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ASSESSMENT OF THE ANTIOXIDANT PROPERTIES OF TOMATO EXTRACTS: A SYNERGISTIC APPROACH USING IN VITRO CHEMICAL TESTS AND CELL-BASED ASSAYS

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The aim of this research was to assess the total antioxidant activity (TAA) of lipophilic (L_{extr}) and hydrophilic (H_{extr}) tomato extracts using in vitro chemical tests and cell-based assays, focusing on possible synergistic actions between tomato antioxidants. Both H_{extr} and L_{extr} were HPLC analysed for their carotenoids, phenolic compounds, and ascorbic acid contents. For the evaluation of TAA, extracts were assayed alone or in combination using in vitro chemical tests (TEAC, FRAP) and cell-based (CAA) assays using human hepatoma (HepG2) and human histiocytic lymphoma (U937) cells. The only carotenoid detected in L_{extr} was lycopene, while a mixture of phenolic compounds (chlorogenic acid, caffeic acid, and rutin) was identified in H_{extr}. Ascorbic acid was not found either in H_{extr} or in L_{extr}. Upon extract combination (1:1, v/v), the FRAP assay revealed additive action between L_{extr} and H_{extr}, whilst a slight synergistic action was observed in TAA as measured by the TEAC assay. Synergistic action was better revealed when TAA was analysed using either U937 or HepG2 cells. This could be explained by the presence of a multiphase media (cell membrane and extra- and intracellular media) that might facilitate the distribution and interaction of antioxidants with different polarities and different mechanisms of action.

Keywords: lycopene, phenolics, total antioxidant activity, cell cultures assay

Nutrition plays a role in the prevention of chronic diseases related to oxidative stress such as cancer, cardiovascular, neurodegenerative, or inflammatory diseases. Epidemiological studies have shown that regular consumption of plant-foods, which are natural sources of dietary antioxidants, is associated with reduced risk of such diseases (Vetrani et al., 2013). In this context, it has been proposed that the antioxidant potential of foods and diets may arise from additive and synergistic actions of the different antioxidant phytochemicals present in the food matrix (Liu, 2003; Zanfini et al., 2010; Wang et al., 2011). Synergism is, in general, the phenomenon in which a number of compounds, when present together in the same system, have a more pronounced effect than that which would be derived from a simple additivity concept (Laguerre et al., 2007). Tomato is one of the most consumed vegetables in the world. In recent decades, its consumption has been associated with the prevention of several diseases mainly due to its content of antioxidants, including carotenes (lycopene as well as β-carotene), ascorbic acid, tocopherol, and phenolic compounds (Periago et al., 2009). Since tomatoes provide a variety of dietary lipo- and hydrophilic antioxidants, in the present study we assessed the antioxidant activity of lipo- and hydrophilic tomato extracts using in vitro chemical tests and cell-based assays, focusing on possible synergistic actions between extracts.

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1. Materials and methods

1.1. Reagents and chemicals

Minimum essential medium with Earle's salts (MEM), RPMI 1640 medium, N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), penicillin, streptomycin, non-essential amino acids, pyruvate, and L-glutamine were purchased from Gibco (Paisley, Scotland, UK). Foetal bovine serum (FBS), 2′,7′-dichlorofluorescin diacetate (DCFHDA), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), *tert*-butylhydroperoxide (*t*BOOH) 70% solution in water were purchased from Sigma-Aldrich (St. Louis, USA). Lycopene standard was purchased from CaroteNature (Lupsingen, Switzerland), β-carotene and phenolic compound standards were purchased from Sigma (St. Louis, EE.UU), dimethylsulphoxide (DMSO) and L-(+)-ascorbic acid were purchased from Merck (Darmstadt, Germany) and 2,4,6-tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka (Buchs, Switzerland). All other reagents were of the highest grade obtainable.

1.2. Preparation of tomato extracts and analysis of bioactive compounds

Tomato lipo- and hydrophilic extracts were prepared from a commercially available tomato concentrate. To obtain the lipophilic tomato extract (L_{extr}), 5 g tomato concentrate was extracted in a mixture of 250 ml hexane and 20 ml water for 30 min using a magnetic stirrer. The hexane upper layer was dried in a rotary evaporator and the resulting extract redissolved in 25 ml DMSO. Similarly, the hydrophilic tomato extract (H_{extr}) was prepared by magnetic stirring of 5 g tomato concentrate in 250 ml distilled water for 30 min. The extract was filtered through Whatman paper No. 1 and concentrated in a rotary evaporator to 25 ml. Finally, the extracts were filtered through a 0.2 µm pore size syringe filter (Millex, Millipore, Cork, Ireland) and stored at –80 ºC until analysed. For the assays, extracts were analysed diluted, undiluted, and combined (Table 1). The content of bioactive compounds in both L_{extr} and H_{extr} tomato extracts was analysed by HPLC. Carotenoids were analysed as described by Böhm (2001), phenolic compounds were analysed according to Navarro-González and co-workers (2011), and vitamin C was assayed by the method of Esteve and co-workers (1995).

