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ARTICLE

Tomato (*Lycopersicon esculentum*) Seeds: New Flavonols and Cytotoxic Effect

Federico Ferreres,[†] Marcos Taveira,[‡] David M. Pereira,[‡] Patrícia Valentão,[‡] and Paula B. Andrade*,[‡]

[†]CEBAS (CSIC) Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, P.O. Box 164, 30100 Campus University Espinardo, Murcia, Spain and [‡]REQUIMTE/Department of Pharmacognosy, Faculty of Pharmacy, Porto University, R. Aníbal Cunha 164, 4050-047 Porto, Portugal

In this study, seeds of *Lycopersicon esculentum* Mill. were analyzed by HPLC/UV-PAD/MS^n-ESI. Fourteen flavonoids were identified, including quercetin, kaempferol, and isorhamnetin derivatives, with 13 of them being reported for the first time in tomato seeds. The major identified compounds were quercetin-3-*O*-sophoroside, kaempferol-3-*O*-sophoroside, and isorhamnetin-3-*O*-sophoroside. A significant cell proliferation inhibition (>80%), against rat basophile leukemia (RBL-2H3) cell line, was observed with this extract (IC_{50} = 5980 μg/mL). For acetylcholinesterase inhibitory activity, a concentration-dependent effect was verified (IC_{20} = 2400 μg/mL). The same behavior was noted regarding antioxidant capacity, evaluated against DPPH (IC_{10} = 284 μg/mL), nitric oxide (IC_{25} = 396 μg/L), and superoxide radicals (IC_{25} = 3 μg/mL).

KEYWORDS: *Lycopersicon esculentum*; seeds; HPLC/UV-PAD/ESI-MS^n; phenolic compounds; antiproliferative activity

INTRODUCTION

The Mediterranean diet is characterized by its high diversity and nutritional value, and *Lycopersicon esculentum* Mill. fruit (tomato) is largely represented, both fresh and as processed products. The consumption of this fruit has been proposed to reduce the risk of several chronic pathological conditions, including cardiovascular diseases, cancer, and Alzheimer's disease (*1*, *2*). These health-promoting effects can be attributed to the high content in antioxidant compounds, namely phenolic compounds, and particularly flavonoids (*3*, *4*). Flavonols, flavanones, and chalcones constitute the main classes of flavonoids in whole ripe tomato fruit. These compounds are usually present as *O*-glycosides, having a sugar moiety that includes glucose, rhamnose, and galactose, but free compounds also occur (*5*, *6*).

The determination of phenolic compounds in seeds has assumed increasing importance because they often constitute a source of specific compounds in high concentration (*6*). Although tomato fruit is widely studied, only a few works focus their attention on the seeds. Some studies reported the phenolic composition of the seed cavity (including columella, placenta tissue, and seeds) (*7*) and that of a mixture of seeds and skin, particularly rutin, rutin apioside, naringenin, naringenin chalcone, and chlorogenic acid (*8*). However, none of them dealt with the molecular speciation of tomato seeds. As far as we know, only 5-caffeoylquinic acid, narigenin, rutin, and myricetin were specifically described in hydroethanolic seeds extract (*9*). This seems to point to the potential of seed extracts as a source of bioactive compounds.

*Corresponding author (telephone + 351 222078934; fax + 351 222003977; e-mail pandrade@ff.up.pt).

High performance liquid chromatography (HPLC) is a robust method for the separation of these compounds, and different options for detection can be coupled. Each class of phenolic compounds has a characteristic spectrum, thus making the use of a photodiode array detector (PAD) very useful, because it yields the full record of the UV−vis spectrum of each compound (*10*). Moreover, the use of HPLC coupled to mass spectrometry (MS) enables the detection of large numbers of parent ions present in a single extract and can provide valuable information on the chemical composition. Currently, the use of HPLC coupled with tandem mass spectrometry (MS/MS) has emerged as a simple and one of the most sensitive methods. Thus, minor or trace constituents, which are difficult to obtain by conventional means, could be detected by mass spectrometry (*11*). This technique has been successfully applied in the determination of phenolics in several vegetable samples (*12*−*15*).

Despite the presence of phenolics and the proven antioxidant activity of tomato fruit in several models (*16*−*18*), as far as we know there are no studies concerning the antioxidant potential of tomato seeds against reactive oxygen or nitrogen species. An overproduction of oxidant reactive species is associated with several deleterious effects, including Parkinson's and Alzheimer's diseases (*19*, *20*), so the antioxidant proprieties of some extracts can contribute to neuroprotection (*21*). With regard to neuroprotective effects, beyond the antioxidant properties, acetylcholinesterase (AChE) inhibitory capacity is also important. One treatment strategy for Alzheimer's disease is to enhance the cholinergic function, which can be achieved by the use of AChE inhibitors (*22*).

The results observed in cell-free systems should be confirmed by cellular models, as some activities may not be verified within

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cells or the concentrations required to scavenge pro-oxidant species may be highly deleterious to them. Cytotoxicity assays are widely used to screen the cell proliferation inhibitory capacity of different extracts, which can be used in the development of therapeutics that rapidly target dividing cancer cells (*23*).

