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Hydrophilic antioxidants from Andean tomato landraces assessed by their bioactivities *in vitro* and *in vivo*

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ABSTRACT

Potential nutraceutical properties of hydrophilic antioxidants in fruits of tomato landraces collected in Andean valleys were characterised. Antioxidant metabolites were measured by HPLC–DAD–MS/MS in mature fruits and their biological activities were assessed by *in vitro* and *in vivo* methods. *In vitro* antioxidant capacities were established by TEAC and FRAP methods. For *in vivo* biological activities we used a procedure based on *Caenorhabditis elegans* subjected to thermal stress. In addition, *Saccharomyces cerevisiae* was also used as a rapid screening system to evaluate tomato antioxidant capacity. All tomato accessions displayed significant differences regarding metabolic composition, biological activity and antioxidant capacity. Metabolite composition was associated with geographical origin and fruit size. Antioxidant activities showed significant association with phenolic compounds, such as caffeoylquinic acids, ferulic acid-*O*-hexosides and rutin. Combination of *in vitro* and *in vivo* methods applied here allowed evaluation of the variability in nutraceutical properties of tomato landraces, which could be applied to other fruits or food products.

1. Introduction

Tomato is widely consumed around the world and its consumption is considered beneficial to reduce the risk of several chronic diseases such as cardiovascular diseases and certain types of cancer (Friedman, 2013; Hollman, Hertog, & Katan, 1996; Viuda-Martos et al., 2014). Human health benefits are associated with antioxidant metabolites present in the fruits, which can be categorized as lipid- (hydrophobic) or water-soluble (hydrophilic). Lycopene, a carotenoid lipid-soluble antioxidant associated with several health benefits, is the major pigment found in ripe tomato fruits (Böhm, 2012; Rao & Agarwal, 2000). In addition, tomato fruits contain phenolic compounds (flavonoids and phenolic acids), which also contribute to a healthy diet (Slimestada & Verheulb, 2009). Phenolics and ascorbic acid (namely vitamin C) are considered the major contributors to total water-soluble antioxidant capacity in fruits and vegetables (Kähkönen et al., 1999; Oroian & Escriche, 2015; Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002).

All tomato species are native to Western South America (Peralta, Spooner & Knapp, 2008), where several landraces of cultivated tomatoes have been selected and maintained by local farmers. Thus, these cultivated tomatoes comprise a valuable resource for breeding, due to their genetic variation. Since 2004, Argentinean tomato landraces, cultivated in Central and Northwestern Andean valleys, have been collected, evaluated and conserved in the National Germplasm Bank, as a resource of public domain (Asprelli et al., 2011; Peralta et al., 2008). Selection during tomato domestication has contributed to reducing genetic diversity in this crop (Kamenetzky et al., 2010). Therefore, wild species together with local landraces are an invaluable source of beneficial compounds for improving cultivars with healthier composition and nutraceutical effects.

The antioxidant capacity of most natural products is mainly attributed to their redox properties (Frankel & Finley, 2008). Several *in vitro* methods have been applied to evaluate the antioxidant capacity of tomato fruits (Fraga, Oteiza, & Galleano, 2014). However, these antioxidant capacity assays do not necessarily reflect

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cellular physiological conditions nor consider absorption, bioavailability and metabolic issues. For these reasons, mammalian cell culture models have been developed to support antioxidant research, prior to assessment in animal models *in vivo* and in human clinical trials (Liu & Finley, 2005). These cell culture assays are simpler than the animal models; however they only address partial biochemical and molecular aspects of the antioxidant capacity. Several lower invertebrates, such as *Caenorhabditis elegans* (*C elegans*), have been proposed as an alternative to animal testing (Doke & Dhawale, 2013). *C. elegans* is a complex multicellular organism widely used to investigate molecular and biochemical mechanisms that could be extrapolated to mammals (Doke & Dhawale, 2013; Kaletta & Hengartner, 2006). In addition, this worm has great advantages, since it is easily reproduced and has a short life cycle. Furthermore, its genome has been fully sequenced and numerous genomic and post-genomic tools are available (Kaletta & Hengartner, 2006).

The aim of this work was to evaluate the potential nutraceutical properties of hydrophilic antioxidants from local tomato landraces collected from Argentine Andean valleys and to identify the main antioxidant compound/s likely responsible for these properties. Thus, the composition and biological activity of hydrophilic antioxidants of 11 Andean tomato landraces compared with those of 3 commercial cultivars and 2 wild-related species were evaluated. Firstly, ascorbic acid and total polyphenols contents were measured followed by the polyphenol composition analysis with HPLC–ESI–QTOF. Secondly, tomato antioxidant capacities were characterized by *in vitro* methods. Lastly, *in vivo* biological activities of fruit extracts were evaluated in terms of their capacity to confer tolerance to thermal stress in a *C. elegans* model and to confer tolerance to oxidative stress in a *Saccharomyces cerevisiae* (*S. cerevisiae*) model. Comparison of all these variables among accessions is shown and discussed. Moreover, relationships between the content of hydrophilic antioxidant compounds and geographical origin and fruit traits, as well as between antioxidant composition and *in vivo* and *in vitro* activities were comprehensively analysed.

2. Materials and methods

2.1. Reagents and materials

Ultra-pure water (<5 μg/L TOC) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Göttingen, Germany). HPLC-grade methanol and formic acid (puriss. p.a. for mass spectroscopy) were obtained from J.T. Baker (Mexico City, Mexico) and Fluka (Buchs, Germany), respectively. Gallic acid was purchased from Riedel-de-Haën (Shanghai, China). Filters (0.45 μm, HAWG04756) were obtained from Millipore (São Paulo, Brazil). ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt); TPTZ (2,4,6-tripyridyl-*s*-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) and Folin–Ciocalteu reagent were purchased from Sigma–Aldrich (Buchs, Switzerland). All other reagents were of analytical grade.

2.2. Tomato germplasm

Seeds of 16 tomato accessions were obtained from the Germplasm Bank of INTA La Consulta Agriculture Experimental Station (see Table 1 in Di Paola Naranjo et al., 2016). Accessions LA 407 of *Solanum habrochaites* and the accession #4739 of *Solanum pimpinellifolium* were multiplied from seeds sent by C. M. Rick Tomato Genetics (University of California, Davis). The other accessions belong to *Solanum lycopersicum*.

