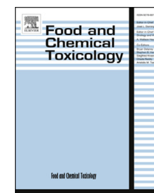
Food and Chemical Toxicology 109 (2017) 386–392

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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



Further insights on tomato plant: Cytotoxic and antioxidant activity of leaf extracts in human gastric cells

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A R T I C L E I N F O

Article history:
Received 12 July 2017
Received in revised form
7 September 2017
Accepted 8 September 2017
Available online 9 September 2017

Keywords:
Tomato leaves
Cytotoxicity
Antioxidant
Gastric cancer
Phytochemicals
By-products

A B S T R A C T

This study focused the toxicity against human gastric adenocarcinoma cells (AGS) and the antioxidant activity of hydromethanol (HME), acetone (AcE) and alkaloid (AE) extracts prepared from the leaves of tomato plant cultivars (Caramba, Valentine, Negro, Abuela, and Anairis). AE, HME and AcE extracts of all cultivars reduced cell viability, IC50 values ranging from 9 ± 2 to 55 ± 11, from 103 ± 25 to 171 ± 29 and from 291 ± 26 to 459 ± 14 μg mL^{-1}, respectively. Moreover, both HME and AcE extracts scavenged $^{\bullet}$NO (IC$_{50}$ values ranged from 0.87 ± 0.12 to 1.54 ± 0.23 and from 0.90 ± 0.01 to 2.23 ± 0.14 mg mL^{-1}, respectively), but only HMEs was able to scavenge $O_2^{\bullet-}$ showing IC$_{50}$ values from 0.12 ± ≤0.01 to 0.43 ± 0.08 mg mL^{-1}. Our results demonstrate that tomato leaves, a by-product of tomato processing industry, are a valuable source of bioactive compounds, providing beneficial properties for human health.
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1. Introduction

Tomato (*Lycopersicon esculentum* Mill., Solanaceae) is among the most consumed vegetables worldwide (Foolad and Panthee, 2012). It is well-known as a health-stimulating fruit because of the antioxidant properties of its bioactive compounds, mainly lycopene and phenolic compounds (Liu et al., 2015). Tomato plant by-products may constitute valuable ingredients for the food, cosmetic and pharmaceutical industries (Figueiredo-González et al., 2016a; Silva-Beltrán et al., 2015; Taveira et al., 2012).

In fact, leaves of tomato plant are a source of phenolic compounds, chlorophylls, carotenoids and alkaloids (Ferreres et al., 2011; Figueiredo-González et al., 2016a; Silva-Beltrán et al., 2015; Taveira et al., 2014, 2012). Phenolic compounds have been described as potent antioxidants due to the proximity of aromatic rings, the presence of free hydroxyl groups on the phenolic rings, and/or the interaction of those with the π-electrons of the benzene ring, to generate free radicals stabilized by delocalization (Hagerman et al., 1998; Nunes et al., 2016; Silva-Beltrán et al., 2015). Moreover, pigments like chlorophylls and carotenoids have already been shown to display antioxidant activity, not only as singlet oxygen quenchers, but also as scavengers of other reactive species (Ferruzzi et al., 2002; Silva-Beltrán et al., 2015; Stajčić et al., 2015).

It has been suggested that antioxidant compounds could decrease the potential stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). The overproduction of both ROS and RNS, and/or an inadequate antioxidant defence, due to an imbalance between pro-oxidant and antioxidant systems, leads to oxidative stress. Under these conditions, reactive species can cause injury to cellular and extracellular macromolecules, including the oxidation of lipids, proteins, nucleic acids and structural carbohydrates (Pandey and Rizvi, 2009; Willcox et al., 2004).

