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In vitro Antitumour Activity of Tomato-Extracted Carotenoids on Human Colorectal Carcinoma

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Abstract

The aim of this research was to establish whether *all-trans* lycopene extracted from fresh and frozen tomatoes is able to inhibit the *in vitro* proliferation of colon cancer cells, to trigger apoptosis by reactive oxygen species modulation and to reveal its influence on NF-kβ signalling, through the p65 transcription factor and expression of two TNF receptors: GITR and CD27. The carotenoid extracts containing *all-trans* lycopene were obtained from fresh (E1) and frozen/thawed (E2) tomatoes (*Lycopersicon esculentum Mill.*), hybrid 'Menhir' F1. DLD-1 and HT-29 human colon adenocarcinoma cell lines were co-cultivated with the two extracts and cytotoxicity, apoptosis, antioxidant activity, reactive oxygen species as well as modulation of NF-kβ signalling pathway were assessed. Tomato extracts E1 and E2 were able to inhibit colon cancer cell growth *in vitro*. E2 contained a higher proportion of *all-trans* lycopene and displayed superior cytotoxicity and a better apoptosis inducing capacity. The two extracts proved antioxidant activity against DPPH radicals and were able to scavenge the reactive oxygen species in the treated tumour cells. This study also showed that lycopene acts mainly through p65 protein and moderately by TNF receptors GITR and CD27 to deactivate the NF-kβ signalling pathway involved in cancer cell proliferation.

Keywords: antioxidant, apoptosis, carotenoid, cytotoxicity, NF-kβ pathway, tomato, tumour

Introduction

Colorectal cancer is the third most common neoplasm in the world, with a high rate of incidence and mortality worldwide (Siegel *et al.*, 2014). Colon adenocarcinoma is related to diet (Ravasco *et al., 2012*), while some natural bioactive compounds such as lycopene proved to have cancer preventing properties (Bohn *et al.*, 2013). Lycopene was intensively studied for its therapeutic potential (Friedman *et al.*, 2009; Lin *et al.*, 2011; Teodoro *et al.*, 2012; Trejo-Solis *et al.*, 2013) and also included in clinical trials (Breemen and Pajkovic, 2008). *All-trans* lycopene, a polyunsaturated hydrocarbon, is an abundant carotenoid contained in tomatoes (Gupta *et al.*, 2013); this compound displays remarkable antioxidant properties and antiproliferative effect against various cancer cell types (Choi *et al.*, 2011) based on its capacity to influence the cell cycle (Teodoro *et al.*, 2012) and activate programmed cells death mechanisms (Soares *et al.*, 2013).

Several studies reveal novel mechanistic approaches regarding the lycopene antitumour properties. Lycopene modulates the ROS production (Makon-Sebastien *et al.*, 2014), possesses a robust antioxidant activity due to its singlet oxygen and free radicals quencher ability, and also protects mammalian cells against lipid peroxidation and oxidative DNA damage (Trejo-Solis *et al.*, 2013). Lycopene mediates apoptosis *via* death receptors, upregulates the anti-apoptotic Bax protein and Fas ligand (Tang *et al.*, 2009) and moreover, downregulates the expression of cyclin D and the antiapoptotic proteins Bcl-2 and Bcl-XL blocking the survival of human colon adenocarcinoma cells (Palozza *et al.*, 2007).

The upregulation of nuclear factor kappa β (NF-kβ) transcription contributes to cancer progression (Linnewiel-

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Hermoni *et al.*, 2014). Lycopene was shown to inhibit NF-kβ activity in various cell lines (Linnewiel-Hermoni *et al.*, 2014; Palozza *et al.*, 2003). This molecule was found to be active in a variety of tumours, and it is a therapeutic target in cancer (Mauro *et al.*, 2009). The efforts to block this transcription factor are focused not only on NF-kβ itself, but also on other molecules from its signalling pathway; the attenuation of NF-kβ signalling (Armoza *et al.*, 2013) is linked to vascular endothelial function (Cenariu *et al.*, 2015) and to the tumour necrosis factor TNF-alpha (Makon-Sebastien *et al.*, 2014). The p65 subunit of NF-kβ is a co-regulator protein, and the activation of NF-kβ transcription factor can be quantified evaluating the extent of nuclear p65 translocation (Lafarge *et al.*, 2007).

GITR, the glucocorticoid-induced tumour necrosis factor receptor-related gene TNFSRSF18 from the TNFR superfamily, is expressed in malignant colon cell lines (Baltz *et al.*, 2007) and has an interesting potential for anti-cancer therapy (Nocentini *et al.*, 2012) by tumour growth reduction and immune response enhancement. Another member of the TNFR family which can activate the NF-kβ pathway (Boursalian *et al.*, 2009) is the transmembrane glycoprotein CD27 or TNFRSF7, which is involved in cell proliferation and survival. The overexpression of CD27 does not influence significantly the NF-kβ p65 protein levels (Yamamoto *et al.*, 1998), but binding to its ligand, CD70, it is able to augment significantly the NF-kβ signalling. Both TNF receptors GITR (Spinicelli *et al.*, 2002) and CD27 (Prasad *et al.*, 1997) display the same apoptosis pathway involving the proapoptotic Siva protein and activate NF-kβ in the same way by TRAF-2 binding.

There are no previous studies regarding lycopene influence on GITR and CD27 membrane markers.