This work aimed to improve the knowledge on the phenolic profile of tomato seeds and to evaluate some of their biological capacities. For these purposes, phenolics were screened for the first time by HPLC/UV-PAD/ESI-MS^n, where ESI denotes electrospray ionization. Cytotoxicity was evaluated by sulforhodamine B assay in RBL-2H3 cells. The antioxidant capacity was assessed against 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide and nitric oxide radicals, and acetylcholinesterase inhibitory capacity was also checked.

MATERIALS AND METHODS

Standards and Reagents. Reference compounds were purchased from various suppliers: quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside were from Extrasynthése (Genay, France). DPPH, β-nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), bovine serum albumin (BSA), nitroblue tetrazolium chloride (NBT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sulfanilamine, AChE (CAS Registry No. 9000-81-1; EC 232-559-3) from electric eel (type VI-s, lyophilized powder), sulforhodamine B (SRB), acetylthiocholine iodide (ATCI), and Tris-HCl were purchased from Sigma (St. Louis, MO). *N*-(1-Naphthyl)ethylenediamine dihydrochloride, sodium nitroprussiate dehydrate (SNP), methanol, trichloroacetic acid, and sulfuric and acetic acids were obtained from Merck (Darmstadt, Germany). NaCl was purchased from José M. Vaz Pereira, S.A. (Sintra, Portugal) and $MgCl.6H_2O$ from Fluka (Buchs, Switzerland). Rat basophile leukemia (RBL-2H3) cell line was acquired from American Type Culture Collection (ATCC). Dulbecco's Modified Eagle Medium (DMEM), phosphate-buffered saline (PBS), and trypsin were from Invitrogen (Gibco). Water was treated in a Milli-Q (Millipore, Bedford, MA) water purification system.

Plant Material and Extraction Procedures. Seeds of *L. esculentum* Mill. 'Bull's Heart' cultivar (RJS, Portugal) were purchased from local commerce and used without any pretreatment.

For phenolics characterization, 0.5 g of seeds was extracted with 2 mL of water/methanol (1:1) by sonication for 1 h, followed by 15 h of maceration and another sonication period (1 h). The resulting extract was centrifuged and filtered.

For the bioactivity assays and phenolics quantification, an aqueous extract was prepared by boiling ca. 6 g of powdered seeds in 600 mL of water, for 30 min. The resulting extract was filtered through a Büchner funnel, frozen, and lyophilized. A yield of 770 mg was obtained. The lyophilized aqueous extract was kept in a desiccator, in the dark, until analysis. For phenolics determination, the seed extract was redissolved in water. The bioactivity assays were performed after the aqueous extract had been redissolved in water or buffer.

HPLC/UV-PAD/ESI-MS^n Phenolic Compound Qualitative Analysis. Chromatographic analyses were carried out on a LiChroCART column (250 × 4 mm, RP-18, 5 μm particle size, LiChrospher100 stationary phase; Merck, Darmstadt, Germany), protected with a LiChroCART guard column (4 × 4 mm, RP-18, 5 μm particle size, Merck). The mobile phase consisted of two solvents: water/acetic acid (1%) (A) and methanol (B), starting with 5% B and using a gradient to obtain 50% at 30 min and 80% at 37 min. The flow rate was 1 mL/min and the injection volume 30 μL. Spectral data from all peaks were accumulated in the range of 240−400 nm, and chromatograms were recorded at 350 nm. The HPLC/UV-PAD/ESI-MS^n analyses were carried out with an Agilent HPLC 1100 series equipped with a PAD and mass spectrometer in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an autosampler (model G1313A), a degasser (model G1322A), and a PAD (model G1315B). The HPLC system was controlled by ChemStation software (Agilent, v. 08.03). The mass spectrometer was an ion trap mass analyzer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full-scan mass covered the range from m/z 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Mass spectrometry data were acquired in the negative ionization mode. MS^n was carried out on the most abundant fragment ion observed in the first-generation mass spectrum. For the compounds present in trace amounts, MS^n was carried in the multiple-reaction monitoring (MRM) mode.

Table 1 shows the most frequent ions that characterize the fragmentation of the flavonoid *O*-glycosides. Other ions were found, but they have not been included due to their low significance on the MS behavior. Most of compounds were hidden by others or in trace amounts, so their UV spectra have not been properly observed.

HPLC-PAD Phenolic Compound Quantitative Analysis. For quantification of phenolic compounds, 20 μL of redissolved seed lyophilized extract (80 mg/mL) was analyzed using a HPLC/PAD unit (Gilson) and a Spherisorb ODS2 (25.0 × 0.46 cm; 5 μm particle size) column. Elution was performed under the conditions described above for phenolics identification. Detection was achieved with a Gilson PAD. Spectral data from all peaks were accumulated in the range of 200−400 nm, and chromatograms were recorded at 350 nm. The data were processed on Unipoint system software (Gilson Medical Electronics, Villiers le Bel, France). Peak purity was checked by the software contrast facilities.

Phenolic compound quantification was achieved by the absorbance recorded in the chromatograms relative to external standards. Because standards of all identified compounds were not commercially available, kaempferol derivatives were quantified as kaempferol-3-*O*-rutinoside, quercetin derivatives as quercetin-3-*O*-rutinoside, and isorhamnetin derivatives as isorhamnetin-3-*O*-rutinoside. Kaempferol-3-*O*-sophoroside and isorhamnetin-3-*O*-sophoroside were quantified together as kaempferol-3-*O*-rutinoside.

