italy) to a fine powder, and kept at –80 °C until analysis.

2.3. Hydrophilic and lipophilic extractions

Each tomato fruit sample consisted of 20-pooled fruits per plot. Hydrophilic and lipophilic fractions were obtained according to Rigano et al. (2014). Briefly, 3 g of tomato frozen powder were extracted with 30 mL of 70% methanol into an ultrasonic bath for 60 min at 30 °C. Subsequently, the mixture was centrifuged at 3500 × *g* using the centrifuge Rotina 420R Hettich

Zentrifugen (Tuttlingen, Germany) for 10 min at 4 °C, and the supernatant was stored at –20 °C until evaluation of total phenolic compounds, HPLC analysis and hydrophilic antioxidant activity (HAA). The pellet was extracted with 10 mL chloroform (100%) using an Ultraturrax IKA T 25 High Speed Homogenizer Cole-Parmer 115VAC (Bunker, Vernon Hills, IL, USA) and centrifuged at 3500 × *g* for 5 min at 4 °C according to a modified procedure reported by Wellburn (1994). The supernatant was collected and stored at –20 °C until the determination of lycopene, total carotenoids, chlorophyll levels and lipophilic antioxidant activity (LAA).

2.4. *Carotenoid and chlorophyll determination*

The determination of total carotenoids and chlorophylls was carried out by reading the absorbance of supernatants from the above extractions at 480, 648, 666 nm using a NanoPhotometer™ Version 2.0 (Schatzbogen, Germany) and then applying specific equations (Wellburn, 1994). The determination of lycopene was performed by reading the absorbance at 503 nm and by using the Lambert Beer equation, with the molar extinction coefficient ε = 152,989 L/mol·cm for lycopene dissolved in chloroform (Naviglio et al., 2008). Results were expressed as mg per 100 g fresh weight (FW). All biological replicates were analysed in triplicate.

2.5. *Ascorbic acid and total phenolic compounds*

Ascorbic acid (AsA) determination was carried out by a colorimetric method (Stevens, Buret, Garchery, Carretero, & Causse, 2006), with modifications previously reported by Rigano et al. (2014). TCA 6% (300 μL) was added to 500 mg of frozen powder. The mixture was vortexed for 10 s, incubated for 15 min on ice, and centrifuged at 16,000 × *g* for 20 min at 4 °C using an Eppendorf Centrifuge 5415R (Hamburg, Germany). Then, 20 μL of 0.4 M phosphate buffer (pH 7.4) and 10 μL of double distilled (dd) H_2O were added to 20 μL of the mixture. Eighty microlitres of colour reagent solution, prepared by mixing solution 1 (31% H_3PO_4, 4.6% (w/v) TCA, and 0.6% (w/v) $FeCl_3$) with solution 2 (4% 2,2′-dipyridil (w/v) made up in 70% ethanol) at a proportion of 2.75:1 (v/v), were added.

The mixture was incubated at 37 °C for 40 min and the absorbance was then read at 525 nm. Three biological replicates were measured for each sample. The standard curve was obtained using 0–70 nmol and the values were converted into milligrams/100 g FW.

Total phenolic compounds were evaluated by using the Folin–Ciocalteu assay (Singleton & Rossi, 1965) with modifications reported by Rigano et al. (2014). Briefly, 62.5 μL of Folin–Ciocalteu's phenol reagent and 250 μL of dd H_2O were added to 62.5 μL of supernatant obtained from the above extractions. After 6 min, 625 μL of 7% Na_2CO_3 solution, 500 μL of dd H_2O were added to the mixture, incubated for 90 min at room temperature and the absorbance was read at 760 nm. Total phenolic content of tomato fruits was expressed as mg gallic acid equivalents (GAE)/100 g FW. Three separated biological replicates and three technical assays for each biological repetition were measured.

2.6. *Identification and quantification of individual phenolic compounds*

Twenty-five millilitres of supernatant obtained from the extractions described in section 2.3 were dried by a rotary evaporator (Buchi R-210, Milan, Italy) and dissolved in 500 μL of 70% methanol. The extract was passed through a 0.45 μm Millipore nylon filter (Bedford, MA, USA). Flavonoids and phenolic acids were separated and quantified by using a HPLC Spectra System SCM 1000 (Thermo Electron Corporation, San Jose, CA, USA) equipped with a Gemini column (3 μm C18, 110 A, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA) and UV-visible detector according to the procedure reported by Rigano et al. (2014). Chromatograms were recorded at 280 nm for gallic acid and chlorogenic acid, and at 365 nm for coumaric acid, ferulic acid, rutin and its derivative, quercetin and naringenin. For quantification, integrated chromatographic peak areas from the tested extracts were compared to the peak areas of known amounts of standard phenolic compounds: gallic acid, chlorogenic acid, coumaric acid, ferulic acid, rutin, quercetin and naringenin. The results were expressed as mg/100 g FW.

2.7. *Antioxidant activity determination*

Hydrophilic antioxidant activity (HAA) was evaluated using both the ferric reducing/antioxidant power (FRAP) method (Benzie & Strain, 1996) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay, while lipophilic antioxidant activity (LAA) was measured according to the ABTS test (Miller & Rice-Evans, 1997).

The FRAP assay was carried out by adding 2.5 mL of acetate buffer, pH 3.6, 0.25 mL of TPTZ solution (10 mM) in 40 mM HCl, 0.25 mL of $FeCl_3 \cdot 6H_2O$ solution (12 mM), and 150 μL of supernatant obtained from the above extraction. After an incubation of 30 min at room temperature the absorbance of the product (ferrous tripyridyltriazine complex) was read at 593 nm. The standard curve was linear between 20 and 800 μM Trolox. Results were expressed as micromoles of Trolox equivalents (TE) per 100 g FW.

ABTS method is based on the reduction of the $ABTS^{\bullet+}$ activity by the antioxidants contained in the sample. A solution of 7.4 mM $ABTS^{\bullet+}$ (5 mL) mixed with 140 mM $K_2S_2O_8$ (88 μL) was prepared, stabilized for 12 h at 4 °C and then mixed with ethanol (1:88, v/v). Subsequently, 100 μL of supernatant obtained from the above extraction were added to 1 mL of diluted $ABTS^{\bullet+}$, incubated for 2.5 min and the absorbance was read at 734 nm. The standard curve was linear between 0 and 20 μM Trolox. Results were expressed as μmol of TE/100 g FW.

2.8. *α-tomatine determinations*

Twenty five millilitres of 2% CH_3COOH (v/v) were added to 10 g of fresh fruit. The suspension was homogenized for 3 min at 16,000 × *g* and then placed into an ultrasonic bath for 30 min. The purification was carried out according to the method reported by Friedman et al. (2009). The residue was dissolved in 1 mL of 50% CH_3OH/0.1% acetic acid, filtered through a 0.45 μm HV membrane (Millipore, Bedford, MA, USA). The extract was

used for HPLC analysis, carried out according to the method reported by Leonardi, Ambrosino, Esposito, and Fogliano (2000) with minor modifications. Absorbance was read at 208 nm. A Gemini column (Phenomenex, Torrance, CA, USA) was used with a mobile phase consisting of 100 mM $NH_4H_2PO_4$ in 32.5% CH_3CN, adjusted to pH 3.5 with H_3PO_4. Elution was performed in isocratic conditions. The concentration was expressed in mg/100 g FW of α-tomatine according to the standard curve, designed over a range of 100–1000 ppm.