1.3. Analysis of total antioxidant activity (TAA)

1.3.1. In vitro *chemical TAA tests.* Two common TAA tests were used in this study: the Trolox Equivalent Antioxidant Capacity (TEAC) assay (Miller et al., 1996) and the Ferric Reducing/Antioxidant Power (FRAP) assay (Benzie & Strain, 1996). Trolox in the range 0–125 µM (TEAC) and 0–250 µM (FRAP) was used for linear calibration (R^2=0.99).

1.3.2. Cell-based antioxidant activity (CAA) tests. The CAA assay is based on prevention of intracellular reactive oxygen species (ROS) production, which is detected using the non-fluorescent compound DCFHDA. In this method, the probe DCFHDA is taken up by cells and deacetylated to DCFH by intracellular esterases (Wolfe & Liu, 2007). 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. For the assay, tBOOH, a widely used model of in vitro oxidative stress (Alía et al., 2005), was used as a free radical generator.

Table 1. Carotenoid and phenolic content of tomato extracts* and their combinations (µM).

Compound	Lipophilic extract (L_{extr})	Hydrophilic extract (H_{extr})	Combined extract (LH_{extr})	Diluted lipophilic extract (Ld_{extr})	Diluted hydrophilic extract (Hd_{extr})
Lycopene	247.17±0.03	nd	123.59	123.59	nd
Total phenolics	nd	48.83	24.42	nd	24.42
Chlorogenic acid		35.48±0.02	17.74		17.74
Caffeic acid		1.94±0.01	0.97		0.97
Rutin		11.41±0.01	5.71		5.71

*For the TAA and CAA assays, extracts were analysed diluted, undiluted, and combined as indicated as follows: L_{extr} and H_{extr} were assayed undiluted, LH_{extr} was obtained by mixing 50% L_{extr} and 50% H_{extr} and then assayed; Ld_{extr} and Hd_{extr} were obtained by diluting 1:1 (v:v) in water before being assayed. For L_{extr} and H_{extr}, results are expressed as mean±SD. In LH_{extr} and Ld_{extr} and Hd_{extr} the amount of bioactive compounds was estimated according to the dilution applied (1:1; v/v). Vitamin C was not detected. This could be explained by the fact that extracts were obtained from a tomato concentrate. Therefore, heating during the concentration process and further storage of the product may have degraded vitamin C; nd: not detected

CAA using human histiocytic lymphoma (U937) cells: U937 cells (American Type Culture Collection, ATCC No. CLR-1593.2, Rockville, MD, USA) were cultured in suspension in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% non-essential amino acids, 1.5 g·l^{-1} $NaHCO_3$, 1 mM pyruvate, 10 mM HEPES, 2 mM L-glutamine, 4.5 g·l^{-1} glucose, 100 U·ml^{-1} penicillin, and 100 mg·ml^{-1} streptomycin, at 37 °C in a humidified atmosphere of 95% air–5% CO_2. For the experiments, cells were washed with saline A buffer pH 7.3 (8.812 g·l^{-1} NaCl, 0.372 g·l^{-1} KCl, 0.336 g·l^{-1} $NaHCO_3$, and 0.9 g·l^{-1} glucose), adjusted to a density of 50 000 cells·ml^{-1} in saline A containing 12.5 µM DCFHDA and incubated for 15 min at 37 ºC. After that, aliquots of 2 ml of the cell suspension were transferred into test tubes and Trolox or tomato extracts were added at the desired concentrations 10 min before exposure to 25 µM *t*BOOH for 30 min at 37 ºC. Fluorescence intensity was measured by flow cytometry (Cytomics FC 500, Beckman Coulter Inc., Fullerton, CA, USA). Trolox in the range 0–10 µM was used for linear calibration (R^2=0.99). Cell viability was checked during flow cytometry analyses using propidium iodide staining. Tomato extracts were tested at 1% final concentration in the cultures. Under these conditions cell viability was always >95%.