Cell Culture. Cells were maintained in DMEM with 15% fetal bovine serum and 2% penicillin, in an incubator with 5% CO_2.

SRB Assay. The method described by Houghton et al. (*23*) was followed, with modifications. Cells were plated with a density of 1×10^4 cells/well and allowed to attach for 24 h at 37 °C, with 5% CO_2. The following day the medium was removed and cells were gently washed with warm PBS. Tomato seed extract redissolved in media was added to the wells in final concentrations ranging from 4 to 8 mg/mL and incubated for 48 h. Three independent assays were conducted, each one in triplicate. Medium was used as a negative control.

After the incubation period, medium was removed, 100 μL of cold 40% trichloroacetic acid was added, and plates were maintained at 4 °C for 60 min. Plates were then washed five times with tap water and allowed to dry. Afterward, 50 μL of 0.4% SRB in 1% acetic acid was added, and plates were incubated for 30 min.

After the incubation period, plates were quickly washed with 1% acetic acid, to remove unbound dye, and allowed to dry. 100 μl of tris-base was added, and after 10 min, the plates were shaken.

Absorbance was read in a multiplate reader, at 492 nm wavelength. OD values were plotted against concentration.

AChE Inhibitory Activity. AChE inhibition was assessed spectrophotometrically in a Multiskan Ascent plate reader (Thermo Electron Corp.), based on Ellman's method, according to a described procedure (*24*). In each well the mixture consisted of AChE, DTNB in buffer A (50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl.6H_2O$), buffer B (50 mM Tris-HCl, pH 8, containing 0.1% BSA), and sample dissolved in a solution of 10% methanol in buffer C (50 mM Tris-HCl, pH 8). Lyophilized enzyme was dissolved in buffer A to make a 1000 U/mL stock solution, and further diluted with buffer B to get 0.44 U/mL enzyme. The absorbance was read at 405 nm. After this step, AChE was added and the absorbance was read again. The rates of reactions were calculated by Ascent Software version 2.6 (Thermo Labsystems Oy). The rate of the reaction before the addition of the enzyme was subtracted from that obtained after enzyme addition to correct eventual spontaneous hydrolysis of substrate. Percentage of inhibition was calculated by comparing the rates of the sample with the control (10% methanol in buffer C). Three experiments were performed in triplicate.

Table 1. t_R, UV, MS: $[M-H]^-$, $-MS^2[M-H]^-$ and $-MS^3[(M-H)\rightarrow(M-H-162/146)]^-$ data of flavonoid-*O*-glycosides from tomato seeds[a]

	Compounds[b]	t_R (min)	UV (nm)	$[M-H]^-$	$-MS^2[M-H]^-$, *m/z* (%)	$-MS^3[(M-H)\rightarrow(M-H-162/146)]^-$, *m/z* (%)				
						−120	−162	−180		$[Aglc-H/2H]^-$
					Flavonol-3-*O*-diglucoside-7-*O*-glucosides					
					−162					
1	Q-3-Soph-7-Glc	15.2	—[c]	787	625(100)	505(5)	463(8)	445(25)		300(100)
2	K-3-Soph-7-Glc	16.6	—[c]	771	609(100)	489(13)	447(5)	429(37)		285(100)
3	Q-3-Gtb-7-Glc	21.8	—[c]	787	625(100)					300(100)
					Flavonol-3-*O*-sophoroside-7-*O*-rhamnosides					
					−146					
4	Q-3-Soph-7-Rh	21.8	—[c]	771	625(100)	505(30)	463(7)	445(30)		300(100)
5	K-3-Soph-7-Rh	23.3	—[c]	755	609(100)		447(27)	429(75)		285(100)
6	I-3-Soph-7-Rh	23.5	—[c]	785	639(100)			459(28)		315(100)
					Flavonol-3-*O*-triglycosides					
					$-MS^2[M-H]^-$					
					−120	−132	−150	−162	−342	
8	K-3-triGlc	26.5	—[c]	771	651(21)			609(100)	429(48)	285(50)
9	Q-3-Pent-Rut	26.5	—[c]	741		609(56)	591(22)			300(100)
					Flavonol-*O*-diglycosides					
					$-MS^2[M-H]^-$					
					−120	−132	−150	−162	−180	
7	Q-3-Soph	24.1	256, 266sh, 298sh, 354	625	505(10)			463(18)	445(28)	300(100)
10	K-3-Soph	26.6	266, 298sh, 350[d]	609	489(6)				429(43)	285(100)
11	I-3-Soph	26.8	—[d]	639	519(11)				459(45)	315(100)
12	I-3-Gtb	27.8	256, 266sh, 304sh, 354	639					459(7)	315(100)
13	Q-3-Rut	28.7	256, 266sh, 299sh, 354	609						301(100)
14	K-3-Pent-Glc	29.3	—[c]	579		447(32)	429(37)			284(100)

[a] Main observed fragments. Other ions were found, but they have not been included. [b] Q, quercetin; K, kaempferol; I, isorhamnetin; Soph, sophoroside (glucosyl(1→2)glucoside); Gtb, gentiobioside (glucosyl(1→6)glucoside); Rut, rutinoside (rhamnosy(1→6)glucoside); Glc, glucoside; Rh, rhamnoside; Pent, pentoside. [c] Compounds hidden by others or in traces. Their UV spectra have not been properly observed. [d] UV spectrum of **10** predominates over the spectrum of **11**.