The percentage of the variations of quantitative parameters among genotypes was calculated by using the following formula:

$$\%\ \text{increase and/or decrease} = [(\text{value in genotype 1} - \text{value in genotype 2})/\text{value in genotype 2}] \times 100.$$

2.9. MTT test

Human renal cortical epithelial cells, HRCE (Innoprot, Derio–Bizkaia, Spain), were cultured in basal medium, supplemented with 2% foetal bovine serum, epithelial cell growth supplement and antibiotics, all from Innoprot. Human HeLa adenocarcinoma cells, rat embryos, heart myoblasts H9c2 and human hepatic carcinoma HepG2 cells were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% foetal bovine serum (HyClone, Thermo Scientific, Logan, UT, USA) and antibiotics, in a 5% CO_2 humidified atmosphere at 37 °C. The growth medium of H9c2 cells was implemented with 2 mM L-glutamine and 2 mM sodium pyruvate. Hydrophilic tomato extracts, obtained as reported above, were concentrated in a rotary evaporator and the residue was dissolved in 5% dimethyl sulphoxide (DMSO) in PBS and then added to the cells (3 mg/mL final concentration) 24 hours after seeding for time course cytotoxicity assays. At the end of incubation, cell viability was assessed by MTT assay as described by Rigano et al. (2014). For each cell line, a calibration curve was obtained by plating an increasing number of cells per well ($0.5–7 \times 10^3$). Three separate analyses were carried out with each extract. Alpha-tomatine was tested at 10 μM. Control experiments were performed either by growing cells in the absence of the extract and by supplementing the cell cultures with identical volumes of extract buffer (5% DMSO in PBS). The method used avoids any possibility of a DMSO effect on the results.

2.10. LDH release assay

The occurrence of necrosis was determined by measuring the release of lactate dehydrogenase (LDH) in the culture medium as described by Sucha et al. (2013). The LDH content of the medium from untreated cells was referred to as spontaneous release, whereas the LDH total cellular content was determined upon cell lysis. The percentage of LDH release was calculated as:

$$\text{LDH release (\%)} = [(\text{experimental} - \text{spontaneous release})/(\text{total content} - \text{spontaneous release})] \times 100.$$

2.11. Determination of membrane cholesterol levels

To determine levels of cholesterol in the surface membrane, the protocol described in Arispe and Doh (2002) was followed. Briefly, cells were grown in 96-well plates for 24 h at 37 °C. Culture medium was removed and cells were incubated for 90 min with filipin (500 μg/mL) in PBS. Filipin was then removed and cells were washed twice with PBS. The fluorescence from each well, indicative of the level of cholesterol on the surface of the cells, was read in an automatic plate reader (Microbeta Wallac 1420, Perkin Elmer, Shelton, CT, USA) with a 355/460 nm filter. Fluorescence intensity was normalized to the cell number. Three separate analyses were carried out for each cell line.

2.12. Western blot analyses and detection of apoptotic nuclei

Cells were plated at a density of 2×10^4 cells cm^{-2} in complete medium for 24 h and then treated for 4 h with 3 mg/mL of tomato extracts or 10 μM α-tomatine. At the end of the incubation, both untreated and treated cells were analysed as described in Galano, Arciello, Piccoli, Monti, and Amoresano (2014) for western blot analyses and apoptotic nuclei staining. Briefly, to prepare cell lysates, cells were scraped off in PBS, centrifuged at $1000 \times g$ for 10 min and lysed in 1% NP-40 in PBS, pH 7.4, containing protease inhibitors. After 30 min of incubation on ice, lysates were centrifuged at $14{,}000 \times g$ for 30 min at 4 °C. Upon determination of total protein concentration in the supernatant by the Bradford assay, samples were analysed by SDS-PAGE and Western blot using specific antibodies directed towards procaspase-3. To normalize to internal standard signals, antibodies against actin were used. To detect apoptotic nuclei, cells were seeded on glass coverslips in 24-well plates for 24 h. Cells were incubated for 16 h with tomato extracts (3 mg/mL) and α-tomatine (10 μM) in complete medium, after which nuclei were stained with 1 mg/mL of Hoechst 33342 for 10 min at 37 °C. Cells were then washed with PBS, fixed for 10 min at room temperature with 2% paraformaldehyde in PBS and mounted in 50% glycerol in PBS. Samples were examined using a Leica 6000 UV microscope and a Leica TCS SP5 confocal microscope, equipped with a Leica application suite software (Leica Microsystems GmbH, Wetzlar, Germany). All images were taken under identical conditions.

2.13. Statistical analysis

All biological replicates of samples were analysed in triplicate. Quantitative results were expressed as the mean value ± SD. Differences among evaluated three different stages of ripening were determined by using SPSS (Statistical Package for Social Sciences) Package 6, version 15.0. Significance was determined by comparing three ripening stages through a factorial analysis of variance (one-way ANOVA) with Duncan *post hoc* test at a significance level of 0.05.

3. Results and discussion

During tomato fruit ripening, coordinated genetic and biochemical events occur (Fraser et al., 2007) that result in changes

in fruit colour, texture and flavour. We characterized the nutritional value of fruits from the cultivated *S. lycopersicum* variety M82, harvested at different ripening stages, by examining their compositional pattern, with particular attention to the different classes of tomato antioxidants and α-tomatine.

3.1. *Content of antioxidant bioactive compounds and antioxidant activity*

Amount of total carotenoid increased during ripening from 0.49 ± 0.02 mg/100 g FW in S1 to 1.29 ± 0.03 mg/100 g FW in S2, with a significant ($p < 0.05$) increase in S3, up to 14.10 ± 0.63 mg/100 g FW (Table 1). In S3, lycopene, on average, constituted 71% of total carotenoids and showed a significant increase compared to S1 and S2 (25 and 9 fold, respectively). An opposite trend was observed for chlorophyll a and chlorophyll b that are enzymatically degraded during tomato ripening (Table 1). The mean value of chlorophyll a was 2.78 mg/100 g FW in S1, with a significant decrease in S2 and S3 (32 and 94 fold, respectively). The mean value of chlorophyll b was 2.16 mg/100 g FW in S1, a level comparable to that obtained in S2, while a decrease of 87% was observed in S3. The decrease of chlorophylls corresponded to the loss of the green colour. Carotenoid and chlorophyll levels reported here are in agreement with data reported by other authors (Kozukue & Friedman, 2003; Raffo et al., 2002). Kozukue and Friedman (2003) found that chlorophyll content decreased by about 25% during stage 2 and by about 75% during successive stages, dropping to a value near zero during the final stage (50 days after flowering).