Seedlings were grown in 150-mL pots until four true leaves developed; they were then transplanted to an experimental field of the Horticulture Institute, Universidad Nacional de Cuyo (Mendoza, Argentina, 32° 50́S, 68° 52́W and 900 metres above sea level). The field trial was conducted from October 2008 to March 2009; plants were distributed according to a randomised design of three replicates with three individuals per replicate (nine plants per accession). The experimental area was protected with an anti-hail mesh, and irrigated periodically to keep available water content constant. At ripe stage defined by colour and firmness, three fruits per plant were harvested from three individual plants around 60 and 65 days after anthesis, and were immediately frozen in liquid nitrogen and kept in polyethylene tubes at −80 °C until use.

2.3. Preparation of tomato extracts

Tomato hydrophilic extracts were obtained as described by Capanoglu, Beekwilder, Boyacioglu, Hall, and De Vos (2008) with minor modifications. Briefly, tomato fruits were ground to a fine powder in liquid nitrogen and 500 mg of the material were extracted with 1.5 mL of 75% (*v*/*v*) aqueous methanol and then sonicated for 15 min. After centrifugation at 7000*g* for 10 min, the supernatant was collected; another 1 mL of 75% (v/v) aqueous methanol was added to the pellet and the extraction procedure was repeated, then both supernatants were combined and adjusted to a final volume of 3 mL with the methanolic extraction solution. The extracts from three different fruits of each accession were used to assess the composition and activity of the antioxidants. For the *in vivo* assays, 1 mL of hydroalcoholic extract from each of the three biological replicates was pooled and dried. Afterwards, the dry residue was dissolved in 200 μL dimethyl sulfoxide (DMSO) and kept at −80 °C until use.

2.4. Ascorbic acid determination

Ascorbic acid concentrations were measured according to the method of Asami, Hong, Barrett, and Mitchell (2003) with minor modifications. One hundred mg of tomato fruit powder (prepared as described in *Preparation of tomato extracts*) were added to 1 mL of 10% (v/v) perchloric acid and 1% (*w*/*v*) metaphosphoric acid solution, and stirred for 30 min at 4 °C. Extracts were centrifuged at 12,000*g* for 10 min at 4 °C. The supernatant was filtered through a 0.45-μm membrane filter and kept at low temperatures and light until HPLC analysis.

The analysis was performed using a Hewlett–Packard series 1100 HPLC system coupled with a UV detector (Agilent Technologies, Santa Clara, CA). Reverse-phase separation was attained using a Phenomenex (Torrance, CA) Luna 5 μm C18 (2) 100 Å (4.6 × 150 mm). The mobile phase was Nanopure water brought to pH 2.2 with sulfuric acid. The flow rate was 1 mL/min and the chromatograms were monitored at 234 nm. All samples were run in triplicate. The linearity range was determined from 12.5 to 250 μg/mL with a 20 μL injection volume (r^2 = 0.999). The results were expressed as mg of ascorbic acid per 100 g fresh weight.

2.5. Total determination of polyphenols

Total polyphenol (TP) content of the hydrophilic extracts was measured by Folin–Ciocalteu according to Arnous, Makris, and Kefalas (2001) with minor modifications. In a 1.5-mL tube, 750 μL of distilled water, 50 μL of hydrophilic extract and 50 μL of Folin–Ciocalteu reagent were added and vortexed. After 1 min, 150 μL of 20% (*w*/*v*) aqueous sodium carbonate were added and the mixture was vortexed and allowed to stand at room temperature in the dark for 120 min. The absorbance was read at 750 nm and the TP concentration was calculated from a calibration curve

using gallic acid as a standard. The linearity range was determined from 7.6 to 250 μg/mL (r^2 = 0.999). The results were expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW) and corrected according to the ascorbic acid contribution.

2.6. HPLC–DAD–ESI–MS/MS analysis

The phenolic compounds from tomato samples were analysed by HPLC–DAD–ESI–MS/MS method using an Agilent Technologies 1200 Series system equipped with gradient pump (Agilent G1312B SL Binary), solvent degasser (Agilent G1379 B) and autosampler (Agilent G1367 D SL+WP). The HPLC system was connected to a photodiode array detector (Agilent G1315 C Starlight DAD) and then to a QTOF mass spectrometer (micrOTOF-Q11 Series, Bruker) equipped with an electrospray ionisation (ESI) interface. HPLC conditions were in accordance with the method described in Llorente et al. (2014).

Tentative identification of phenolic compounds was based on their retention times, elution order, UV–Vis spectra and MS fragmentation spectra as compared with phenolic standards, in addition to those reported in the literature. For this purpose, nine commercially available standards: caffeic acid and ferulic acid (Extrasynthèse, Genay, France), myricetin, kaempferol, *p*-coumaric acid and naringin (Fluka, United Kingdom), chlorogenic acid, naringenin and rutin (Sigma–Aldrich, Steinheim, Germany) were used. Polyphenol quantification was based on external calibration curves from available phenolic standards, by using the mass peak areas obtained from the extracted ion chromatograms, at concentrations between 0.78 and 100 μg/mL. When the corresponding standards were not available, the quantification was performed using an external standard with a similar structure to the compound in question. The extracts from tomato fruits and the standards were filtered (0.45 μm) and injected in HPLC–ESI–MS/MS system. The linear analytical range was between 0.015 and 7.00 mg/mL (r^2 > 0.98). Coefficient of variation (CV) was below 10%. LOD ranged from 0.003 to 0.050 mg/L, whereas LOQ varied from 0.009 to 0.185 mg/L.

2.7. In vitro antioxidant capacity

The *in vitro* antioxidant capacity was measured using both Trolox equivalent antioxidant capacity (TEAC) and ferric reducing ability of plasma (FRAP) assays.

2.7.1. TEAC assay

This assay was performed according to Re et al. (1999) with minor modifications. Twenty-five μL of hydrophilic extract or Trolox standard were mixed with 1 mL of diluted $ABTS^{\bullet+}$ solution according to Re et al. (1999) and vortexed for 10 s; absorbance was measured at 734 nm after 4 min of reaction at 30 °C. The results were obtained by interpolating absorbance on a calibration curve obtained with Trolox (0.03–0.50 mM, r^2 = 0.999) and expressed as mmol of Trolox equivalent per 100 g of fresh weight.

2.7.2. FRAP assay

This assay was performed in accordance with Benzie and Strain (1998), with minor modifications. The working FRAP reagent was prepared as required by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) and 2.5 mL 20 mM $FeCl_3.6H_2O$ solutions. A total of 100 μL of hydrophilic extract was added to 3 mL of FRAP reagent and absorbance was measured at 593 nm after incubation at room temperature for 6 min, by using FRAP reagent as a blank. The results were obtained by interpolating absorbance on a calibration curve obtained with Trolox (0.03–1.00 mM, r^2 = 0.999) and expressed as mmol of Trolox equivalent per 100 g FW.