DPPH• Scavenging Assay. The antiradical activity of the extract was determined spectrophotometrically in a plate reader Multiskan Ascent (Thermo Electron Corp.), at 515 nm, as before (*12*). The reaction mixture consisted of aqueous extract and a methanolic solution of 150 mM DPPH•. The plate was incubated for 30 min at room temperature after the addition of DPPH•. Three experiments were performed in triplicate.

Superoxide Radical Scavenging Assay. Antiradical activity was determined spectrophotometrically at 562 nm, in a plate reader working in kinetic function, by monitoring the effect on reduction of NBT induced by superoxide radical. Superoxide radicals were generated in a NADH/PMS system, according to a described procedure (*12*). All components were dissolved in phosphate buffer (19 mM, pH 7.4). Three experiments were performed in triplicate.

Nitric Oxide Scavenging Assay. Antiradical activity was determined spectrophotometrically in a 96-well plate reader according to the described procedure (*12*). The reaction mixtures in the sample wells consisted of extract and SNP, and plates were incubated at room temperature for 60 min, under light exposure. Griess reagent was then added, and 10 min later the absorbance was determined at 540 nm. Three experiments were performed in triplicate.

Statistical Analysis. Comparisons of data from different groups were performed using a one-way ANOVA test. A p value of <0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

HPLC/UV-PAD/ESI-MS^n Phenolic Compound Qualitative Analysis. The HPLC/UV-PAD/ESI-MS^n screening of tomato seed hydromethanolic extract showed a chromatogram (**Figure 1**) in which two major peaks (compounds **7** and **10** + **11**) can be observed, besides other compounds present in small amounts. The structures of the compounds indicated in the chromatogram were characterized, except compounds **A** and **F**. The UV spectra of the latter compounds point to hydroxycinnamic acids, although this could not be confirmed with their mass spectra.

The mass spectrum of compounds **1**−**14** indicated the presence of flavonoid-*O*-glycosides. In the MS^2 fragmentation of compounds **7**−**14** or in the MS^3 of compounds **1**−**6** the aglycon-related ion ($[Aglc-H/2H]^-$) (300/301 quercetin, 285 kaempferol, and 315 isorhamnetin) (**Table 1**) was the base peak or an abundant one. According to the MS fragmentation profile, these compounds can be divided into three different groups. The MS^2 fragmentation of the deprotonated molecule ($[M-H]^-$) from the first group (compounds **1**−**6**) shows the base peak, almost the unique ion, corresponding to the loss of a hexose (162 u, **1**−**3**) or a deoxyhexose residue (146 u, **4**−**6**). This MS^2 fragmentation results from the loss of glycosylation in position 7, which is characteristic of flavonol-3-*O*-glycoside-7-*O*-glycosides (*25*) and also agrees with the fragmentations observed in the majority of the Brassicacea glycosides, published before by our group (*12*−*15*). Additionally, the presence of several compounds with substitution in the 3,7-position and never in the B-ring was already reported. Thus, we think that biosynthetically these compounds have the same pattern of substitution. Moreover, the MS fragmentation is similar to that of the compounds with this substitution type. The resulting ion corresponds to the aglycone with the glycosidic fraction in the 3-position (dihexosides). In the MS^3 fragmentation of these ions ($MS^3[(M-H)\rightarrow(M-H-162/146)]^-$) it can be observed, besides the ion $[(M-H)-120]^-$ resulting from the loss of a hexose residue in positions 0−3, ions that involve the cleavage of the interglycosidic bond, with the loss of a hexose residue (162 u) and/or a hexose (180 u) (**Table 1**). This behavior is typical of a 1→2 bond