Ascorbic acid (AsA) is one of the main water-soluble tomato antioxidants, together with phenolic compounds. Literature is contrasting on changes in AsA content during ripening; some authors have observed no clear trend during ripening while others have found that AsA content increases slightly only in the last stage of ripening (Cano, Costa, & Arnao, 2003; García-Valverde et al., 2013). Here we found that AsA levels increase in S2 and decrease again in S3 when the fruit is fully ripe (Table 2). AsA showed a mean value of 12.98 ± 0.28 mg/100 g FW in S1 while in S2 a mean significant increase ($p < 0.05$) of 62% was found. In S3, AsA content decreased again to 14.47 ± 1.40 mg/100 g FW, a value not significantly different from that detected in S1.

Results obtained from the Folin–Ciocalteu assay are reported in Table 2. The mean value of total phenolics in S1 was 21.23 ± 0.40 mg GAE/100 g FW, with an increase of 26% in S2. A mean decrease of 37% was reported in S3 compared to S2.

Single phenolic compounds were identified by HPLC analyses (Table 3). A significant decrease in the content of gallic acid was observed in S3 compared to S2 and S1. Chlorogenic acid and coumaric acid showed a slight decrease from S1 to S2, followed by a significant increase in S3. Ferulic acid, instead, showed its maximum level in S2. No significant changes in the sum of the detected phenolic acids were observed from S1 to S2, whereas a slight increase was found in S3. When we analysed the flavonoid content, no significant change in pentosil rutin was observed between S1 and S2, whereas a significant increase was observed in S3 (Table 3). Rutin trend revealed a significant mean decrease in S2 compared to S1 (about 41%) and a successive significant increase (about 30%) in S3 compared to S2. The flavonoid quercetin was found only in S3, whereas naringenin, which is the main detected flavonoid, was not detectable in S1 and reached its maximum level in S2. A typical chromatogram with identified polyphenols is reported in Fig. 1.

Our results are in agreement with those previously reported that demonstrated that flavanones and their glycosides, as well as many flavonol derivatives, increase during ripening (Mintz-Oron et al., 2008; Moco et al., 2007). In addition, a maximum level of naringenin during the intermediate stage of ripening was observed by Raffo et al. (2002). The declining content of naringenin in the last stage of ripening could be associated with its involvement in fruit defence mechanisms against ROS, which are produced in high amounts during climacteric peak as a consequence of respiration increasing rate (Yamasaki, Sakihama, & Ikehara, 1997). The fact that naringenin is the main flavonoid is interesting also from a functional point of view, since it has been reported that this flavonoid protects peripheral lymphocytes of diabetic mice from DNA-damage and reduces oxidative stress (Oršolić et al., 2011).

Lipophilic (LAA) and hydrophilic (HAA) antioxidant activity was calculated by using ABTS and FRAP assays (Tables 1 and 2). Tomato HAA is mainly conferred by AsA and soluble phenolic compounds, while LAA is mainly conferred by carotenoids (García-Valverde et al., 2013). LAA was higher in S3 when compared to the other ripening stages, with a mean value of 130.07 ± 5.19 μmol TE/100 g FW, which represents a 3 fold

Table 2 – Ascorbic acid (mg/100 g FW), total phenolics (mg GAE/100 g FW) and hydrophilic antioxidant activity (HAA) (μmol TE/100 g FW) in analysed stages of ripening in M82.[a]

Stage	AsA	Total phenolics	HAA (FRAP)	HAA (ABTS)
S1	12.98 ± 0.28[a]	21.23 ± 0.40[b]	35.00 ± 4.25[a]	108.79 ± 2.04[a]
S2	21.08 ± 1.20[b]	26.89 ± 0.48[c]	187.43 ± 18.07[c]	150.34 ± 5.51[b,c]
S3	14.47 ± 1.40[a]	16.97 ± 1.14[a]	125.14 ± 12.62[b]	139.36 ± 2.62[b]

[a] Values are means ± SD (n = 9). Values with different letters are significantly different ($p < 0.05$).

Table 1 – Lycopene, total carotenoids, chlorophyll a, chlorophyll b (mg/100 g FW) and lipophilic antioxidant activity (LAA) (μmol TE/100 g FW) in analysed stages of ripening in M82.[a]

Stage	Lycopene	Total carotenoids	Chlorophyll a	Chlorophyll b	LAA
S1	0.40 ± 0.06[a]	0.49 ± 0.02[a]	2.78 ± 0.21[c]	2.16 ± 0.31[b]	39.60 ± 1.17[a]
S2	1.12 ± 0.05[a]	1.29 ± 0.03[a]	1.88 ± 0.11[b]	2.00 ± 0.16[b]	80.06 ± 1.40[b]
S3	10.04 ± 0.48[b]	14.10 ± 0.63[b]	0.15 ± 0.01[a]	0.28 ± 0.02[a]	130.07 ± 5.19[c]

[a] Values are means ± SD (n = 9). Values with different letters are significantly different ($p < 0.05$).

Table 3 – Concentration of phenolic compounds in the extracts of M82 at the three analysed stages of ripening (mg/100 g FW).

Stage	Gallic acid	Chlorogenic acid	Coumaric acid	Ferulic acid	Sum of phenolic acids	Pentosil rutin	Rutin	Quercetin	Naringenin	Sum of flavonoids
S1	0.35 ± 0.01[b]	0.66 ± 0.10[a]	0.71 ± 0.08[a]	0.03 ± 0.005[a]	1.75 ± 0.19[a]	0.16 ± 0.06[a]	0.63 ± 0.07[b]	n.d.	n.d.	0.79 ± 0.13[a]
S2	0.35 ± 0.04[b]	0.47 ± 0.15[a]	0.53 ± 0.10[a]	0.07 ± 0.007[c]	1.42 ± 0.29[a]	0.11 ± 0.04[a]	0.37 ± 0.10[a]	n.d.	6.23 ± 0.43[b]	6.71 ± 0.7[c]
S3	0.24 ± 0.04[a]	1.02 ± 0.10[b]	0.79 ± 0.12[a,b]	0.04 ± 0.001[b]	2.09 ± 0.26[a,b]	0.28 ± 0.08[b]	0.53 ± 0.05[a,b]	0.22 ± 0.05	2.57 ± 0.43[a]	3.6 ± 0.61[b]

[a] Values are means ± SD (n = 9). Values with different letters are significantly different ($p < 0.05$). n.d., not detected.