It is evident that the oxidative stress induced by such species can be involved in the multistage process of carcinogenesis, by both genetic and epigenetic mechanisms (Franco et al., 2008). Gastric cancer is the third most common cause of death by cancer worldwide (Fitzmaurice et al., 2017). Although many risk factors have been addressed, the causative and protective agents of gastric cancer remain to be clarified (Zhou et al., 2016). Nevertheless, it is well-kwon that the reduction of reactive species may be beneficial in the management of cancer (Valko et al., 2006). In fact, previous studies have reported a positive correlation between the antioxidant capacity of plants and their anti-proliferative effect, pointing to the potential role of phenolics, carotenoids and steroidal alkaloids in inhibiting cancer cells growth (Abrahim et al., 2012; Chen

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http://dx.doi.org/10.1016/j.fct.2017.09.018
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et al., 2015; Gawlik-Dziki et al., 2013; Stajčić et al., 2015).

In our previous work (Figueiredo-González et al., 2016a) we determined the chemical composition (phenolic compounds, chlorophylls, carotenoids and alkaloids) of extracts obtained from tomato leaf of different cultivars. The results suggested that they are valuable sources, inhibiting key enzymes related with Alzheimer's disease and diabetes mellitus. The objective of the present work was to evaluate the antioxidant activity of different extracts (hydromethanol, acetone and selective alkaloid extraction) prepared with the leaves of five different tomato cultivars (Caramba, Valentine, Negro, Abuela and Anairis) against ROS and RNS with biological significance (superoxide radical anion ($O_2^{\bullet -}$), hypochlorous acid (HOCl) and nitric oxide ($^{\bullet}$NO)), as well as against lipid peroxidation, using non-cellular models. According to our literature survey, the antioxidant capacity of the selected cultivars has not been reported elsewhere. In addition, we investigated the cytotoxic effects of these extracts against human gastric adenocarcinoma cells (AGS). To our knowledge, no data is available on the cytotoxic effects of HME and AcE extracts prepared from tomato plant leaf on gastric cancer cells. Furthermore, only one work had assessed the effect of an AE obtained from the leaves of two other tomato cultivars (Bull's heart and Cherry cultivars) on the viability of AGS cells (Taveira et al., 2014).

2. Materials and methods

2.1. Chemicals and standards

Phenazine methosulphate (PMS), nitrotetrazolium blue chloride (NBT), sodium nitroprusside dehydrate (SNP), sulphanilamide, naphthylethylenediamine dihydrochloride, Trizma®hydrochloride (Tris–HCl), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), linoleic acid, *ortho*-phosphoric acid 85%, L-ascorbic acid, sodium borohydride, and (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Methanol, acetone, and glacial acetic acid were acquired to Chem-Lab NV (Zedelgem, Belgium). Potassium dihydrogen phosphate, ammonia solution 25%, iron (II) sulfate ($FeSO_4 \cdot 7H_2O$), 2-propanol, dimethyl sulfoxide (DMSO), and sodium hypochlorite solution were obtained from Merck (Darmstadt, Germany). Quercetin-3-*O*-rutinoside (95.0%) was from Extrasynthèse (Genay, France), and ion-exchange SPE cartridge (sulphonic acid bonded silica with H^+ counterion (SCX)) was purchased from Supelco (Bellefonte, USA).

Reagents for cell culture were obtained from Invitrogen (Gibco Laboratories; Lenexa, KS): Dulbecco's modified Eagle's medium (DMEM; 4.5 g L^{-1} glucose, with L-glutamine and pyruvate), Hanks' Balanced Salt Solution (HBSS), foetal bovine serum (FBS), antibiotic (10,000 U mL^{-1} penicillin, 10,000 μg mL^{-1} streptomycin) and trypsin–EDTA.

2.2. Plant material and preparation of extracts

The *L. esculentum* selected cultivars in this work were Caramba, Valentine, Negro, Abuela and Anairis, which are among the most consumed in Galicia (Spain). The leaves from the five cultivars were collected in June of 2015, from tomato plants grown in a greenhouse in A Coruña, Galicia (Spain). The plant material was washed with distilled water, and lyophilized for later use (−57 °C, 100 mTorr, 96 h).