An important genetic alteration in colorectal cancer is the mutation of Kras gene, which is a member of the Ras subfamily of GTPase proteins, having an important role in cellular signal transduction. Palozza *et al.* (2010) demonstrated that lycopene treatment in colon cancer changes the cytoplasmatic accumulation of Ras by translocation from cell membranes to cytosol and reduces the Ras-dependent activation of NF-kβ. Previously, the effect of lycopene was described on HT-29 Kras wild type human colon carcinoma (Guil-Guerrero *et al.*, 2011; Palozza *et al.*, 2007; Soares *et al.*, 2013), and also on K-ras mutant tumours (Palozza *et al.,* 2010), but no reports were found until now regarding the lycopene effect on the highly proliferative DLD-1, Kras mutant (Khan *et al.*, 2014) colon adenocarcinoma cell line. The colorectal carcinoma cells constitutively express NF-kβ p65 protein (Damnjanovic *et al.*, 2014; Liu *et al.*, 2012; Liu *et al.*, 2014) and GITR (Baltz *et al.*, 2007), therefore the two malignant colon cell populations are suitable to study the implication of these molecules in lycopene antitumour activity.

The aim of this research was to establish whether *all-trans* lycopene extracted from fresh and frozen tomatoes is able to inhibit the *in vitro* proliferation of colon cancer cells, to trigger apoptosis by reactive oxygen species modulation and to reveal its influence on NF-kβ signalling, through the p65 transcription factor and expression of two TNF receptors: GITR and CD27. The carotenoid extracts containing *all-trans* lycopene were obtained from fresh (E1) and frozen/thawed (E2) tomatoes (*Lycopersicon esculentum Mill.*), hybrid 'Menhir' F1. This hybrid is suitable for cultivation both for production cycle I and II. The fruits weigh 160-190 grams/fruit (average values), with fast and simultaneous ripening ability and a great yield/earliness/taste ratio. Our study proved an adequate total carotenoid content for both extracts (E1-11.76 mg/100 g f.w., E2-8.89 mg/100 g f.w) the main carotenoid being *all-trans* lycopene (E1-6.46 mg/100 g f.w., E2-6.78 mg/100 g f.w.). The extracts also contained smaller amounts of β-carotene and γ-carotene and other minor components, like lycopene epoxide, *cis*-isomers of lycopene and the colourless carotenoids phytoene and phytofluene. All experiments were performed in triplicate, and repeated three times. IC50 was assessed with serial dilutions of the extracts that were made taking into consideration the concentration of *all-trans* lycopene (between 0.5 μM and 200 μM *all-trans* lycopene in culture medium). Subsequently, only the IC50 was used for future investigations. The antitumour activity of the extracts was studied on DLD-1 and HT-29 human colon adenocarcinoma cell lines.

Materials and Methods

Carotenoid extraction

The carotenoid extracts were obtained from fresh (E1) and frozen/thawed (E2) tomatoes (*Lycopersicon esculentum Mill.*), hybrid 'Menhir' F1, organically cultivated in greenhouse tunnels and harvested at full maturity in August 2014.

Tomato samples were extracted three times with a mixture of light petroleum/ethyl acetate/methanol (1:1:1, v/v/v) (Breithaupt and Schwack, 2000). The extracts were transferred in a separation funnel and water was added to allow phase separation. The organic phase was filtered over anhydrous sodium sulphate, evaporated to dry and kept at -20°C until HPLC analysis.

The carotenoid residue was transferred quantitatively in methyl-*tert*-butyl ether (MTBE), filtered through PTFE 0.45 μm filters and subjected to HPLC-PDA analysis. Separation of carotenoids was performed using a Shimadzu LC20 AT high performance liquid chromatograph (HPLC) with a SPD-M20A diode array detector and an YMC C30 column (24 cm x 4.6 mm, 5 μm). The mobile phases were methanol (solvent A) and MTBE (solvent B) and the linear gradient was: at 0 min - 100 % A (0 % solvent B) to 60 min - 0 % A (100 %) 62 min 100 % A, followed by equilibration of column for 10 min (Carillo-Lopez *et al.*, 2014). The flow rate was fixed at 1.0 ml/min and the DAD detector was set at 470 nm.

Identification of carotenoids in samples was carried out by comparing the retention time and the UV-Vis absorption spectrum of each compound with those of available standard compounds. Standard compounds β-carotene and lycopene were provided by LGC Standards, UK. The other compounds were tentatively identified according to their UV-VIS absorption spectra. Calibration curves were made with the standard compounds by plotting peak area against concentration for five concentrations ranging from 1-50 μg/ml.

In vitro testing

In vitro testing was performed on human tumoural colon cell lines: DLD-1 and HT-29, both purchased from the European Cell Culture Collection. DLD-1 cells were grown in RPMI-1640,

while HT-29 were cultivated in McCoy's 5 cell culture media; both supplemented with fetal bovine serum (FCS) and glutamine (all media and supplements from Sigma Aldrich, St Louis, MO, USA).