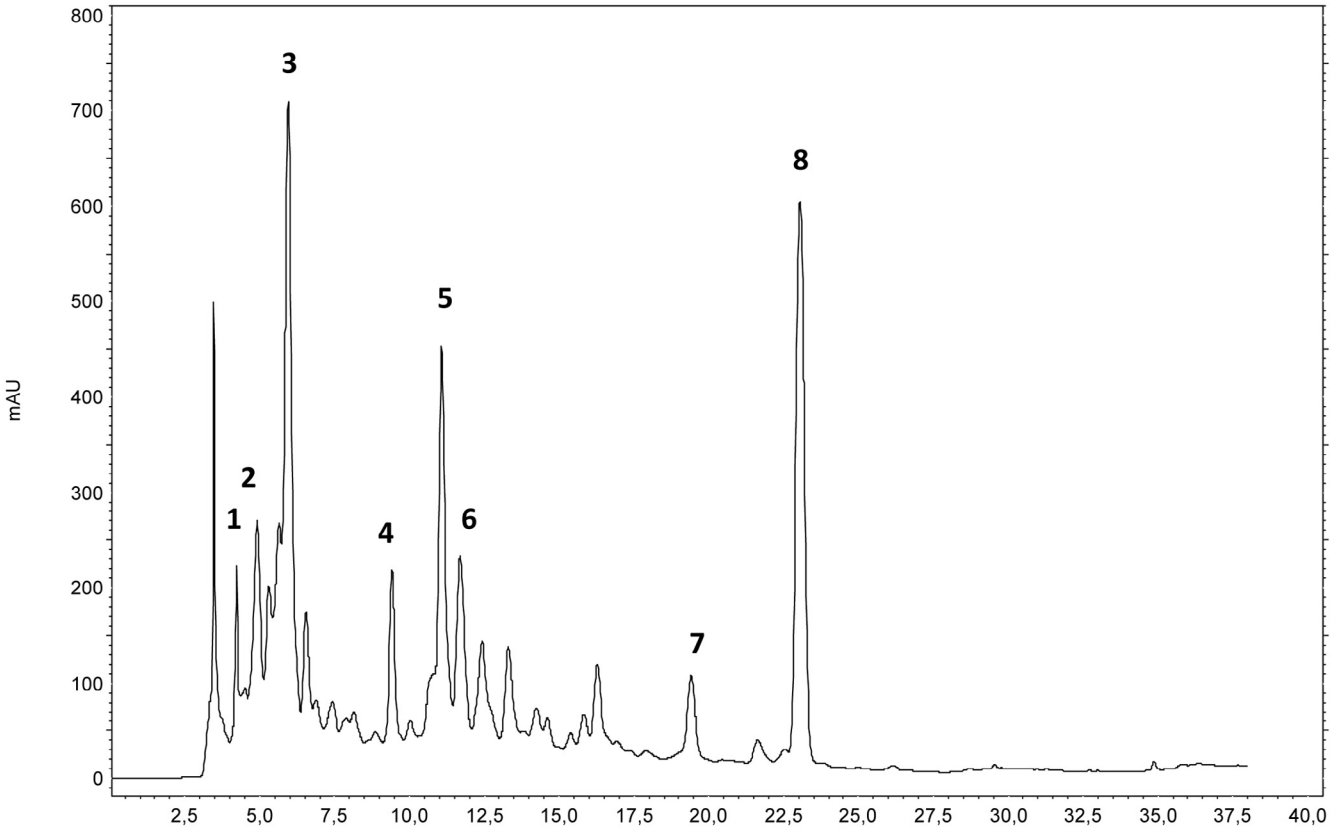

Fig. 1 – Typical HPLC chromatographic profile (λ = 365 nm) of phenolic compounds in M82 tomato fruit at the S3 ripening stage. 1: gallic acid; 2: chlorogenic acid; 3: coumaric acid; 4: pentosil rutin; 5: rutin; 6: ferulic acid; 7: quercetin; 8: naringenin.

increase compared to S1 and a 1.6 fold increase compared to S2. These results are in agreement with those reported by other authors who found variations ranging from 15 to 88 μM TE/100 g FW in LAA during tomato fruit ripening (Cano et al., 2003; Raffo et al., 2002).

The hydrophilic antioxidant activity (HAA) was determined by FRAP and ABTS methods (Table 2). FRAP test revealed the highest HAA in S2 with a mean value of 187.43 ± 18.07 μmol TE/100 g FW, whereas this value was about 5 and 0.6 fold lower in S1 and S3, respectively. Accordingly, ABTS test revealed a significant mean increase of HAA in S2 (about 38%) compared to S1, and a subsequent decrease in S3 (about 7%) compared to S2. The antioxidant potential of tomatoes varied with the assay method used, as the two methods measure different antioxidative effects (Choi et al., 2011, 2014).

Cano et al. (2003) did not observe significant differences in HAA during ripening, while Ilahy, Hdider, Lenucci, Tlili, and D'Alessandro (2011) found the highest level of HAA at the green stage of ripening and the lowest value at the red-ripe stage. These differences can be related to environmental factors, time of harvest, as well as to the different analytical methods used (Dumas, Dadomo, Di Lucca, & Grolier, 2003).

3.2. *α-tomatine content determination and effects of tomato extracts on cell viability*

It is known that immature green tomatoes are rich in glycoalkaloids, with α-tomatine being the most abundant and toxic species that decreases during the ripening process (Friedman et al., 2009). We analysed the α-tomatine content