For each cultivar, an hydromethanol extract (HME) was prepared as follows: powdered leaves with mean particle size below 910 μm (ca. 400 mg) were sonicated with 20 mL of methanol:water (50:50, v/v) (30 min), followed by stirring maceration (120 min, 300 rpm) at room temperature and further sonication (30 min). The extract obtained was filtered under vacuum, concentrated to dryness and stored at −20 °C, protected from light. The same procedure was followed with acetone to obtain the acetone extract (AcE).

For alkaloid extracts (AE), powdered leaves (ca. 200 mg) were sonicated with 4 mL of 5% acetic acid (30 min), followed by stirring maceration (120 min, 300 rpm) at room temperature and further sonication (30 min). Then, the extract was centrifuged (12,000 g, 4 °C, 10 min) and the supernatant was directly loaded into a SCX cartridge, previously activated with 10 mL of methanol followed by 10 mL of 5% acetic acid. The sorbent was washed with 20 mL of 5% methanol and then the retained compounds were eluted with 20 mL of 2.5% ammonium in methanol. The resulting alkaloids-purified extract was concentrated to dryness and stored at −20 °C protected from light.

All extractions were performed in triplicate. The yield of the extraction varied from 26 ± 1.0 to 34 ± 0.5% for HME, from 4 ± 0.7 to 5 ± 1% for AcE and from 4 ± 0.2 to 5 ± 0.5% for AE.

2.3. Antioxidant activity

2.3.1. Superoxide radical anion ($O_2^{\bullet -}$)

The methodology was described by Valentão et al. (2001). Briefly, the dry extracts (5 mg mL^{-1}) were dissolved in potassium phosphate buffer (19 mM, pH 7.4) and serial dilutions were prepared. $O_2^{\bullet -}$ was generated by the NADH/PMS/O_2 system. The scavenging activity was determined by monitoring the effect on the reduction of NBT induced by superoxide radicals, in the presence and absence of tomato leaf extracts, using a Multiskan Ascent plate reader (Thermo Electron Corporation) working in kinetic function during 2 min at 560 nm. The assay was performed three times ($n = 3$), each assay in triplicate, and results were expressed as IC_{50} values. Quercetin-3-*O*-rutinoside was used as positive control.

2.3.2. Nitric oxide ($^{\bullet}$NO)

The activity of AcE, HME and AE extracts was determined using the method described by Sousa et al. (2008). Sodium nitroprusside was incubated with extracts (5 mg mL^{-1}) at five different concentrations for 60 min, at room temperature, under direct light. After this period, Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamine in 2% phosphoric acid) was added to each well. The mixture was then incubated at room temperature for 10 min, and the absorbance of the chromophore formed was read at 560 nm. The IC_{50} values were calculated from three independent assays, performed in triplicate. Quercetin-3-*O*-rutinoside was used as positive control.

2.3.3. Hypochlorous acid (HClO)

The effect against HClO was assessed according to a previously reported procedure (Czerwińska et al., 2012). HClO was used as oxidant of TNB into DTNB. TNB was synthetized by reaction of DTNB and sodium borohydride. NaOCl solution (0.05%, 150 μL, pH 6.2) and extracts (50 μL, 2.5 mg mL^{-1}) were dissolved in potassium phosphate buffer (50 mM, pH 6.6) and added to 96-well plates. The reaction mixture was incubated for 3 min in the dark. Then TNB (0.1 mM, 30 μl) was added and the mixture was further incubated for 3 min. The absorbance was read at 412 nm. Initial TNB was calculated without addition of the tested extracts and without NaOCl.

2.3.4. Lipid peroxidation

Peroxidation of fatty acyl groups was determined following the methodology proposed by Ferreres et al. (2012) with modifications to be carried out as a microassay. The reaction mixture contained 125 μL of linoleic acid (20 mM in ethanol), 75 μL of Tris-HCl

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