2.8. In vivo biological activity

2.8.1. C. elegansthermotolerance assay

The survival assay was inspired by the procedure described by Kampkötter et al. (2007), which was adapted and optimised to characterise the properties of tomato fruits to revert mortal stresses in living organisms. Wild-type *Bristol N2* strain was obtained from the Caenorhabditis Genetics Center (CGC). Worms were reproduced and maintained at 20 °C on nematode growth medium (NGM) plates using *Escherichia coli* OP50 as food source. For thermotolerance assays, age-synchronised worms were obtained by isolating eggs through the sodium hypochlorite method (Stiernagle, 2006). When worms reached the young adult stage (three days after worms synchronisation), at least 30 young adults were transferred to plates containing fresh medium supplemented with serial dilutions of the tomato fruit extracts. The indicated 1:10 serial dilutions of tomato extracts were obtained starting from 1:100 dilution of the DMSO-dissolved extracts (prepared as aforementioned) in the same solvent.

Each experiment was performed using the extracts in triplicate and 1% (*v*/*v*) DMSO as a vehicle control. After incubation at 20 °C for 18 h, worms were subjected to heat stress by treatment at 37 °C for 5 h 40 min. Worms were scored as dead when they failed to move in response to touch with a platinum wire.

Survival rate was dependent on extract dilution and in most accessions an inverted U-curve was obtained where one side is produced by toxic effects of high doses of extract and the other side is produced by the thermotolerance activity. Therefore, a dose–response curve (log μg tomato *vs*% worms rescued from death with respect to control) was plotted for each accession with extract dilutions showing no toxic effects, and thermotolerance activity was calculated (survival rate relative to control) and expressed as the effective dose (ED50).

Moreover, thermotolerance activity of pure commercial standards of chlorogenic acid and rutin was evaluated at the same concentrations as those found in the accessions. Likewise, mixtures of compound rutin and chlorogenic acid, in the same ratios as those found in these accessions, were assessed. The commercial compounds were dissolved in DMSO and evaluated using the same procedure as that for the extracts. The thermotolerance activity was expressed as the survival rate relative to control.

2.8.2. S. cerevisiae oxidative stressassay

The survival assay was performed in accordance with Baroni, Di Paola Naranjo, García-Ferreyra, Otaiza, and Wunderlin (2012). *S. cerevisiae* cells were grown in liquid YPD (yeast extract, peptone, dextrose) medium, using an orbital shaker at 28 °C and 160 rpm (the flask: medium ratio was 5:1). Yeast cells at the exponential phase (A_{600}: 0.5–0.7) were transferred to fresh medium (A_{600}: 0.2) containing H_2O_2 (0.75 mM) with or without tomato extracts. Yeast cells were then incubated for 1 h at 28 °C and spun at 160 rpm. The optimal extract dose was determined in adaptive treatments, by exposing cells to increased concentrations of tomato extracts. The selected concentration (13.9 μg/mL final concentration) was the minimum concentration improving cell growth as compared with yeast exposed to H_2O_2 alone (0.75 mM). Two control groups were used: a control plate (untreated cells) and tomato extract control plate (yeast exposed to extract alone, without addition of H_2O_2)

Cell viability was analysed by plating the cells on solid YPD medium, after proper dilution. The plates were incubated at 28 °C for 72 h. The number of colonies observed in the control plate (untreated cells) was set as 100%. The number of colonies in each plate was between 150 and 200. *S. cerevisiae* activity was expressed as the survival percentage with regard to the control and

calculated as: (% survival in yeast exposed to extract and H_2O_2) – (% survival in yeast exposed to H_2O_2).

2.9. Statistical analysis

Three biological replicates (three fruits of different plants) were used for all the analysis methods. In the case of Folin–Ciocalteu, TEAC and FRAP analyses, three technical replicates per biological replicate were measured. For the *in vivo* assays, three technical replicates were evaluated from the three pooled biological replicates as described in *Preparation of tomato extracts*. Statistical analyses were carried out using Infostat Software Package (Di Rienzo et al., 2013). Data are expressed as mean ± SD and were analysed using ANOVA test with $p < 0.05$. When significant differences were found by ANOVA, the Scott and Knott test was used. For multivariate analysis, principal component analysis (PCA) and hierarchical cluster analysis (complete linkage clustering method) were performed in Infostat Software; canonical analysis correlation and multiple regression analysis were performed in Statistical software (StatSoft, 2005).

3. Results

3.1. Total polyphenols and ascorbic acid

Fig. 1 shows mean content of total polyphenols (TP) and ascorbic acid for each tomato accession. TP and ascorbic acid mean contents ranged from 37 to 147 mg/100 g GAE and from undetectable values to 54.2 mg/100 g, respectively. Wild species *S. habrochaites* and *S. pimpinellifolium* showed the highest TP content in comparison with all *S. lycopersicum* accessions. In contrast to TP contents, fruits from *S. habrochaites* showed undetectable levels of ascorbic acid, whereas *S. pimpinellifolium* showed the highest levels, followed by *S. lycopersicum* accessions. The ratio of TP and ascorbic acid content in these species is in agreement with those reported by Kavitha et al. (2014). However, in that study, antioxidant contents reported for commercial cultivars and wild species were lower than those obtained in this research (20–96 mg/100 g GAE and 4–25 mg/100 g for TP and ascorbic acid, respectively). Among *S. lycopersicum* accessions, Andean landrace #572 showed the highest levels of TP and ascorbic acid, which were equivalent to the values detected in the wild species. By contrast, accession #3842 had the lowest levels of both TP and ascorbic acid.