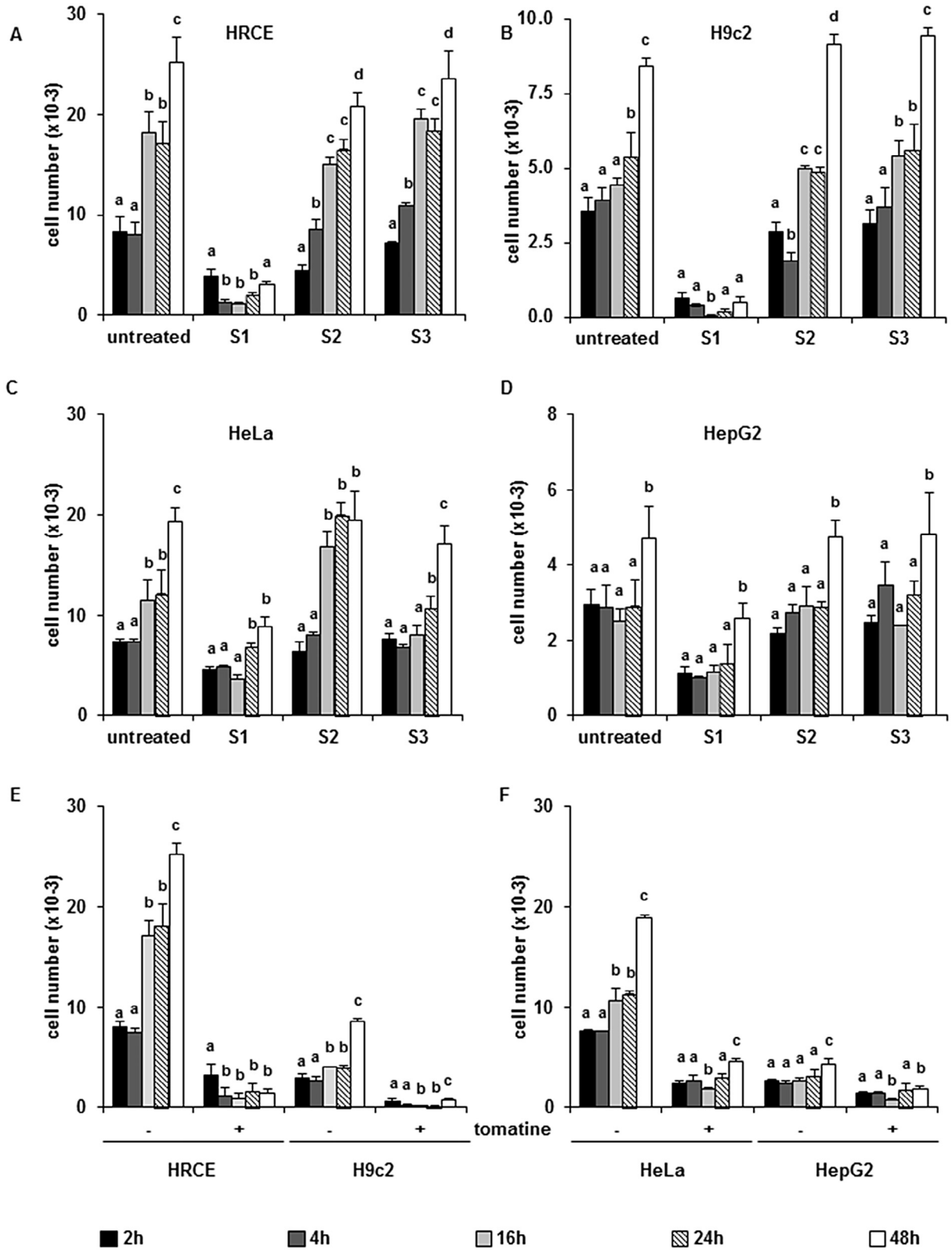

Fig. 2 – Time-course of the effects of tomato extracts and α-tomatine on the viability of normal (A, B, E) and cancer (C, D, F) cell lines. HRCE (A), H9c2 (B), HeLa (C) and HepG2 (D) cells were incubated in the absence, untreated, or in the presence of tomato extracts, S1, S2, S3 (3 mg/mL) for different lengths of time: 2 h (black bars), 4 h (dark grey bars), 16 h (light grey bars), 24 h (hatched bars) and 48 h (white bars). Normal (E) and cancer (F) cells were incubated in the absence or in the presence of 10 μM α-tomatine as reported above. Cell numbers were determined by MTT assay. Data shown are the means ± SD of at least three independent experiments. Values with different letters are significantly different ($p < 0.05$) within the same treatment.

Table 4 – α-tomatine content (mg/100 g FW) in analysed stages of ripening in M82.[a]

Stage	α-Tomatine (mg/100 g FW)
S1	20.30 ± 1.33[c]
S2	14.59 ± 1.15[b]
S3	0.51 ± 0.05[a]

[a] Values are means ± SD (n = 9). Values with different letters are significantly different ($p < 0.05$).

in S1, S2 and S3 tomato extracts (Table 4). Alpha-tomatine levels were high in S1 fruits (20.30 ± 1.33 mg/100 g) and then decreased down to 0.51 mg/100 g in S3 fruits. Changes in the levels of α-tomatine during tomato ripening have been widely studied by several authors and it has been demonstrated that they are dependent on genotype, tissue, and growth conditions. Results reported in this work are in accordance with those reported by other studies (Itkin et al., 2011; Kozukue, Han, Lee, & Friedman, 2004; Mintz-Oron et al., 2008).

We then analysed the biological activity of S1, S2 and S3 hydrophilic extracts on two normal cell lines, HRCE and H9c2 cells (Fig. 2A, B), and two human cancer cell lines, HeLa and HepG2 cells (Fig. 2C, D). We selected 3 mg/mL of tomato extracts since we recently observed that a high concentration of S3 tomato extracts (12 mg/mL) exerts a cytotoxic effect due to an overdose of phenolic acids and flavonoids (Rigano et al., 2014). In addition, at the concentration of 3 mg/mL, α-tomatine content in S1 extracts was about 10 μM, a value known to inhibit cell proliferation (Sucha et al., 2013). Cells were treated with the tomato extracts for different lengths of time (2 h, 4 h, 16 h, 24 h, 48 h). At the end of the incubation, cell viability was assessed by the MTT reduction assay, as an indicator of metabolically active cells. The values are the average of four independent experiments, each carried out with triplicate determinations. As shown in Fig. 2A–D, no cytotoxic effects were observed when cells were treated with S2 and S3 extracts. Interestingly, S1 extracts were found to have a cytotoxic effect both on normal and cancer cells, but more pronounced on normal cells. The toxic effect of S1 extracts was very evident after 2–16 h on all the cell lines analysed. Noteworthy, cancer cells were able to recover, as indicated by the increase of their cell number overtime (24–48 h; Fig. 2C, D), whereas this effect was not observed on normal cells (Fig. 2A, B). The observation that cancer cells were able to recover from the treatment with S1 extracts might be due to their high metabolic rate and to a substantial decrease in the availability of α-tomatine in the culture medium because of its binding with cholesterol, according to Sucha et al. (2013).

To confirm that the toxic effect of S1 extracts was due to the presence of α-tomatine, we performed cytotoxicity experiments by using 10 μM α-tomatine, which corresponds to the α-tomatine content in S1 extracts when tested at 3 mg/mL. As shown in Fig. 2E, F, α-tomatine induced a significant cytotoxic effect on both normal and cancer cells, similar to that observed with S1 extracts, suggesting that α-tomatine is mainly responsible for S1 extracts' cytotoxic effect.

It is interesting to note that even though S2 extracts contained a significant amount of α-tomatine, they did not affect cell viability. The difference between S1 and S2 toxicity may be due to the presence of highly active antioxidants in S2 extracts, as reported in Table 2. We can hypothesize that although α-tomatine appears to be cytotoxic to cells, the presence of a high amount of hydrophilic antioxidants in the S2 extracts and/or of other nutrients not detected in this study, such as triterpenoid glycosides and esculentin, may exert a protective effect on the tested cell lines. In this context, it is noteworthy that the consumption of red tomatoes containing high levels of α-tomatine, grown in the mountains of the Andes, does not affect human health (Rick, Uhlig, & Jones, 1994). Accordingly, even though α-tomatine is present both in S1 and S2 extracts, only S1 tomato extracts were found to inhibit cell proliferation, where the antioxidant capacity is lower than in other stages. We hypothesize that the higher antioxidant levels in S2 extracts may contribute to counteract the negative effect of α-tomatine. This hypothesis is supported by the observation that low doses of mature tomato extracts show a proliferative effect on several cells (Choi et al., 2011; Rigano et al., 2014).

It is known that α-tomatine binds to the cholesterol present in the outer leaflet of the plasma membrane with consequent membrane disruption. Moreover, the presence of cholesterol in membranes is known to induce changes in the structure and fluidity of the phospholipid bilayer. Thus, we evaluated cholesterol content in the plasma membrane of the cell lines under study by using filipin as a fluorescent probe (Fig. 3). We found that both cancer cell lines (HeLa and HepG2) showed lower levels of membrane associated cholesterol compared to normal cells (HRCE and H9c2). This result is in agreement with the decrease in cholesterol concentration observed in leukaemic murine thymocyte membranes compared to the normal murine cells (Andoh, Okazaki, & Ueoka, 2013 and references therein).