CAA using human Caucasian hepatocyte carcinoma (HepG2) cells: HepG2 cells (European Collection of Cell Cultures; ECACC No. 85011430, CAMR, Salisbury, Wiltshire, UK) were routinely grown in MEM supplemented with 10% (v/v) FBS, 100 U·ml^{-1} penicillin, 100 mg·ml^{-1} streptomycin, 1% non-essential amino acids, and 2 mM L-glutamine, in a humidified atmosphere of 95% air–5% CO_2 at 37 °C. For the experiments, cells were seeded onto 24-well plates at a density of 200 000 cells per well and grown to confluence. On the treatment day, cells were preloaded with serum-free MEM containing 12.5 µM DCFCDA for 15 min. After that, media was replaced by serum-free MEM containing known concentrations of Trolox or tomato extracts 10 min before exposure to 250 µM *t*BOOH for 30 min at 37 ºC. Fluorescence intensity was measured in a Fluostar Galaxy fluorimeter (BMG LabTechnologies, Offenburg, Germany). Trolox in the range 0–4 µM was used for linear calibration (R^2=0.99). After the whole treatment process, cell viability was checked by the MTT assay (Mosmann,

1983). Tomato extracts were tested at 0.2% final concentration in the culture media. Under these conditions cell viability was always >95%.

1.4. Establishment of synergistic, additive, or antagonistic effects

To study the antioxidant nature of extract combinations (synergistic, additive, or antagonistic), the theoretical TAA of the combined extract (LH_t) was calculated as the following sum:

$$_{TAA}LH_t = _{TAA}Ld_{extr} + _{TAA}Hd_{extr}$$

Then, $_{TAA}LH_t$ was compared to the experimentally measured TAA of the combined extract ($_{TAA}LH_{extr}$) so that when:

$$_{TAA}LH_{extr} < _{TAA}LH_t \rightarrow \text{Antagonism}$$

$$_{TAA}LH_{extr} = _{TAA}LH_t \rightarrow \text{Addition}$$

$$_{TAA}LH_{extr} > _{TAA}LH_t \rightarrow \text{Synergy}$$

1.5. Statistics

Data were analysed by Statistical Package SPSS 15.0 version for Windows (SPSS Inc., Chicago, IL, USA). Comparisons between the means were analysed by one-way analysis of variance (ANOVA) followed by Tukey's test, considering a P value <0.05 statistically significant.

2. Results and discussion

As evidenced by Figure 1, an additive effect between lipo- and hydrophilic antioxidants was observed when TAA was measured by the FRAP assay ($_{TAA}LH_{extr} = _{TAA}LH_t$) (Fig. 1A), while a slight synergistic action is observed when using the TEAC test ($_{TAA}LH_{extr} > _{TAA}LH_t$). This was revealed by a 0.9-fold increase of $_{TAA}LH_{extr}$ with respect to $_{TAA}LH_t$ showing significant differences in a pair-wise comparison (Fig. 1B). Stronger synergistic effects were observed when tomato extracts were tested in cell-based assays, as 1.5-fold and 5-fold increases of $_{TAA}LH_{extr}$ with respect to $_{TAA}LH_t$ were observed in HepG2 and U937 cells, respectively (Figs 1C and 1D).

Cooperative effects between antioxidants and plant-food extracts have been previously reported in different chemical systems. In a recent study of major antioxidants and antioxidant activity in eight tomato cultivars, hydrophilic antioxidant activity determined by the ABTS assay had far more significant impact (83%) on total antioxidant activity. The analysis of two-component mixtures showed significant synergism between lycopene-lutein, lycopene-beta-carotene, and alpha-tocopherol-beta-carotene. However, ascorbic acid proved to be the most efficient antioxidant (Kotíková et al., 2011).

However, to our knowledge, studies using cell-based assays to assess synergistic action between antioxidants are still not commonly used (Murakami et al., 2003; Blasa et al., 2011). Cell-based assays are considered to provide a more biologically relevant protocol to measure TAA than chemical tests, as they address issues of uptake, distribution, and metabolism. Furthermore, cell models allow the use of relatively low concentrations of oxidant and avoidance of using excessive levels of antioxidants (Wolfe & Liu, 2007). It should be noted that, in our study, the final concentrations in the cultures were in the range c.a. 0.25–2.5 μM for lycopene and c.a. 0.05–0.5 μM for total phenolics. Such concentrations are physiologically attainable under a usual diet (Manach et al., 2004; Jacob et al., 2008).