3.2. Phenolic composition of tomato accessions

Hydrophilic tomato extracts were analysed by HPLC–DAD–MS and 16 phenolic compounds were identified according to their retention times, fragmentation patterns data (mass spectrometry) and UV–Vis spectra data (see Table 2 and Figs 1 and 2 in Di Paola Naranjo et al., 2016) and quantified (Fig. 2). All of the identified compounds had been previously reported in tomato (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010; Larbat, Paris, Le Bot, & Adamowicz, 2014; Moco et al., 2006; Vallverdú-Queralt, Jáuregui, Di Lecce, Andrés-Lacueva, & Lamuela-Raventós, 2011). Chlorogenic acid (CGA) and rutin (quercetin-3-*O*-rutinoside) were the most abundant components. In addition, other caffeoylquinic acids, such as cryptochlorogenic acid (cryptoCGA), neochlorogenic acid (neoCGA), a chlorogenic acid isomer (CGA isomer) and trihydroxycinnamoylquinic acid (TriOH-CQA), were also found in all accessions. In contrast, naringenin-chalcone was present in 14 out of 16 cultivars studied. Additionally, we detected five other compounds corresponding to caffeic acid-*O*-hexoses (CaffAH) and two coumaric acid-*O*-hexoses (CAH), which can be found as different constitutional isomers in tomatoes (Gómez-Romero et al., 2010). These isomers were named with consecutive numbers according to their chromatographic retention times (see Table 2 in Di Paola Naranjo et al., 2016). Indeed, structural variations can arise from linkage of hexose moiety to 3-hydroxy, 4-hydroxy and carboxylic acid positions; glucose, galactose or mannose being the most common plant hexoses (Gómez-Romero et al., 2010). We have also identified, in all accessions, caffeoylhexaric acid (Caff-Hex) and ferulic acid-*O*-hexoside (FAH). Consequently, for all phenolic compounds identified, quantitative analysis was achieved by using the respective standards or closely related derivatives of the same phenolic family.

As described above, phenolic profile showed a high level of variation among tomato accessions (Fig. 2). To gain insight into the antioxidant composition among accessions, principal component analysis (PCA) was applied and the accessions were grouped in the PCA biplot graphic according to the result of hierarchical cluster analysis (Fig. 3). Five groups were identified (Fig. 3a). Cluster one contained the wild species *S. habrochaites* (accession LA407) showing the highest content of CGA, cryptoCGA, the CaffAH1, 2 and 4 isomers and FAH (Fig. 3b). The second cluster contained landrace accessions #565 and #557 that showed the highest levels of TriOH-CQA and both CAH isomers. The third cluster was formed by the other wild species *S. pimpinellifolium* (accession #4739) showing also high levels of CGA acid and cryptoCGA as well as high rutin level. Cluster four, which is shown to overlap with cluster 5 in the biplot of PC1 vs PC2, corresponded to landrace accession #572 with the highest levels of rutin, naringenin chalcone and neoCGA (Fig. 2). The last cluster (cluster 5) contained the remaining accessions characterised by a high level of CaffAH3 and 5 isomers, CGA isomer and Caff-Hex.

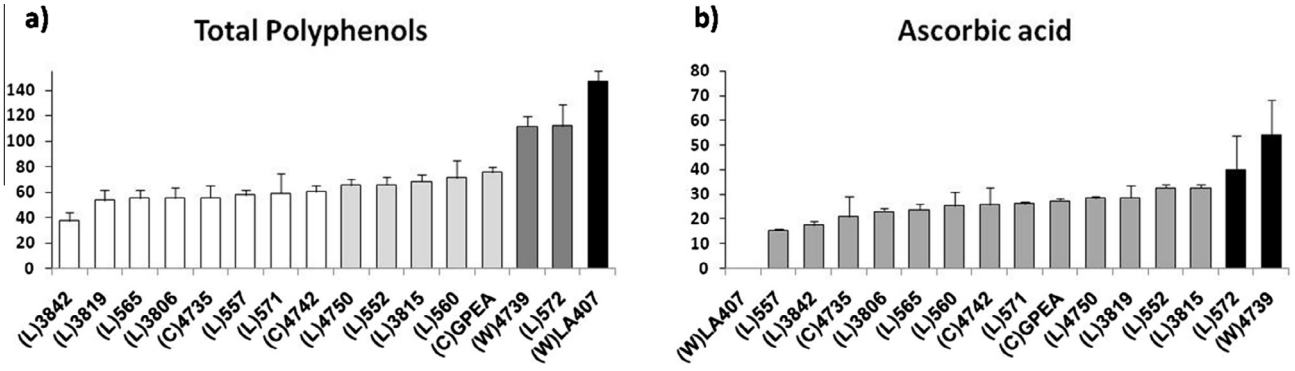

Fig. 1. Hydrophilic antioxidant content of tomato fruit from landrace accessions (L), commercial accessions (C) and wild tomato accessions (W). (a) Total polyphenol (TP) expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW) and (b) ascorbic acid expressed as mg per 100 g FW. Significant differences ($p < 0.05$, Scott and Knott test) between tomato accessions are represented by different grey tones in the bars.