Cells were removed using enzymatic methods at subconfluency, resuspended and seeded into 96-well microplates, about 15x 10^3 cells/well in 190 µl media, and on 6-well plates, respectively, 2.5x10^5 cells in 2.85 ml media (Nunclon delta surface plates from Thermo Fischer Scientific, Waltham, MA, USA). After 24-hours of incubation, once cells were attached to the surface and the proliferation begun, wells were treated with the extracts and with the reference compound, an *all-trans* lycopene standard, MW 536.87 (Fluorochem Ltd, Hadfield, UK). The treatment was made using a quantity of 10 µl solution/well in 96-well plates, and 150 µl in 6-well plates. The fresh tomatoes (E1) and frozen tomatoes (E2) extracts as well as the *all-trans* lycopene standard solution were dissolved in tetrahydrofuran (THF, Sigma), obtaining 2mM *all-trans* lycopene stock solutions. Stock solutions were diluted in PBS, to obtain serial dilutions for treatment; final concentrations in cell culture media were between 0.5 µM and 200 µM *all-trans* lycopene. These serial dilutions were used in order to establish the IC50. After establishing the IC50 for each of the extracts as well as for the lycopene standard compound, the following experiments were performed with the IC50 concentration.

Cytotoxicity

The cytotoxicity of the compounds was measured in colorimetry using the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, from Sigma Aldrich), following a protocol described before (Fischer-Fodor *et al.*, 2014). In living cells, the MTT is transformed into insoluble formazan as a result of the mitochondrial enzymatic activity; formazan crystals were solubilized in dimethylsulphoxyde (Titolchimica, Italy), and the 96-well plates were measured with a Synergy 2.0 microplate reader (from BioTek Company, Winooski, USA) at 570nm wavelength. Three independent measurements were performed for every compound, on both cell lines. The absorbance values reflect the number of viable cells; IC50 values were calculated using the sigmoidal dose-response response relationship, in the 95% confidence interval. As reference we used THF treated cells, and as positive control the standard aqueous lycopene solution. The toxicity of THF was also measured separately, using the same series of THF concentrations as for extracts solubilisation and compared to untreated cells; none of the cell lines were affected by THF in the studied concentration range (0.001-2.5% THF in PBS).

Apoptosis

For apoptosis testing the Alexa Fluor 488 Annexin V/Dead cell apoptosis kit (Invitrogen, Eugene, USA) was employed. Cells treated with the two extracts as well as with the lycopene standard at the IC50 concentration were incubated with fluorescent-labelled Annexin V to mark the apoptotic cells, than treated with propidium iodide (PI) solution to show dead cells, as described before (Miklasova *et al.*, 2014). Samples were analyzed by flow cytometry, with a FACS Canto II Flow cytometer, (BD Biosciences, San Jose, CA, USA) using the 488-nm, blue, air-cooled, 20-mW solid-state excitation laser. Fluorescence was detected using the 530/30 filter for Alexa Fluor 488 and 575/26 filter for PI. As reference we used THF treated cells.

Membrane and intracytoplasmatic markers assessment

For membrane and intracytoplasmatic markers assessment, NF-kβ primary antibody and PE-labelled secondary antibody were acquired from R&D Systems, while the Inside Stain kit for cell permeabilization, GITR-APC and CD27-FITC antibodies were from Miltenyi Biotech, Bergisch Gladbach, Germany. Cells treated with the two extracts as well as with the lycopene standard at the IC50 concentration were stained according to the manufacturer's protocol. As reference we used THF treated cells. The samples were analyzed by flow cytometry, with a FACS Canto II Flow cytometer, (BD Biosciences, San Jose, CA, USA) using the 488-nm, blue, air-cooled, 20-mW solid state excitation laser and the 530/30 filter for FITC as well as the 585/42 filter for PE. The 633-nm, red, 17-mW HeNe excitation laser and the 660/20 filter was used to detect APC.

Antioxidant activity

The antioxidant activity was evaluated by the extract's capacity of quenching the 2,2- diphenyl-1-picrylhydrazyl radical (DPPH, from Sigma Aldrich) as described earlier (Tamokou *et al.*, 2013). The phenomenon was highlighted by an oxidoreductive colour change when 5 µl of the 2mM extract reacted with pink-purple 0.0025% DPPH solution, and was measured at 520 nm using the UV/VIS spectrophotometer (V-530 equipment from Jasco). The reference median extinction of unreacted DPPH was 0.7817 at 520 nm.

Reactive oxygen species

Assessment of reactive oxygen species (ROS) in cell cultures treated with the IC50 concentration of the tomato extracts was made using the CM-H2DCFDA General Oxidative Stress Indicator kit (Life Technologies, Carlsbad, Germany). The active compound of the kit, the acetyl ester of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate diffuses into the cells, undergoes a transformation into a highly fluorescent 2',7'-dichlorofluorescein (DCF) adduct, due to the interactions with intracellular esterases and intracellular glutathione oxidation. The treated cells were removed from cell culture plates and washed with phosphate buffered saline (PBS), counted and an equal quantity of 4.5x10^5 HT-29, and 5.5 x10^5 DLD-1 cells were subjected to staining. The cells were incubated with 10 µM CM-H2DCFDA solution for 30 minutes, and then washed twice with Hepes solution (buffers from Sigma Aldrich). The samples were processed in triplicate; fluorescence measurement at 485 nm excitation/528 nm absorption was performed with a Synergy 2 equipment (BioTek Company, Winooski, USA).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5 software (from GraphPad Software, La Jolla, USA). All analyses were performed in triplicate and repeated three times. Data obtained for the two extracts was compared with each other and with that obtained for cells treated only with THF as well as treated with the *all-trans* lycopene standard. Various statistical tests were performed, as mentioned for each analysis in particular.