Indeed, an essential difference between normal and cancer cells is the decrease in the concentration of cholesterol in cancer

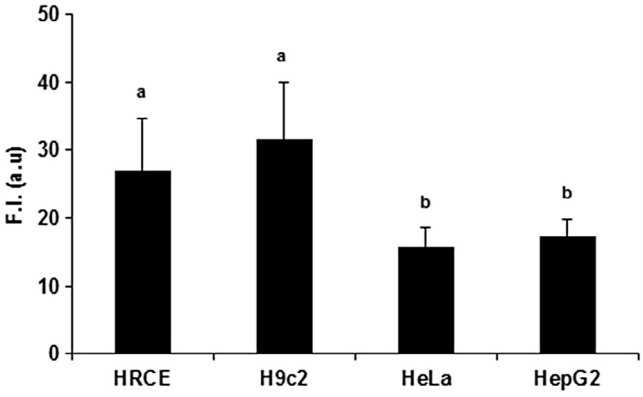

Fig. 3 – Cholesterol content on the cell membrane. HRCE, H9c2, HeLa and HepG2 cells were grown for 24 h at 37 °C. Culture medium was removed and cells were incubated for 90 min with filipin (500 μg/mL) in PBS. The fluorescence intensity (F.I.) (a.u.) from each cell line, indicative of the level of cholesterol on the cell surface, was normalized to each cell number. Three separate analyses were carried out for each cell line. Data shown are the means ± SD. Values with different letters are significantly different ($p < 0.05$).

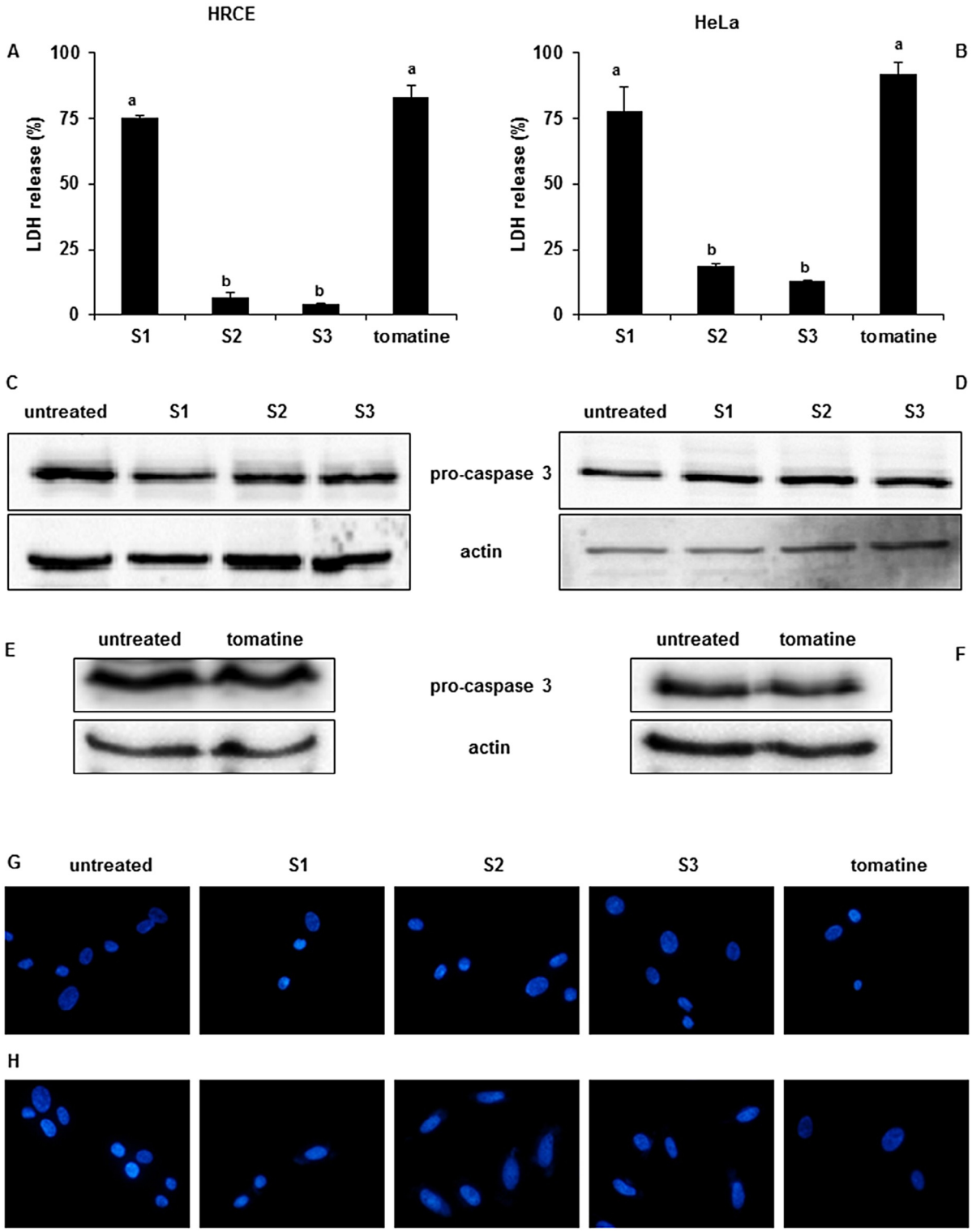

Fig. 4 – Analysis of cell death mechanism induced by tomato extracts and α-tomatine on human cells. LDH release from HRCE (A) and HeLa (B) cells upon treatment with tomato extracts (3 mg/mL) and 10 μM α-tomatine for 16 h at 37 °C. The % of LDH release was calculated as described in the Materials and Methods section. Three separate analyses were carried out for each cell line. Data shown are the means ± SD. Values with different letters are significantly different ($p < 0.05$).
(C–F) Western blot analysis of the apoptotic marker procaspase-3 in HRCE (C, E) and HeLa (D, F) cells untreated or treated with tomato extracts (C and D) or α-tomatine (E and F). Western blots were performed using antibodies directed towards procaspase-3. Endogenous actin was used as an internal standard. (G, H) Hoechst staining of HRCE (G) and HeLa (H) cells untreated or treated with tomato extracts and α-tomatine. All images were acquired at the same magnification.

cell membranes with a considerable change of their physicochemical properties, such as fluidity (Andoh et al., 2013 and references therein).

Sucha et al. (2013) reported that α-tomatine is not biotransformed in human breast cancer cells, but its concentration significantly decreased in solution since cholesterol forms an insoluble precipitate with α-tomatine. Starting from these observations, we hypothesized that the mechanism involved in the cytotoxicity of S1 extracts resides on the membrane-disruptive effect of α-tomatine upon binding to cholesterol on the cell membrane. This event finally leads to cell death.