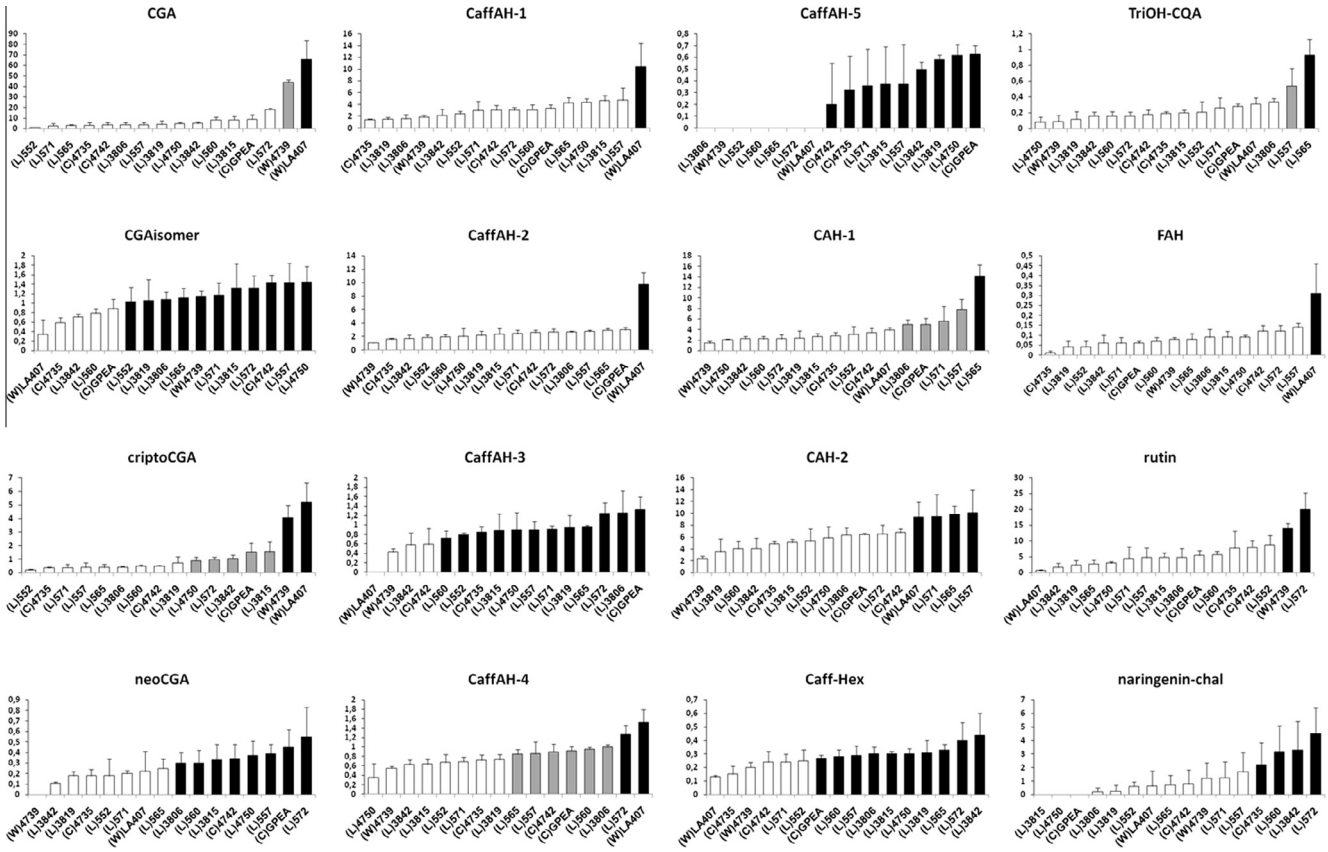

Fig. 2. Phenolic composition of tomato fruit from landrace accessions (L), commercial accessions (C) and wild tomato accessions (W). Phenolic compounds are expressed as mg per 100 g of fresh weight. Significant differences ($p < 0.05$, Scott and Knott test) between tomato accessions are represented by different grey tones in the bars. Caff-Hex: caffeoylhexaric acid; CGA: Chlorogenic acids; TriOH-CQA: Trihydroxycinnamoylquinic acid; CAH: coumaric acid-*O*-hexoside; CaffAG: caffeic acid-*O*-hexoside; FAH: ferulic acid-*O*-hexoside.

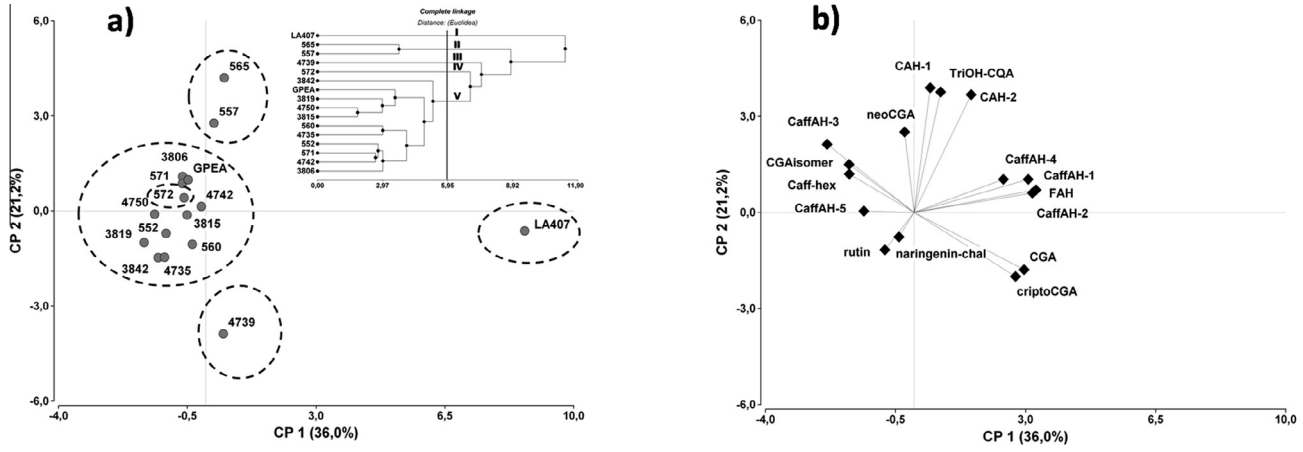

Fig. 3. Principal component analysis of tomato accessions. Bi-plot of 16 tomato accessions grouped (defined by ellipses) according to cluster analysis (a) and bi-plot of antioxidant metabolite variables (b).

As expected, the composition of antioxidant compounds in wild species was clearly different from that found in *S. lycopersicum* accessions. Therefore, to evaluate associations between antioxidant composition and fruit colour, morphology and size (defined by fruit weight), we have focused the analysis only on *S. lycopersicum* accessions (see Fig 3 in Di Paola Naranjo et al., 2016). Small fruits (fruit weight lower than 40 g) were distinguished from bigger fruits across principal component 1 (PC1) (see Fig. 3a in Di Paola Naranjo et al., 2016), which is consistent with the higher levels of most antioxidant metabolites (see Fig. 3b in Di Paola Naranjo et al., 2016). We have also evaluated possible associations between antioxidant composition and accession origin and altitude (see Fig. 4 in Di Paola Naranjo et al., 2016). PCA analysis showed two separated groups in PC1, one of them grouped fruits from accessions collected in Argentine northwestern locations (Jujuy, Salta and Catamarca) with the highest altitude and, in the other group, those collected from Argentine central locations with lower altitude (Mendoza and La Plata) and commercial accessions (see

Fig. 4a in Di Paola Naranjo et al., 2016). Fruit derived from Jujuy, Salta and Catamarca accession was grouped because of its high levels of most antioxidants (see Fig. 4b in Di Paola Naranjo et al., 2016). PC2 showed that accessions collected in Jujuy displayed a differentiated composition of Caff-Hex and CAH isomers as well as TriOH-CQA.

3.3. In vitro antioxidant capacity of tomato cultivars

Antioxidant capacity was measured by both FRAP and TEAC *in vitro* assays. FRAP assay provides a reliable method to evaluate antioxidant capacity in food as it is usually correlated with its reducing capacity (Benzie & Strain, 1998). Likewise, TEAC assay measures the ability of an antioxidant to scavenge free radicals such as $ABTS^{\bullet+}$ (Re et al., 1999). Fig. 4a shows the results from these measurements. The most clear differences in both TEAC and FRAP assays were those displayed by the wild species in comparison with those from *S. lycopersicum* accessions, in agreement with those reported by Kavitha et al. (2014). The results of both methods showed that the two wild tomato accessions had the highest *in vitro* antioxidant capacity followed by the Andean accession #572. This is in agreement with the high levels of TP observed in these accessions (Fig. 1).