Results and Discussion

Carotenoid composition

Fresh tomatoes (E1) had a total carotenoid content of 11.76 mg/100 g fresh weight. The major carotenoid was lycopene, with smaller amounts of β-carotene and γ-carotene. Frozen tomatoes (E2) had a total carotenoid content of 8.89 mg/100 g, most of

Table 1. Carotenoid composition of fresh and frozen tomatoes

Peak no.	Compound	Retention time (min)	Fresh (E1) mg/100 g f.w.	Frozen (E2) mg/100 g f.w.
1	β-Carotene	28.4	3.11	0.95
2	γ-Carotene	39.3	1.32	0.33
3	Lycopene epoxide	44.3	0.25	0.18
4	*All-trans* Lycopene	48.8	6.46	6.78
5	*Cis*-Lycopene	49.3	0.61	0.64

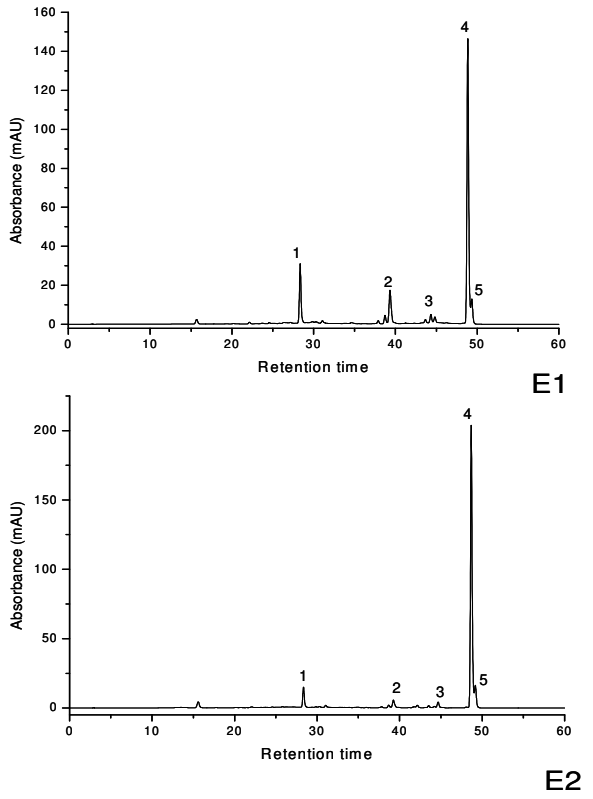

Fig. 1. Chromatograms showing HPLC-PDA separation of carotenoids in fresh (E1) and frozen (E2) tomato extracts. Each peak represents a carotenoid compound. For peak identification see Table 1

Table 2. Half inhibitory concentrations for *all-trans* lycopene from tomato extracts E1 and E2, and the reference purified compound; median values of 3 independent measurements on DLD-1 and HT-29 colon cancer cell lines. Differences between the two extracts as well as between each of the extracts and the *all-trans* lycopene standard are statistically significant (unpaired t-test, one tailed p value <0.05). When THF alone was added, it had no inhibitory effect, similar to untreated cells

Parameters	IC50 values (μM)		
	Extract 1	Extract 2	*All-trans* lycopene
DLD-1			
Median value	65.66[a]	37.24[b]	9.21[c]
SEM	4.28	1.87	1.12
HT-29			
Median value	455.37[a]	242.70[b]	19.32[c]
SEM	11.84	6.90	0.91

Note: On the same row, the same superscript letter following 2 values shows no statistically significant difference between them, while a different letter signifies a statistically significant difference (unpaired t-test, one tailed p value <0.05).

which was also lycopene. Some other minor compounds were present in both extracts, like lycopene epoxide, *cis*-isomers of lycopene and the colourless carotenoids phytoene and phytofluene (Table 1, Fig. 1).

Cytotoxicity

Extract 2 (E2) displayed a superior inhibitory effect against tumour cells having lower IC50 values than extract 1 (E1), and both were less toxic than the purified *all-trans* lycopene solution (Table 2). Statistically significant differences were found between the two extracts as well as between each of the extracts and the *all-trans* lycopene standard (unpaired t-test, one tailed p value <0.05).

Apoptosis

The K-ras mutant DLD-1 cells and the K-ras wild type HT-29 colon cancer cells were subjected to a 24-hours treatment with the *all-trans* lycopene containing extract 1 and 2, an the results were compared with the same concentration of pure lycopene in aqueous solution. The percent of apoptotic cells was better for E1 and E2 when compared to the reference compound, in both cell lines, (one-way analysis of variance, Bonferroni multiple comparison test, 95% confidence interval, p<0.001, very significant). The same amount of positive reference compound induced more necrosis in both cell lines and the proportion of apoptotic/necrotic cells is better for the extracts (Fig. 2 and 3). Instead, the late apoptotic stage represented by the double coloration was better represented when the standardized lycopene compound was used (one-way analysis of variance, p<0.001). This indicates that the transition of cells towards the late apoptotic stages is faster for the pure *all-trans* lycopene as for E1 and E2, where the same amount of *all-trans* lycopene is present in a combination with other isomers. Since E2 contains slightly more *all-trans* lycopene than E1, the overall survival of cells in both cell lines was correlated with the proportion of *all-trans* lycopene from the extracts. The extracts capacity to trigger apoptosis was more efficient in HT-29 cell line, even if the IC50 values in HT-29 were noticeably bigger (Table 2), indicating that the *all-trans* lycopene induced cell death occurred by other cell death mechanisms as well.