As it is reported that cell membrane disruption induces necrosis (Do et al., 2003; Dong, Saikumar, Weinberg, & Venkatachalam, 1997), we evaluated the release of the enzyme LDH. This cytosolic enzyme is rapidly released into the cell culture medium upon membrane destabilization, making it a suitable marker of early necrosis and the late stage of apoptosis (Andoh et al., 2013; Chan, Moriwaki, & De Rosa, 2013). We chose HeLa cells as a cancer cell line and HRCE cells as normal cells to evaluate necrosis upon treatment with tomato extracts. Cells were treated with tomato extracts for 16 h at 37 °C, since at this time point we observed the maximum of the cytotoxic effect of S1 extracts. The results are reported in Fig. 4A, B as the average of three independent experiments carried out with triplicate determinations. A consistent LDH release was measured for both cell lines after treatment with S1 extracts and α-tomatine. No significant LDH release was observed after treatments with S2 and S3 extracts.

Furthermore, no caspase-3 activation was observed after treatment with either tomato extracts or α-tomatine (Fig. 4C–F), as the intensity level of procaspase-3, normalized to endogenous actin, was unvaried in all the treated samples and in both cell lines. This result was also confirmed by the absence of apoptotic nuclei in treated cells (Fig. 4 G, H). All these results indicate that S1-induced cell death is an apoptotic independent mechanism and might be due to binding of α-tomatine to cell membrane cholesterol, which leads to the loss of cell integrity resulting in necrosis. Our results are in agreement with recent studies on the effects of α-tomatine on cells, as reported by Sucha et al. (2013). However, to the best of our knowledge, this is the first report on the correlation between the cytotoxicity of tomato extracts containing high levels of α-tomatine and cholesterol levels on normal and cancer cell membranes.

4. Conclusions

The results of the present study indicated that significant changes in the composition of chlorophylls, carotenoids, phenolics and glycoalkaloids, occur in tomato during ripening. Here we confirmed that some of the most significant changes in composition occur at the breaker stage, where carotenoids start to appear, phenolic compounds reach maximum levels and glycoalkaloids begin to be degraded. The observed changes suggest that it may be possible to select tomato fruit with maximum concentration of health-promoting compounds. In particular, the high content of bioactive compounds in tomato fruits at the breaker stage indicates that they have a potential use for the nutraceutical and food industry. A high cytotoxic effect of α-tomatine in green tomato extracts on normal and cancer cell lines was also demonstrated, whereas, in breaker tomatoes, α-tomatine detrimental effect was masked by the presence of higher concentration of molecules endowed with high antioxidant power. We found that α-tomatine induced cell death is a caspase-independent mechanism and resides on α-tomatine's ability to disrupt cell membrane integrity by binding to cholesterol on the plasma membrane, thus leading to necrosis. Although many studies have reported the cytotoxic action of α-tomatine on cancer cells, our study is the first to describe this phenomenon by using tomato extracts at different ripening stages on both normal and cancer cells. This work strongly points to the need for additional studies on animals and humans in order to better define the possible beneficial roles of the different tomato compounds, including α-tomatine and phenolics.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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REFERENCES

Abete, I., Perez-Cornago, A., Navas-Carretero, S., Bondia-Pons, I., Zulet, M. A., & Martinez, J. A. (2013). A regular lycopene enriched tomato sauce consumption influences antioxidant status of healthy young-subjects: A crossover study. *Journal of Functional Foods*, 5, 28–35.

Andoh, Y., Okazaki, S., & Ueoka, R. (2013). Molecular dynamics study of lipid bilayers modeling the plasma membranes of normal murine thymocytes and leukemic GRSL cells. *Biochimica et Biophysica Acta*, *1828*, 1259–1270.

Arispe, N., & Doh, M. (2002). Plasma membrane cholesterol controls the cytotoxicity of Alzheimer's disease Abeta P (1–40) and (1–42) peptides. *The FASEB Journal*, 16, 1526–1536.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Cano, A., Costa, M., & Arnao, M. B. (2003). Hydrophilic and lipophilic antioxidant activity changes during on-vine ripening of tomatoes (*Lycopersicon esculentum Mill.*). *Postharvest Biology and Technology*, *28*, 59–65.

Chan, F. K., Moriwaki, K., & De Rosa, M. J. (2013). Detection of necrosis by release of lactate dehydrogenase activity. *Methods in Molecular Biology*, *979*, 65–70.

Choi, K. M., Lee, Y. S., Shin, D. M., Lee, S., Yoo, K., Lee, M. K., Lee, J., Kim, S. Y., Lee, Y., Hong, J., Yun, Y., & Yoo, H. (2013). Green tomato extract attenuates high-fat-diet-induced obesity through activation of the AMPK pathway in C57BL/6 mice. *Journal of Nutritional Biochemistry*, *24*, 335–342.

Choi, S. H., Kim, D. S., Kozukue, N., Kim, H. J., Nishitani, Y., Mizuno, M., Levin, C. E., & Friedman, M. (2014). Protein, free amino acid, phenolic, β-carotene, and lycopene content, and antioxidative and cancer cell inhibitory effects of 12 greenhouse-grown commercial cherry tomato varieties. *Journal of Food Composition and Analysis*, *34*, 115–127.

Choi, S. H., Kim, H., Kim, H., Lee, I., Kozukue, N., Levin, C. E., & Friedman, M. (2011). Free amino acid and phenolic contents and antioxidative and cancer cell-inhibiting activities of extracts of 11 greenhouse-grown tomato varieties and 13 tomato-based foods. *Journal of Agricultural and Food Chemistry*, *59*, 12801–12814.

Choi, S. H., Lee, S. H., Kim, H. J., Lee, I. S., Kozukue, N., Levin, C. E., & Friedman, M. (2010). Changes in free amino acid, phenolic, chlorophyll, carotenoid, and glycoalkaloid contents in tomatoes during 11 stages of growth and inhibition of cervical and lung human cancer cells by green tomato extracts. *Journal of Agricultural and Food Chemistry*, *58*, 7547–7556.

Do, T. N., Rosal, R. V., Drew, L., Raffo, A. J., Michl, J., Pincus, M. R., Friedman, F. K., Petrylak, D. P., Cassai, N., Szmulewicz, J., Sidhu, G., Fine, R. L., & Brandt-Rauf, P. W. (2003). Preferential induction of necrosis in human breast cancer cells by a p53 peptide derived from the MDM2 binding site. *Oncogene*, *22*, 1431–1444.

Dong, Z., Saikumar, P., Weinberg, J. M., & Venkatachalam, M. A. (1997). Internucleosomal DNA cleavage triggered by plasma membrane damage during necrotic cell death. Involvement of serine but not cysteine proteases. *American Journal of Pathology*, *151*, 1205–1213.

Dumas, Y., Dadomo, M., Di Lucca, G., & Grolier, P. (2003). Effects of environmental factors and agricultural techniques on antioxidant content of tomatoes. *Journal of the Sciences of Food and Agriculture*, *83*, 369–382.

Fraser, P. D., Enfissi, E. M. A., Halket, J. M., Truesdale, M. R., Yu, D., Gerrish, C., & Bramley, P. M. (2007). Manipulation of phytoene levels in tomato fruit: Effects on isoprenoids, plastids, and intermediary metabolism. *The Plant Cell*, *19*, 3194–3211.