3.4. In vivo assay of biological activity

3.4.1. C. elegans thermotolerance assay

Incubation of *C. elegans* worms at 37 °C generates a thermal stress and ROS accumulation that is lethal within a few hours (Kampkötter et al., 2007). In our assay, worms are exposed to 37 °C for 5 h 40 min, which causes death of more than 50% of the population incubated in the DMSO control. For each trial, worms were pre-incubated with either DMSO (control) or tomato extracts dissolved in DMSO (prepared as described under *Materials and Methods*). A dose–response curve (log µg *vs* survival rate with respect to the control) was plotted (see Fig. 5 in Di Paola Naranjo et al., 2016) and the effective dose (ED50) was calculated for each accession (Fig. 4b). A very broad range of thermotolerance activity (from 0.02 to 313 µg ED50) with significant difference among accessions can be noted. Accession #4739 (*S. pimpinellifolium*), Andean landrace accessions #557, #4750, #4742, #552, LA407 (*S. habrochaites*) and #3806 (commercial accession) showed the lowest ED50, and therefore, the strongest thermotolerance activity, whereas commercial cultivar GPEA and landrace accessions #3842, #3819, #572 and #571 showed significantly higher ED50 values, and, therefore, the lowest thermotolerance activity.

3.4.2. S. cerevisiae oxidative stressassay

We next turned our attention to other method to test the biological activity of our tomato collection. We used a *S. cerevisiae* model under oxidative stress caused by hydrogen peroxide treatment (Baroni et al., 2012). Yeast cells showed sensitivity to H_2O_2 (0.75 mM) and only 52% of the yeast population were able to survive oxidative stress. A pre-treatment of yeast cells with tomato fruit extracts partially suppressed the damage triggered by the oxidant and increased the survival rate with respect to the control (Fig. 4b). Except for the Andean landrace accessions #565, #3819, #571, #560 and the commercial accession #GPEA and #3806, the other accessions increased significantly the survival rate with respect the control (yeast exposed only to H_2O_2). Among these

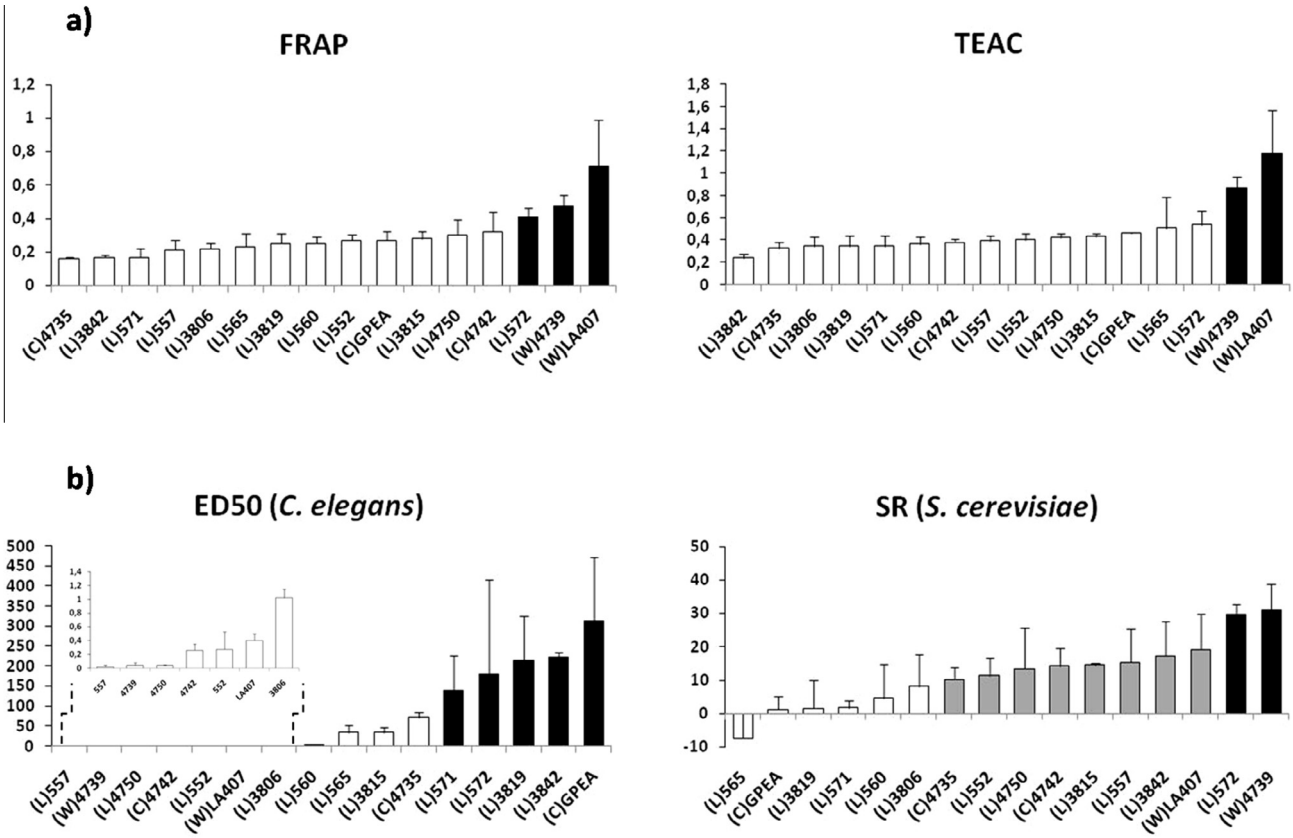

Fig. 4. Antioxidant activities of hydrophilic tomato extract from landrace accessions (L), commercial accessions (C) and wild tomato accessions (W). (a) *In vitro* antioxidant capacity TEAC and FRAP expressed as mmol of Trolox equivalent per 100 g of fresh weight. (b) *In vivo* activities in *C. elegans* and *S. cerevisiae*. *C. elegans* activity expressed as effective doses (ED50) of tomato (µg) that rescued from death 50% of dead worm population. *S. cerevisiae* activity is expressed as the survival rate relative to control (survival rate, SR). Significant differences ($p < 0.05$, Scott and Knott test) between tomato accessions are represented by different grey tones in the bars.

accessions, #4739 (*S. pimpinellifolium)* and the Andean landrace #572 showed the highest survival rate of the treated yeasts.

Similar to antioxidant composition, we evaluated putative associations between antioxidant activities and fruit traits such as colour, size and shape (see Fig. 6 in Di Paola Naranjo et al., 2016). Contrary to antioxidant composition, antioxidant activities showed no associations with these traits, and no association between antioxidant activities and altitude or geographical origin of accessions (see Fig. 7 in Di Paola Naranjo et al., 2016).