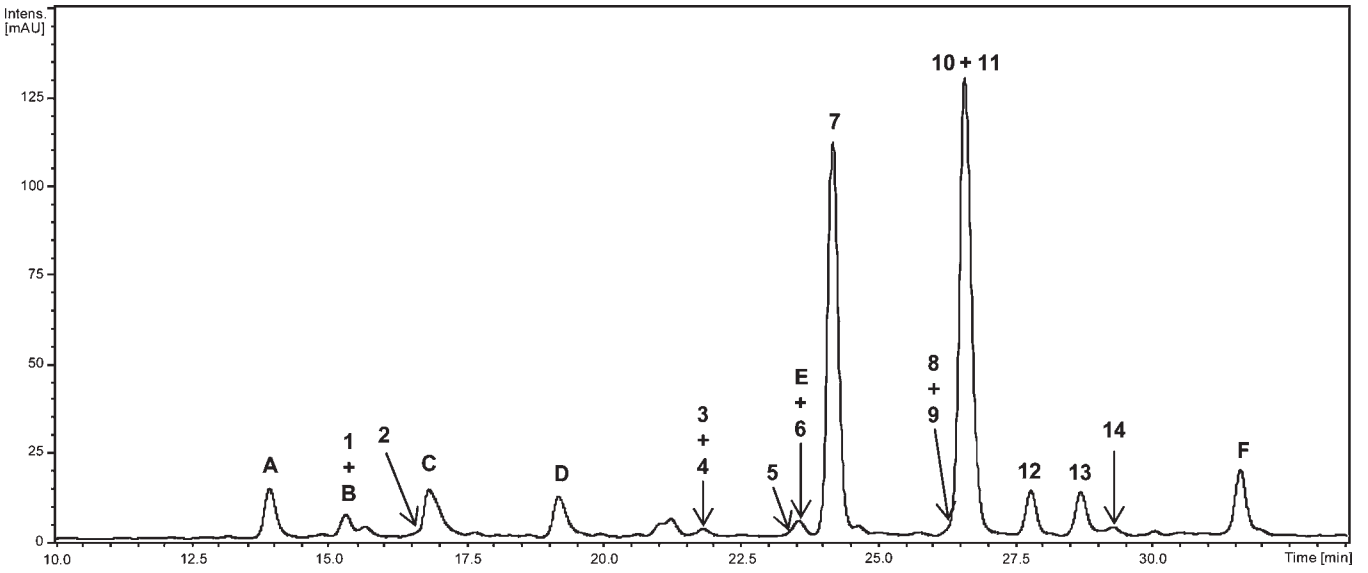

Figure 1. HPLC phenolic profile of tomato seeds, detection at 350 nm. Compounds: (**1**) quercetin-3-*O*-sophoroside-7-*O*-glucoside; (**2**) kaempferol-3-*O*-sophoroside-7-*O*-glucoside; (**3**) quercetin-3-*O*-gentiobioside-7-*O*-glucoside; (**4**) quercetin-3-*O*-sophoroside-7-*O*-rhamnoside; (**5**) kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside; (**6**) isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside; (**7**) quercetin-3-*O*-sophoroside; (**8**) kaempferol-3-*O*-(2-sophorosyl)glucoside or kaempferol-3-*O*-glucosyl-(1→2‴)-glucosyl-(1→2″)-glucoside; (**9**) quercetin-3-*O*-(2-pentosyl)rutinoside or quercetin-3-*O*-(2-pentosyl, 6-rhamnosyl)glucoside; (**10**) kaempferol-3-*O*-sophoroside; (**11**) isorhamnetin-3-*O*-sophoroside; (**12**) isorhamnetin-3-*O*-gentiobioside; (**13**) quercetin-3-*O*-rutinoside; (**14**) kaempferol-3-*O*-(2-pentosyl)glucoside. Compounds **A**−**F** are nonidentified compounds.

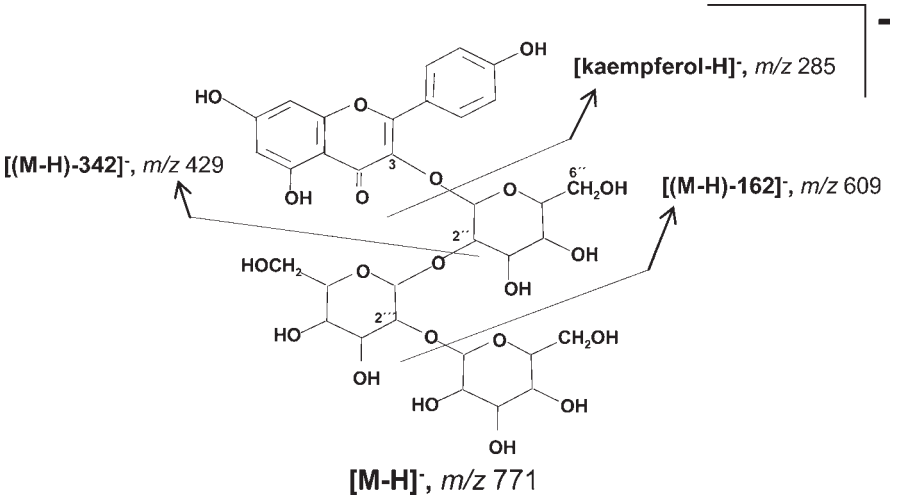

Figure 2. Fragmentation pattern of compound **8**.

(compounds **1**, **2**, **4**−**6**) versus a 1→6 bond (compound **3**), in which these ions are absent or present in low abundance (*25*). Considering that the hexoses detected in different parts of the fruit from diverse tomato cultivars are glucosides (*26*), we can tentatively assign the compounds to quercetin-3-*O*-sophoroside-7-*O*-glucoside (**1**), kaempferol-3-*O*-sophoroside-7-*O*-glucoside (**2**), quercetin-3-*O*-gentiobioside-7-*O*-glucoside (**3**), quercetin-3-*O*-sophoroside-7-*O*-rhamnoside (**4**), kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (**5**), and isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside (**6**).