The antioxidant activity (AOA)

The antioxidant capacity of the *all-trans* lycopene-containing extracts was expressed as percent of DPPH radical inhibition, related to unreacted DPPH. AOA values for *all-trans* lycopene containing extracts E1 and E2 are statistically significant compared with the basal DPPH value, and markedly exceed the reference *all-trans* lycopene antioxidant properties (Fig. 4). The higher antioxidant capacity of the extracts, compared to pure lycopene, can be due to presence of other carotenoids or tocopherols.

Reactive oxygen species (ROS) reduction

In DLD-1 cells, extract E2 reduced considerably the ROS level, while E1 had no statistically significant influence; both compounds ROS scavenging capacity was below the pure lycopene (one-way analysis of variance, Bonferroni multiple post-test, p<0.01). In HT-29 cells the ROS reduction was significant for E1, E2 and the reference lycopene, the extract E2 having similar action as the pure compound (Fig. 5). The ROS reduction was more efficient following the cells treatment with E2, being associated with a higher cytotoxicity (Table 2) and a larger proportion of cells in late apoptosis (Figs. 2 and 3).

Table 3. The modulation of NF-kβ, GITR and CD27 positive cells within colon cancer cell populations. The percent of PE-stained NF-kβ, APC-GITR and FITC labelled CD27 was calculated by FACS Diva 6.1 software after analyzing 10000 events. All determinations were performed in triplicate; the values shown represent medians of the three determinations, and standard deviation (SD) is shown next to each value. The graphical representation of these results is shown in Figs. 6 and 7

	THF % (±SD)	Extract 1 % (±SD)	Extract 2 % (±SD)	Reference *all-trans* lycopene % (±SD)
DLD-1 cells				
NF-kβ$^+$	30.9^a (±0.2)	27.8^b (±0.3)	27.7^b (±0.3)	5.1^c (±0.2)
GITR$^+$	3.6^a (±0.1)	5.4^a (±0.1)	17.5^b (±0.2)	18.7^b (±0.3)
CD27$^+$	0.2^a (±0.1)	1.5^a (±0.1)	1.8^a (±0.1)	17.1^b (±0.1)
NF-kβ$^+$ GITR$^+$	21.2^a (±0.2)	43.7^b (±0.3)	29.6^c (±0.2)	71.9^d (±0.3)
NF-kβ$^+$ CD27$^+$	0.3^a (±0.1)	12.5^b (±0.1)	6.0^c (±0.2)	74.1^d (±0.2)
GITR$^+$ CD27$^+$	0.5^a (±0.1)	9.6^b (±0.1)	5.7^b (±0.1)	85.4^c (±0.2)
HT-29 cells				
NF-kβ $^+$	19.4^a (±0.1)	8.8^b (±0.1)	13.1^b (±0.1)	33.1^c (±0.3)
GITR$^+$	4.8^a (±0.1)	11.0^b (±0.2)	7.6^c (±0.2)	2.8^d (±0.1)
CD27$^+$	0.0^a (±0.0)	0.0^a (±0.0)	0.0^a (±0.0)	3.1^b (±0.2)
NF-kβ$^+$ GITR$^+$	6.9^a (±0.1)	5.2^a (±0.3)	5.3^a (±0.1)	21.7^b (±0.4)
NF-kβ$^+$ CD27$^+$	0.0^a (±0.0)	0.1^a (±0.0)	0.0^a (±0.0)	9.1^b (±0.2)
GITR$^+$ CD27$^+$	0.0^a (±0.0)	0.0^a (±0.0)	0.0^a (±0.0)	9.4^b (±0.2)

Note: On the same row, the same superscript letter following 2 values shows no statistically significant difference between them, while a different letter signifies a statistically significant difference (unpaired t-test, one tailed p value <0.05).