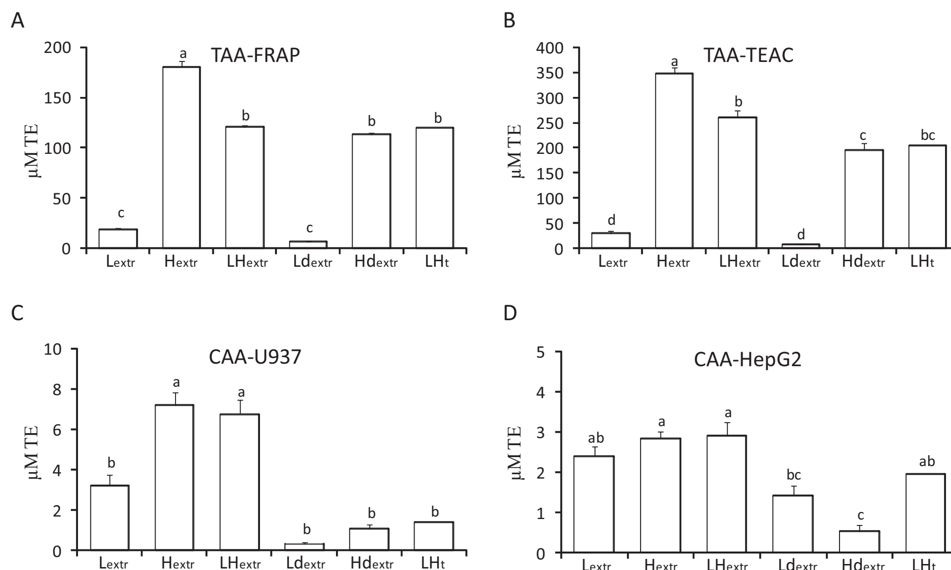

Fig. 1. Total antioxidant activities of tomato extracts and their combinations as measured by in vitro chemical tests and cell-based assays. Results (mean±SEM of at least three separate experiments) are expressed as μM Trolox Equivalentes (TE). Lipophilic extract (L_{extr}), hydrophilic extract (H_{extr}), combined extract (LH_{extr}), diluted lipophilic extract (Ld_{extr}), diluted hydrophilic extract (Hd_{extr}), theoretical total antioxidant activity of the combined extract (LH_t). (A) TAA-FRAP; total antioxidant activity measured by the FRAP assay. (B) TAA-TEAC; total antioxidant activity measured by the TEAC assay. (C) CAA-U937; cell-based antioxidant activity measured using U937 cells. (D) CAA-HepG2: cell-based antioxidant activity measured using HepG2 cells. Different superscript letters mean statistical significance ($P<0.05$)

Synergism between antioxidants may arise via different mechanisms: (i) via regeneration reactions, (ii) via sacrificial oxidation (where one antioxidant, by radical scavenging, protects the other one) or (iii) by exertion of different mechanisms of action (singlet oxygen quenchers and chain-breaking antioxidants, metal chelators), thus prolonging the antioxidative effect of each other. Also, in multiphase media, the interaction of antioxidants with different polarities, which are thus distributed in different phases or solvents, could induce synergy (e.g. ascorbate-induced regeneration of α-tocopherol) (Laguerre et al., 2007). In line with this, the synergistic action observed in cell-based assays could rely on the presence of a multiphase media (cell membrane and extra- and intracellular media) that might facilitate the distribution and interaction of lycopene and phenolics in preventing oxidation by means of different antioxidant mechanisms (e.g. radical scavenging, metal chelation).

The contribution of L_{extr} to TAA measured in cell systems could be explained by the affinity of carotenoids for lipid compartments together with the characteristics of the cell-based assays. The organic hyperoxide tBOOH, used herein as oxidative stressor, decomposes to other alkoxyl and peroxyl radicals in a reaction aided by metal ions that leads to damage in lipophilic cell compartments (Alía et al., 2005). Acting as chain-breaking antioxidants, carotenoids have been shown to be capable of inhibiting free radical reactions involving species such as peroxyl radicals, therefore playing an important role in the protection of cellular membranes and lipoproteins against oxidative damage (Sies & Stahl, 1995).

Phenolics can exert antioxidant activity mainly by either free-radical scavenging or metal chelation (García-Alonso et al., 2007; Laguerre et al., 2007). In this manner, the phenolic compounds contained in the H_{extr} may have displayed antioxidant activity by protecting cell membranes from internal and/or external oxidation via scavenging of *t*BOOH-derived peroxyl radicals and/or by inhibiting the generation of further free radicals by Fenton-type reactions. Interaction of polar phenolics with inner or outer cell membranes by hydrogen bonding has been suggested as playing a role in antioxidant protection (Wolfe & Liu, 2007).

3. Conclusions

We herein report synergistic action between the carotenoid (lycopene) and hydrophilic phenolic fractions of tomato. Synergism is better revealed when using cell-based assays, which are considered more physiologically relevant than the commonly used chemical tests.

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