3.5. Relationships among tomato metabolite composition, in vitro antioxidant activities and in vivo biological activities

To better understand possible associations between antioxidant composition and biological activity of tomato fruit extracts, we performed canonical correlation analyses (Fig. 5). *In vitro* antioxidant capacities (TEAC and FRAP) correlated significantly with the set of metabolites ($r = 0.83$, $p = 0.0001$) (Fig. 5a). The correlation within variables showed CGA, cryptoCGA and CaffAH2 as the metabolites that most contributed to *in vitro* activities, whereas CaffAH3 contributed negatively to *in vitro* activities (Fig. 5a). For *in vivo* antioxidant activities, canonical correlation analyses also showed a strong and significant correlation between *in vivo* activities in *C. elegans* and *S. cerevisiae* and antioxidant metabolites ($r = 0.94$, $p = 0.00001$) (Fig. 5b). The correlation matrix showed CGA and FAH as the metabolites that most contributed to thermotolerance in *C. elegans*, whereas rutin and CGA most contributed to oxidative stress tolerance in *S. cerevisiae* (Fig. 5b). On the other hand, Caff-Hex, CaffAH5 and CaffAH3 were the metabolites that most negatively contributed to thermotolerance, and CAH and triOH-CQA2 to oxidative stress tolerance (Fig. 5b).

Although phenolics and ascorbic acid are probably the major contributors to the total water-soluble antioxidant capacity in fruits and vegetables (Kähkönen et al., 1999; Oroian & Escriche, 2015; Ou et al., 2002), in our study only phenolic contents correlated to the antioxidant activities measured. To support the abovementioned associations, multiple regression analyses were performed for each activity. For antioxidant *in vitro* capacity, a significant linear relationship was observed for TEAC ($r^2 = 0.75$, $p = 0.000001$) and FRAP ($r^2 = 0.33$, $p = 0.00002$), where CGA was a positive contributor to both these activities (TEAC = 0.0123 * CGA + 0.329) (FRAP = 0.007 * CGA + 0.221). This compound was found in high concentration in the wild species and in the landrace accession #572 (Fig. 2). For *C. elegans* thermotolerance activity, a significant correlation was obtained ($r^2 = 0.63$, $p = 0.000002$) with CGA, triOH-CQA2 and FAH as positive contributors to this activity and CAH-2 as a negative contributor (ED50 = 394 * CaffHex + 295 * CaffAH(4) + 49 * CaffAH(5) + 31 * cryptoCGA + 45 * CAH(1) + 22 * naringenin − 1000 * FAH − 602 * TriOHCQA(2) − 5.878 * CGA − 346). For oxidative stress tolerance in *S. cerevisiae*, a significant correlation was also observed ($r^2 = 0.47$, $p = 0.000002$) with CGA isomer, rutin and CaffAH3 as positive contributors and CAH 1 as a negative contributor to this activity (survival rate = 7.9 * CGA isomer + 1 * rutin − 14 * CaffAH(3) − 1.1 * CAH(1) − 13).

Likewise, we detected significant correlations (Spearman correlation) among antioxidant activities; *S. cerevisiae* biological activity correlated with both methods (FRAP: $r = 0.43$, $p = 0.002$; TEAC: $r = 0.30$, $p = 0.04$), whereas *C. elegans* biological activity only significantly correlated with FRAP ($r = -0.33$, $p = 0.02$; TEAC $r = -0.25$, $p = 0.09$).

In the present results, the chlorogenic acids and rutin were associated with *in vivo* activities by statistical tests. To experimentally confirm these associations, we evaluated the thermotolerance activity in *C. elegans* exerted by the pure compounds (chlorogenic acid and rutin) at the same concentrations found in the wild species (chlorogenic acid: 44 and 65 mg/100 g FW; rutin: 14 and 0.66 mg/100 g FW for the accessions #4740 and LA407, respectively) and in the landrace accession #572 (chlorogenic acid: 18 mg/100 g; rutin: 20 mg/100 g FW) (three accessions with highest levels of these compounds, except for LA407 that showed the lowest level of rutin, see Fig. 2). Both phenolic compounds showed a significant increase in the survival rate of worms to thermal stress at different concentrations (Fig. 6). Interestingly, accession #572 showed a high *in vivo* activity in *S. cerevisiae* model but a low thermotolerance activity in *C. elegans* model (Fig 4b). However, chlorogenic acid and rutin at the same concentration as this accession showed a significantly high thermotolerance. We next evaluated the thermotolerance activity of chlorogenic acid and rutin mixtures at the same levels of their corresponding tomato accessions (Fig. 6). Only the mixture corresponding to wild species showed a significantly higher thermotolerance, whereas the mixture corresponding to accession #572 did not contribute to thermotolerance. This result shows an antagonist effect of rutin and chlorogenic acid at certain levels that could explain the low thermotolerance activity observed in accession #572.

4. Discussion and conclusion

We present here an in-depth characterisation of a highly valuable germplasm collection of tomato in terms of its nutraceutical properties. Particularly, the hydrophilic antioxidant composition of ripe fruits was evaluated *in vitro* and *in vivo*. The antioxidant composition in tomato fruits seems to be related to the evolutionary history of the species and domestication of the crop. The antioxidant composition of wild species *S. habrochaites* and *S. pimpinellifolium* clearly differed from that of *S. lycopersicum* accessions (Figs. 1–3). The genetic divergences among these species have been settled (Koenig et al., 2013) and previous studies have reported metabolic difference among these species (Quadrana et al., 2012; Top et al., 2014 and Quadrana et al., 2014). Moreover, an important diversity in antioxidants composition was found among *S. lycopersicum* accessions (Figs. 1 and 2). Other studies involving cultivar groups of local tomato varieties have found similar results (Figàs et al., 2015). Cultivated tomatoes have been selected for a better taste, aroma, colour and size, especially landraces preferred by local farmers for their own consumption. In previous studies, the composition of secondary metabolites (such as antioxidants) and their heritability have been associated to fruit traits (Alseekh et al., 2015). Here, we found associations of antioxidant composition with fruit size but not with colour and shape (see Fig. 3 in Di Paola Naranjo et al., 2016). We also found associations of antioxidant composition with the origin-altitude site of accessions; accession from high altitudes showed the greatest abundance of antioxidant metabolites (see Fig. 4 in Di Paola Naranjo, et al., 2016). Present results have exposed the genetic diversity among these accessions, where the antioxidants production could be playing an important role in the survival in and adaptability to changing environment. For instance, antioxidant phenolic metabolites are key players to protect fruits against solar radiation (González et al., 2014). Although for this study all accessions were grown in the same environment (Mendoza, 700 m above sea level), the higher antioxidant content observed in the northwest accessions could be related to an improved genetic response to environmental conditions.