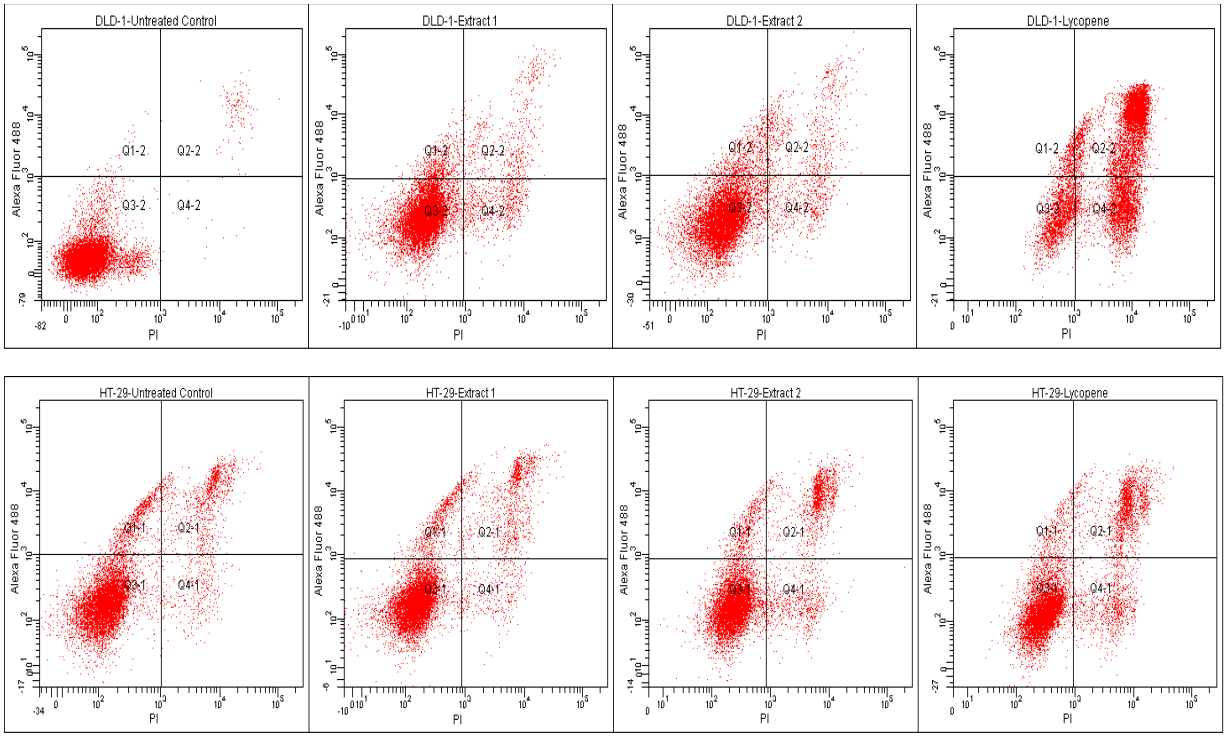

Fig. 2. Histogrames obtained by flow cytometry showing apoptosis versus necrosis in the two colon cancer cell populations: DLD-1 (upper row) and HT-29 (lower row)

The upper left quadrand contains apoptotic cells stained with Alexa Fluor 488, while the lower right quadrand contains necrotic cells stained with Propidium Iodide (PI). A fraction of the colon cancer cells display both colorations (upper right quadrant), which denotes that they are in a transition phase towards late apoptosis. Cells shown in the lower left quadrant are viable, negative for both colorations. From left to right: controls, *all-trans* lycopene-containing extract 1, extract 2 and the reference pure *all-trans* lycopene

Deactivation of NF-kβ pathway

The two extracts (E1 and E2) inhibited the intracellular NF-kβ expression (Table 3). The inhibitory capacity of E1 and E2 was lower than that of the pure *all-trans* lycopene in DLD-1 cells, but was superior in HT-29 cells.

In both cell lines, the surface epitope TNFRSF18/GITR was overexpressed following the treatment with E1 and E2 (Fig. 6), although the tendency of double positive NF-kβ$^+$GITR$^+$ cells expression was divergent: enhanced in DLD-1 cells and diminished in HT-29 cells (unpaired t-test, one tailed p value <0.05).

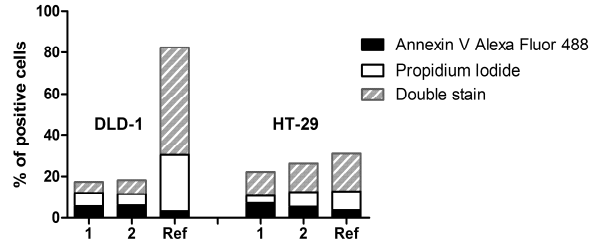

Fig. 3. Grouped representation of the proportion of early and late apoptosis and necrosis which occurs in the colon cancer cells treated with *all-trans* lycopene from tomato extracts E1 and E2, and the positive reference *all-trans* lycopene (abbreviated as Ref). All analyses were performed in triplicate. The average values show apoptosis being statistically higher for E1 and E2 when compared to the reference compound, in both cell lines, while the late apoptotic stage and necrosis is more significant in the standardized all-trans lycopene (one-way analysis of variance, Bonferroni multiple comparison test, 95% confidence interval, $p<0.001$, very significant)

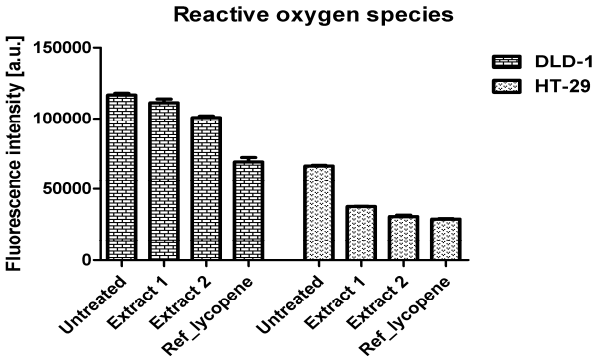

Fig. 5. Reactive oxygen species level in treated colon tumour cells displayed as fluorescence intensity of CM-H2DCFDA. The *all-trans* lycopene from tomato extracts E1 and E2 was compared to THF treated cells, and with a reference compound, the pure lycopene. All analyses were performed in triplicate. The average values for DLD-1 show that E2 had a statistically significant influence, while E1 did not, and both extracts had a lower capacity to scavenge ROS than pure lycopene. For HT-29, the ROS reduction was statistically significant for E1, E2 and the reference lycopene, E2 being almost as effective as the pure compound (one-way analysis of variance, Bonferroni multiple post-test, $p<0.01$)