The MS^2 $[M - H]^-$ fragmentation of another group of compounds, also triglycosides (compounds **8** and **9**), is different from the one previously discussed. In their fragmentation, besides the aglycon-related ions, other ions resulting from the rupture of the interglycosidic bond of the triglycoside were observed. Thus, in compound **8** (kaempferol-trihexoside) it was noted the presence of the *m*/*z* 429 ion, which results from the combined loss of a hexose residue (162 u) and a hexose (180 u) (**Figure 2**). This water loss shows that the dihexosyl is bound to another sugar, instead of a phenolic hydroxyl (*27*). This behavior suggests that a dihexosyl part should not be linked to the hydroxyl in the 6-position of the hexose because, as referred to above when the 1→2 bond versus 1→6 bond was compared, this bond is difficult to be break. Thus, it should be in another position, probably 2″. On the other hand, the presence of ions at *m*/*z* 609 (base peak), resulting from the loss of 162 u from the $[M - H]^-$, indicates that the dihexosyl should also have a 1→2 interglycosidic linkage (**Figure 2**). Therefore, compound **8** can be tentatively identified as kaempferol-3-*O*-(2-sophorosyl)glucoside or kaempferol-3-*O*-glucosyl-(1→2‴)-glucosyl-(1→2″)glucoside. The other compound (**9**) exhibits a $[M - H]^-$ ion at *m*/*z* 741, and in its MS^2 fragmentation the base peak corresponds to the radical quercetin aglycon anion (*m*/*z* 300 $[\text{quercetin} - 2H]^-$).Thus, it corresponds to a quercetin triglycoside (quercetin + pentose + rhamnose + hexose, 741 = 301 + 132 + 146 + 162), substituted only at one phenolic hydroxyl group (*25*). Other important ions result from the loss of a pentose

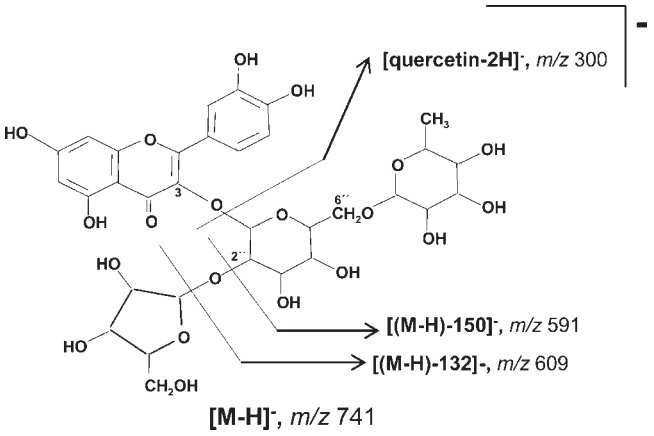

Figure 3. Fragmentation pattern of compound **9**.

residue (132 u) or a pentose (150 u), which, as referred to above, indicates that the pentose is linked to the hydroxyl from a sugar moiety and is different from the 6″-position of hexose (**Figure 3**). In the $MS^3[(M-H)\rightarrow(M-H-132)]^-$ event (results not shown in **Table 1**), no ions involving cleavage of the interglycosidic bond in the rhamnohexosyl part are noted, with only the aglycone-related ion and the ion at *m*/*z* 343 being detected, which corresponds to an internal cleavage of the hexose in the positions 0−3. These data indicate that compound **9** can be a quercetin rutinoside substituted with a pentose. Thus, compound **9** can be tentatively identified as quercetin-3-*O*-(2-pentosyl)rutinoside or quercetin-3-*O*-(2-pentosyl, 6-rhamnosyl)glucoside. In fact, it agrees with quercetin-3-*O*-(2″-*O*-β-apiofuranosyl-6″-*O*-α-rhamnopyranosyl-β-glucopyranoside), reported before in tomato (*28*).

The other compounds (**7** and **10**−**14**) are diglycosides of flavonoids for which the MS^2 fragmentations present, as base peak, the aglycone-related ion, as the last group of compounds, with only one substitution in the flavonol skeleton (**Table 1**). For compounds **7**, **10**, **11**, and **14** characteristic MS^2 fragmentation from diglycosides with interglycosidic linkage 1→2 are observed, having several ions corresponding to the loss of a hexose residue (162 u) and/or a hexose (180 u) (**7** + **10** and **11**) and 132 and 150 (132 + 18) pentosyl (**14**) in considerable abundance. Thus, these compounds can be identified as quercetin-3-*O*-sophoroside (**7**), kaempferol-3-*O*-sophoroside (**10**), isorhamnetin-3-*O*-sophoroside (**11**), and kaempferol-3-*O*-(2-pentosyl)glucoside (**14**). For compound **13** these ions are not observed, whereas for compound **12** (isomer of **11**) the ion at *m*/*z* 459 ($[M-H-180]^-$) has a relative abundance of 7 versus 45% of compound **11**, indicating that the interglycosidic bonds of these compounds are 1→6. Therefore, these compounds can be identified as isorhamnetin-3-*O*-gentiobioside (**12**) and quercetin-3-*O*-rutinoside (**13**). Within these characterized compounds, only one, quercetin-3-*O*-rutinoside (**13**), was described in tomato seeds (*9*). Moreover, quercetin-3-*O*-sophoroside-7-*O*-rhamnoside (**4**) and kaempferol-3-*O*-sophoroside-7-*O*-rhamnoside (**5**) were described in tomato fruit (*26*, *29*).

HPLC-PAD Phenolic Compound Quantitative Analysis. The phenolic composition of *L. esculentum* seed aqueous lyophilized extract was similar to that of the hydromethanolic one. To get a better characterization of the extract used in the biological assays, phenolic compounds were quantified by HPLC-PAD.