Friedman, M., Levin, C. E., Lee, S. U., Kim, H. J., Lee, I., Byun, J., & Kozukue, N. (2009). Tomatine-containing green tomato extracts inhibit growth of human breast, colon, liver, and stomach cancer cells. *Journal of Agricultural and Food Chemistry*, *57*, 5727–5733.

Galano, E., Arciello, A., Piccoli, R., Monti, D. M., & Amoresano, A. (2014). A proteomic approach to investigate the effects of cadmium and lead on human primary renal cells. *Metallomics*, *6*, 587–597.

García-Valverde, V., Navarro-Gonzalez, I., Garcia-Alsonso, J., & Periago, M. J. (2013). Antioxidant bioactive compounds in selected industrial processing and fresh consumption tomato cultivars. *Food and Bioprocess Technology*, *6*, 391–402.

Ilahy, R., Hdider, C., Lenucci, M. S., Tlili, I., & D'Alessandro, G. (2011). Antioxidant activity and bioactive compound changes during fruit ripening of high-lycopene tomato cultivars. *Journal Food Composition and Analysis*, *24*, 588–595.

Itkin, M., Rogachev, I., Alkan, N., Rosenberg, T., Malitsky, S., Masini, L., Meir, S., Iijima, Y., Aoki, K., De Vos, R., Prusky, D., Burdman, S., Beekwilder, J., & Aharoni, A. (2011). Glycoalkaloid metabolism is required for steroidal alkaloid glycosylation and prevention of phytotoxicity in tomato. *The Plant Cell*, *23*, 4507–4525.

Kozukue, N., & Friedman, M. (2003). Tomatine, chlorophyll, β-carotene and lycopene content in tomatoes during growth and maturation. *Journal of the Science of Food and Agriculture*, *83*, 195–200.

Kozukue, N., Han, J. S., Lee, K. R., & Friedman, M. (2004). Dehydrotomatine and alpha-tomatine content in tomato fruits and vegetative plant tissues. *Journal of Agricultural and Food Chemistry*, *52*, 2079–2083.

Leonardi, C., Ambrosino, P., Esposito, F., & Fogliano, V. (2000). Antioxidative activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. *Journal of Agricultural and Food Chemistry*, *48*, 4723–4727.

Li, F., Li, S., Li, H. B., Deng, G. F., Ling, W. H., Wu, S., Xu, X. R., & Chen, F. (2013). Antiproliferative activity of peels, pulps and seeds of 61 fruits. *Journal of Functional Foods*, *5*, 1298–1309.

Li, H., Deng, Z., Liu, R., Loewen, S., & Tsao, R. (2013). Carotenoid compositions of coloured tomato cultivars and contribution to antioxidant activities and protection against H_2O_2-induced cell death in H9c2. *Food Chemistry*, *136*, 878–888.

Miller, J. N., & Rice-Evans, C. A. (1997). Factors influencing the antioxidant activity determined by the $ABTS^{\bullet+}$ radical cation assay. *Free Radical Research*, *26*, 195–199.

Mintz-Oron, S., Mandel, T., Rogachev, I., Feldberg, L., Lotan, O., Yativ, M., Wang, Z., Jetter, R., Venger, I., Adato, A., & Aharoni, A. (2008). Gene expression and metabolism in tomato fruit surface tissues. *Plant Physiology*, *147*, 823–851.

Moco, S., Capanoglu, E., Tikunov, Y., Bino, R. J., Boyacioglu, D., Hall, R. D., Vervoort, J., & De Vos, R. C. H. (2007). Tissue specialization at the metabolite level is perceived during the development of tomato fruit. *Journal of Experimental Botany*, *58*, 4131–4146.

Naviglio, D., Pizzolongo, F., Ferrara, L., Naviglio, B., Aragòn, A., & Santini, A. (2008). Extraction of pure lycopene from industrial tomato waste in water using the extractor Naviglio®. *African Journal of Food Science*, *2*, 37–44.

Niranjana, R., Gayathri, R., Mol, S. M., Sugawara, T., Hirata, T., Miyashita, K., & Ganesan, P. (2015). Carotenoids modulate the hallmarks of cancer cells. *Journal of Functional Foods*, doi:10.1016/j.jff.2014.10.017; (in press).

Oršolić, N., Gajski, G., Garaj-Vrhovac, V., Dikić, D., Prskalo, Z. Š., & Sirovina, D. (2011). DNA-protective effects of quercetin or naringenin in alloxan-induced diabetic mice. *European Journal of Pharmacology*, *656*, 110–118.

Raffo, A., Leonardi, C., Fogliano, V., Ambrosino, P., Salucci, M., Gennaro, L., Bugianesi, R., Giuffrida, F., & Quaglia, G. (2002). Nutritional value of cherry tomatoes (Lycopersicon esculentum Cv. Naomi F1) harvested at different ripening stages. *Journal of Agricultural and Food Chemistry*, *50*, 6550–6556.

Raiola, A., Rigano, M. M., Calafiore, R., Frusciante, L., & Barone, A. (2014). Enhancing the health-promoting effects of tomato fruit for biofortified food. *Mediators of Inflammation*, *2014*, 139873. doi:10.1155/2014/139873.

Rick, C. M., Uhlig, J. W., & Jones, A. D. (1994). High alpha-tomatine content in ripe fruit of Andean Lycopersicon esculentum var. cerasiforme: Developmental and genetic aspects. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 12877–12881.

Rigano, M. M., Raiola, A., Tenore, G. C., Monti, D. M., Del Giudice, R., Frusciante, L., & Barone, A. (2014). Quantitative trait loci pyramiding can improve the nutritional potential of tomato (Solanum lycopersicum) fruits. *Journal of Agricultural and Food Chemistry*, *62*, 11519–11527.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology Viticulture*, *16*, 144–158.

Stevens, R., Buret, M., Garchery, C., Carretero, Y., & Causse, M. (2006). Technique for rapid small-scale analysis of vitamin C levels in fruit and application to a tomato mutant collection. *Journal of Agricultural and Food Chemistry*, *54*, 6159–6165.

Sucha, L., Hroch, M., Rezacova, M., Rudolf, E., Havelek, R., Sispera, L., Cmielova, J., Kohlerova, R., Bezrouk, A., & Tomsik, P. (2013). The cytotoxic effect of α-tomatine in MCF-7 human adenocarcinoma breast cancer cells depends on its

interaction with cholesterol in incubation media and does not involve apoptosis induction. *Oncology Report*, *30*, 2593–2602.

Tohge, T., Alseekh, S., & Fernie, A. R. (2014). On the regulation and function of secondary metabolism during fruit development and ripening. *Journal of Experimental Botany*, *65*, 4599–4611.

Weaver, J., Briscoe, T., Hou, M., Goodman, C., Kata, S., Ross, H., McDougall, G., Stewart, D., & Riches, A. (2009). Strawberry polyphenols are equally cytotoxic to tumorigenic and normal human breast and prostate cell lines. *International Journal of Oncology*, *34*, 777–786.

Wellburn, A. R. (1994). The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of Plant Physiology*, *144*, 307–313.

Yamasaki, H., Sakihama, Y., & Ikehara, N. (1997). Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. *Plant Physiology*, *115*, 1405–1412.