CD27 is not constitutively expressed in colorectal cells; there are few data regarding CD27 gene expression (Fan *et al.*, 2004). We obtained very low basal values in THF treated cells. In DLD-1 cell line, CD27 was overexpressed following both E1 and E2 treatments, accompanied by an increase of double positive NF-kβ$^+$CD27$^+$ and GITR$^+$CD27$^+$ cells, without correlations between the data sets (Wilcoxon signed rank test, $p>0.05$). The overexpression of CD27 was related to an increased apoptosis (Fig.6), but there was no statistically significant correlation between the two data sets (nonparametric Spearman correlation, two-tailed p value >0.5). In HT-29, only the pure *all-trans* lycopene was able to increase the CD27 expression, together with a strong increase in NF-kβ$^+$CD27$^+$ and GITR$^+$CD27$^+$ cells ratio (Table 3, Fig. 7).

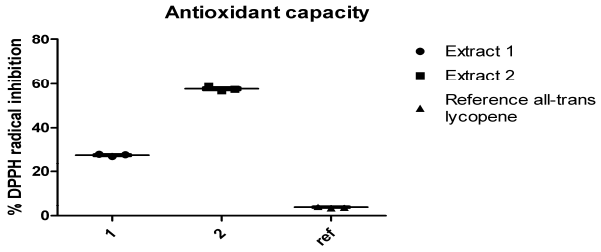

Fig. 4. The *all-trans* lycopene-containing tomato extracts antioxidant activity measured by DPPH radical inhibitory capacity. All analyses were performed in triplicate. The average values for E1 and E2 are statistically significant compared with the basal DPPH value, and markedly exceed the reference all-trans lycopene antioxidant properties (unpaired t-test, one tailed p value <0.05)

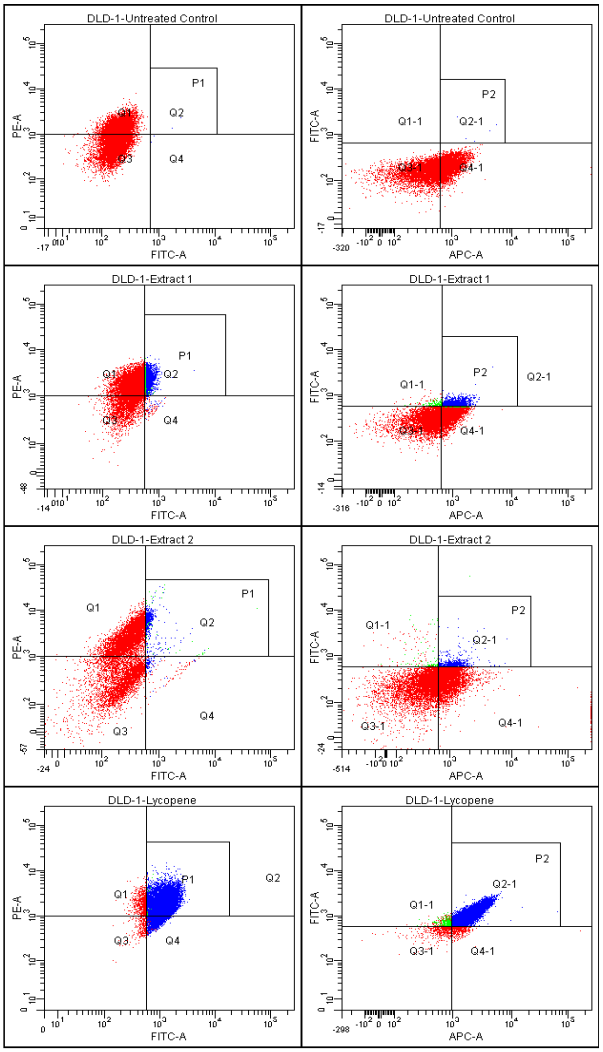

Fig. 6. Distribution of intracellular NF-kβ and membrane epitopes GITR and CD27 in DLD-1 K-ras mutant cancer cell line after the treatment with E1, E2 and the reference pure *all-trans* lycopene. From top to bottom: THF treated cells, cells treated with E1, E2 and purified lycopene; fluorescent staining: NF-kβ PE, GITR APC and CD27 FITC. All analyses were performed in triplicate. For precise values and statistic interpretation, see Table 3