A total content of 20657 mg/100 g phenolic compounds was found in the aqueous lyophilized extract (**Table 2**). The compounds in highest amounts were quercetin-3-*O*-sophoroside (**7**) and kaempferol-3-*O*-sophoroside (**10**) plus isorhamnetin-3-*O*-sophoroside (**11**), representing ca. 29.2 and 59.1% of total phenolics,

Table 2. Phenolic Composition of Tomato Seed Aqueous Lyophilized Extract

flavonoid[a]	mg/100 g (dry basis)[b]
1 + **B**	328 ± 0.110
2	nq
3 + **4**	354 ± 1.06
5	nq
6 + **E**	151 ± 0.618
7	$603 \times 10^1 \pm 15.6$
8 + **9**	nq
10 + **11**	$122 \times 10^2 \pm 51.8$
12	738 ± 2.54
13	840 ± 7.58
14	nq
$\sum^c$	206×10^2

[a] Identity of compounds as in **Figure 1**. [b] Results are expressed as means (standard deviation) of three determinations. nq, not quantified. [c] Sum of the determined flavonoids.

respectively. All other compounds were found in smaller amounts (**Table 2**).

These compounds are secondary metabolites that are present in most plant seeds and grains (*14*, *30*, *31*) and play vital roles in defense against pathogens and predators and contribute to physiological functions, such as seed maturation and dormancy (*30*). At the subcellular level, phenolic compounds may accumulate in vacuoles or in the cell walls. In this particular case, all of the flavonoids present in tomato seeds are diglycosides or triglycosides, which are mainly confined to hydrophilic regions such as vacuoles and apoplasts (*32*).

Cytotoxicity. The SRB assay relies on the uptake of the negatively charged pink aminoxanthine dye, sulforhodamine B, by basic amino acids in the cells. The greater the number of cells, the higher amount of dye is taken up and, after fixing, when the cells are lysed, the released dye will give a more intense color and higher absorbance (*23*).

Tomato seed aqueous extract displayed a concentration-dependent cell proliferation inhibition with an IC_{50} of 5980 μg/mL. At the highest tested concentration (8 mg/mL), the inhibition reached 80% and, due to solubility issues, no concentrations above 8 mg/mL could be tested.

Due to the chemical complexity of tomato seed aqueous extract, it is not easy to establish which compounds are responsible for the displayed activity. Quercetin derivatives account for some 37% of the total amount of flavonoids. The aglycon has been implied in growth-inhibitory effects on several malignant tumor cell lines in vitro. These included NK/Ly ascites tumor cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, and MKN-28), colon cancer cells (COLON 320 DM), human breast cancer cells, human squamous and gliosarcoma cells, ovarian cancer cells, Ehrlich ascites cells, and the cell lines L1210 and P-388, which are leukemia cells like RBL-2H3 (*33*).

Although several molecules and pathways have been proposed as targets of flavonoids, the precise mechanisms by which these compounds exert their cancer-protective effects are still poorly understood. Most studies concerning cytotoxic effects of flavonoids employ the free aglycon. However, in natural extracts the predominant compounds are, usually, their glycosylated derivatives. It could be assumed that sterically hindered molecules, such as di- and triglycosylated flavonoids present in this extract, do not manage to pass through the cell membrane in high amounts. Nevertheless, some simpler molecules, such as quercetin-3-*O*-sophorose, kaempferol-3-*O*-sophorose, or isorhamnetin-3-*O*-sophorose, could be able to cross the membrane. In fact,

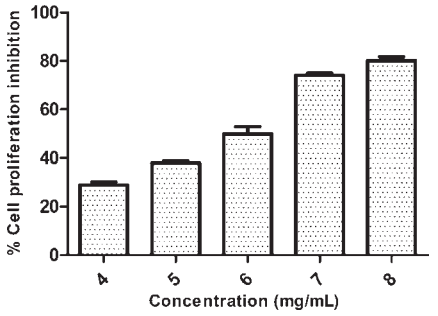

Figure 4. Cell proliferation inhibitory capacity of tomato seed aqueous extract in the SRB assay. Values show mean ± SE from three experiments performed in triplicate.

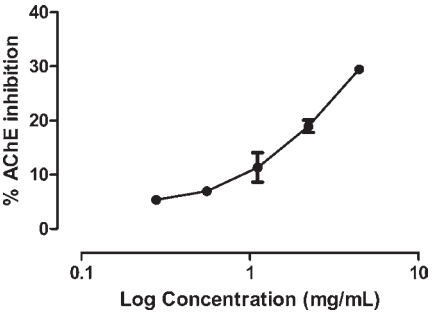

Figure 5. AChE inhibitory effect of tomato seeds aqueous extract. Values show mean ± SE from three experiments performed in triplicate.

quercetin-3-*O*-glucoside exhibited potent antiproliferative activity against MCF-7 cells (*34*). In addition to the identified compounds the role of the unidentified compounds **A**−**F** cannot be ignored. Moreover, the presence of glycoalkaloids, present in tomato plant (*35*) and which could also contribute to this activity, was discarded by the general alkaloid precipitation tests, applying Dragendorff's (solution of potassium bismuth iodide), Mayer's (potassiomercuric iodide solution), and Bertrand's (silicotungstic acid solution) reagents in the extract, purified according to Friedman and co-workers (*35*).