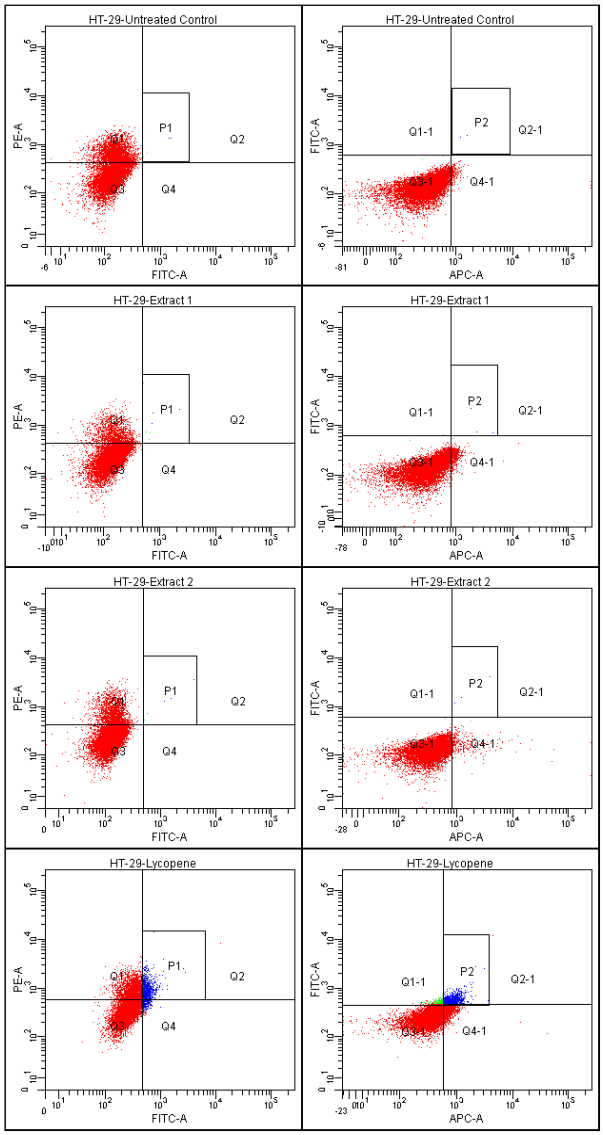

Fig. 7. Expression of NF-kβ, GITR and CD27 positive cells in HT-29 population treated with *all-trans* lycopene-containing tomato extracts. From top to bottom: THF treated cells, cells treated with E1, E2 and purified lycopene; fluorescent staining: NF-kβ PE, GITR APC and CD27 FITC. All analyses were performed in triplicate. For precise values and statistic interpretation, see Table 3

No correlation was found between NF-kβ, GITR and CD27 expression in the two colon tumour cell populations; the activated p65 NF- kβ values were slightly reduced in DLD-1 K-ras mutant cells, despite GITR and CD27 augmentation. The reduction of p65 NF- kβ by E1 and E2 was more pronounced in K-ras wild-type HT-29 cells, where it was accompanied by NF-kβ$^+$ GITR$^+$ decrease, even if the NF-kβ$^-$ GITR$^+$ cells proportion had a raising tendency.

Thus, the two extracts acted divergently in the two cell lines: in HT-29 cells, E1 and E2 possessed superior capacity to inhibit NF-kβ signalling than the reference purified compound, while in DLD-1 this trend was opposite. This suggests that the NF-kβ p65 activation was not inhibited in the K-ras wild type colon cells, despite better cytotoxicity (Table 2) and more efficient apoptosis triggering (Fig. 3). Other cell death mechanisms were involved in this case, that need further investigation.

The inhibitory effect of lycopene is dose-related (Teodoro *et al.*, 2012) which is in concordance with our findings. Although E2 was obtained from frozen tomatoes, we consider that the freezing process did not interfere with the extract's cytotoxicity or apoptosis inducing ability. It was rather the fact that it contained a slightly higher proportion of *all-trans* lycopene than E1 that made it display superior cytotoxicity and a better apoptosis inducing capacity.

The K-ras mutant DLD-1 cells proliferation was stronger affected by *all-trans* lycopene than the K-ras wild type, in concordance with previous studies on HCT-116 cells (Palozza *et al.*, 2010), proving the lycopene interference with Ras-dependent signalling. Despite their common K-ras mutation on codon 13 (G13D), HCT116 and DLD-1 genetic profiles are not identical: the BRCA2 mutation is present only on HCT-116, not on DLD-1 (Khan *et al.*, 2014) and they contain different mutation of PIK3CA: HCT116 has an H1047R alteration in exon 20, while DLD-1 contains an E545K alteration in exon 9 (Samuels *et al.*, 2005). Both cell lines are BRAF-wild type, different from HT-29 which displays BRAF, SMAD4, PIK3CA3 (phosphoinositide-3-kinase, catalytic, alpha polypeptide) and TP53 mutant protein sequences (Ikediobi, 2008), but no K-ras mutations.

Lycopene reduces the reactive oxygen species (Palozza *et al.,* 2012) in normal, but also in tumour cells, resulting in a dual outcome: ROS reduction protects the tumour cell from DNA damages, and regulates the redox signal in cancer by controlling Nrf2, modulation ROS-production enzymes, MAP kinases and inhibiting transcription factors, such as NF-kβ.

The extracts 1 and 2 inhibited the intracellular NF-kβ expression, in concordance with the previously communicated data (Armoza *et al.*, 2013; Linnewiel-Hermoni *et al.*, 2014; Mauro *et al.*, 2009; Palozza *et al.*, 2010).

Conclusions

Tomato extracts E1 and E2 were able to inhibit colon cancer cell growth *in vitro*; E2 contained a higher proportion of *all-trans* lycopene and displayed superior cytotoxicity and a better apoptosis inducing capacity. The two extracts proved antioxidant activity against DPPH radicals, and their capacity to scavenge the reactive oxygen species in the treated tumour cells followed the same trend. The *all-trans* lycopene rich extracts capacity to inhibit intracellular NF-kβ p65 protein opens the perspectives of antitumour applications. The concomitant examination of three molecules involved in the NF-kβ transcription events enlighten that lycopene was able to modulate their expression, in both tumoural cell lines. This study opens new perspectives regarding in vivo studies concerning the antitumoural effect of lycopene and other tomato-extracted carotenoids on colorectal carcinoma.

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