It is hypothesized that the anticancer activities of phytochemicals in fruits and vegetables result from additive and synergistic effects of a complex mixture of molecules (*36*). Therefore, the interest in using phytochemicals in therapeutics can arise not only from their isolated bioactivities but also from the combined effect of these compounds with others, such as medicines used in disease.

Bioactive compounds can offer additive or synergistic interaction through different biochemical targets. It is believed that phenolics can exert their effects on the different signaling pathways such as mitogen-activated protein kinases (MAPK), activator protein-1 (AP-1), or nuclear factor-κB (NF-κB), either separately or sequentially, as well as possibly interacting between/among these pathways (*37*), which can offer complementary and overlapping mechanisms of action.

AChE Inhibitory Activity. AChE is the principal enzyme involved in the hydrolysis of acetylcholine, a neuromediator. The screening of AChE inhibitors can have therapeutic applications in senile dementia, ataxia, myasthenia gravis, and Alzheimer's and Parkinson's diseases (*38*). The effect of tomato seed aqueous extracts on the enzyme activity was assessed for the first time. Tomato seeds exhibited a concentration-dependent AChE inhibitory capacity (**Figure 4**), and the IC_{20} obtained corresponded to 2400 μg/L. This activity can be, in part, explained by the presence of flavonoids, especially quercetin derivatives, which have been described in the literature as AChE inhibitors (*39*).

Antioxidant Capacity. Reactive species of oxygen (ROS) and nitrogen (RNS) are highly reactive molecules, constantly produced through numerous biological reactions. Reactive species lead to oxidative stress, which plays a determinant role in the development of chronic diseases. Therefore, diet-derived antioxidants could be important in providing protection against these diseases.

The DPPH$^{\bullet}$ assay provides basic information on the antiradical activity of extracts. In this assay, tomato seeds exhibited a weak concentration-dependent antioxidant potential (**Figure 5**).

Against nitric oxide, tomato seeds also provided protection in a concentration-dependent way, with an IC_{25} = 396 μg/mL.

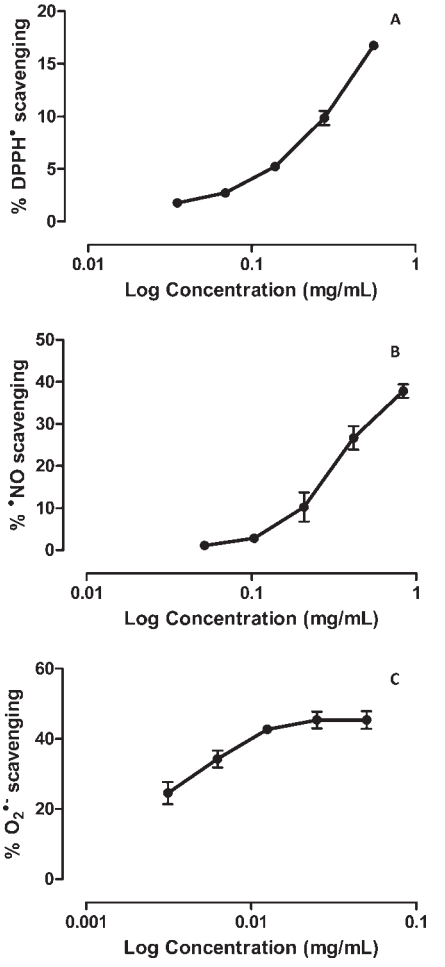

Figure 6. Effect of tomato seed aqueous lyophilized extracts against (**A**) DPPH, (**B**) nitric oxide, and (**C**) superoxide radical. Values show mean ± SE from three experiments performed in triplicate.

The same was observed against superoxide anion (IC_{25} at 3 μg/mL) (**Figure 6**). The scavenging of these two radicals can be of major importance due to their role in the formation of other reactive species, such as peroxynitrite, which can be extremely deleterious to cells (*40*).

The total antioxidant activity of extract results from the interaction between the several constituents, which may include synergistic or additive effects. Phenolic compounds such as those present in the extract, quercetin, kaempferol, and isorhamnetin glycosides, were already described as having antioxidant proprieties in several systems (*41*, *42*). The antioxidant potential exhibited by seeds can be explained by the need to protect their

storage lipids from oxidation and to ensure their viability, especially important during their germination when oxygen demand is high (*43*).

In conclusion, this work suggests that tomato seeds constitute a potential source of valuable secondary metabolites, such as antioxidant phenolic compounds. From a nutritional point of view, the determined compounds were present in the aqueous extract obtained after boiling for 30 min, which resembles the cooking procedure, and the biological activity was reported for this extract. Extracts obtained from tomato seeds can be an alternative to synthetic antioxidants in the food industry and also potentially used as a food supplement and source of bioactive compounds. With regard to cytotoxicity, the aqueous extract revealed a promising activity, and other studies should be performed to confirm the cell proliferation inhibitory potential in other carcinogenic cell lines and to elucidate the mechanism of action. Tomato seeds are often considered as waste in the production of tomato derivatives, and some consumers remove them from the fruit before use. According to the potential health benefits of tomato seeds reported in this work, the consumption of extracts from this vegetal material should not be ignored.

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