Antioxidants may generate a protective effect against chronic human diseases by activating different mechanisms to neutralise the production of ROS and thereby contributing to nutraceutical properties of food. A current challenge is to identify nutraceutical properties of food and measure their ability to reverse oxidative processes. For this purpose, here we showed that the potential nutraceutical properties of tomato fruits can be easily characterised by a method based on the thermotolerance displayed by

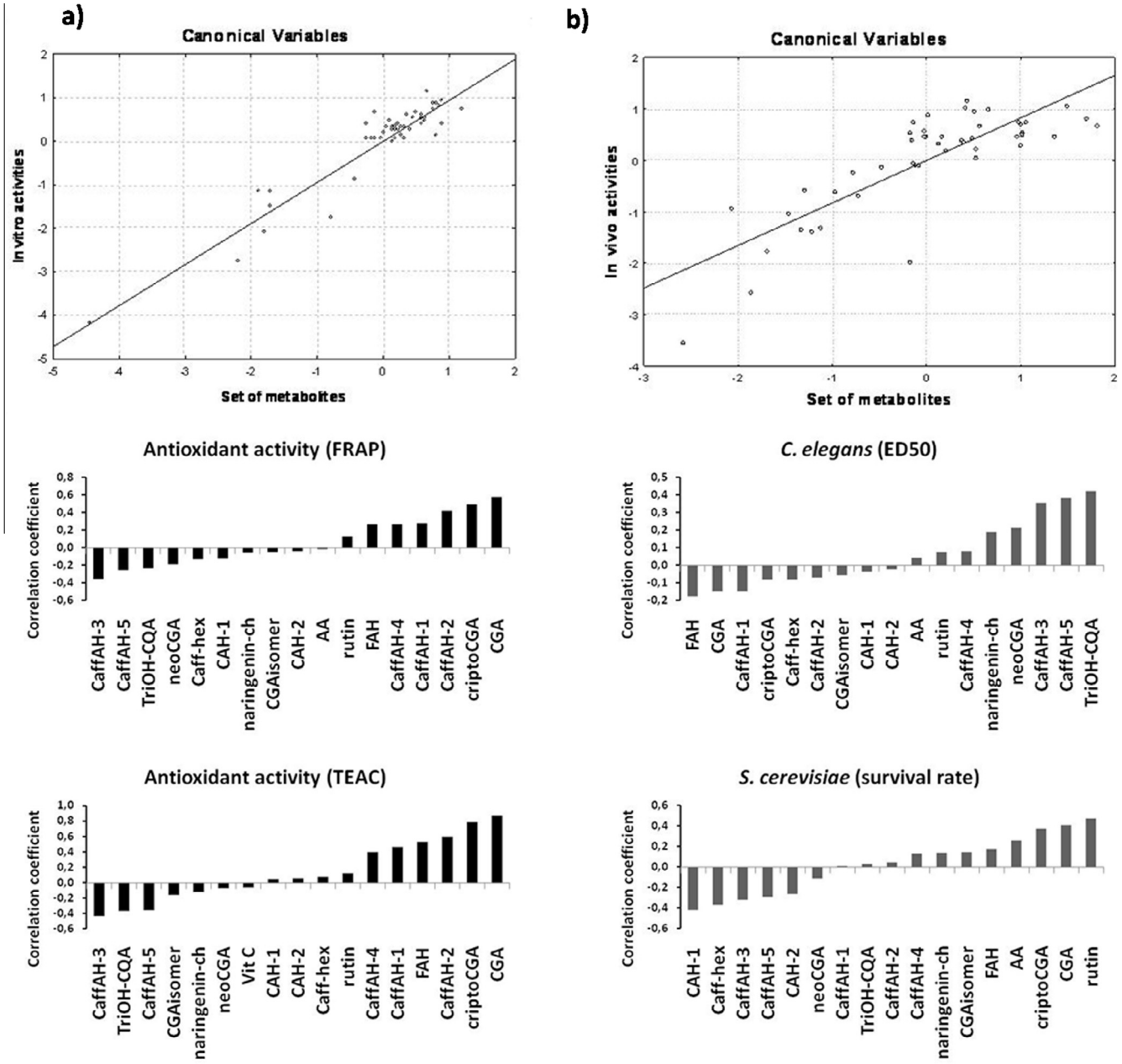

Fig. 5. Canonical correlation between antioxidant activities and antioxidant metabolites. (a) Linear regression plot between *in vitro* antioxidant activities and metabolites and correlation coefficients of each metabolite that contributed to canonical correlation. (b) Linear regression plot of *in vivo* antioxidant activities and total metabolites and correlation coefficients of individual metabolites that contributed to canonical correlation.

C. elegans when incubated with tomato extracts. The antioxidant activity obtained by *in vivo* assay with *C. elegans* was in agreement with a previously described method based on tolerance to hydrogen peroxide of *S. cerevisiae* (Baroni et al., 2012). We identified eight tomato accessions (wild species *S. habrochaites* LA 416 and *S. pimpinellifolium* #4739 and landrace accessions, #557, #4750, #552 and #3815, and commercial accessions #4735, #4742) with the highest impact on tolerance to lethal stresses. Furthermore, landrace accessions #560, #565 and commercial cultivar #3806 showed only a high thermotolerance, while landrace accessions #572 and #3842 showed only a high tolerance to oxidative stress. Contrary to antioxidant composition, antioxidant activities were not associated with fruit traits and altitude of places of origin (see Figs. 6 and 7 in Di Paola Naranjo, et al., 2016). However, they were significantly associated with antioxidant metabolites. The main compounds associated with tolerance to oxidative stress in *S. cerevisiae* were chlorogenic acid isomer and rutin. On the other hand, the main compounds associated with reversion upon thermal stress in *C. elegans* were ferulic acid-*O*–hexoside, trihydroxycinnamoylquinic acid and chlorogenic acid. Rutin and chlorogenic acid were tested pure with doses equivalent to those found in the fruits; both compounds displayed significant thermotolerance activity in *C. elegans* (Fig. 6). However, the thermotolerance activity of rutin/chlorogenic mix was strongly dependent on their ratio (Fig. 6). This result exposed the dependence and complexity of nutraceutical properties on the interaction of antioxidant metabolites in a complex food matrix. Selection and breeding of new tomato varieties with high chlorogenic acid content could contribute to develop new varieties with enhanced properties for human health. However, strategies for improving the content of

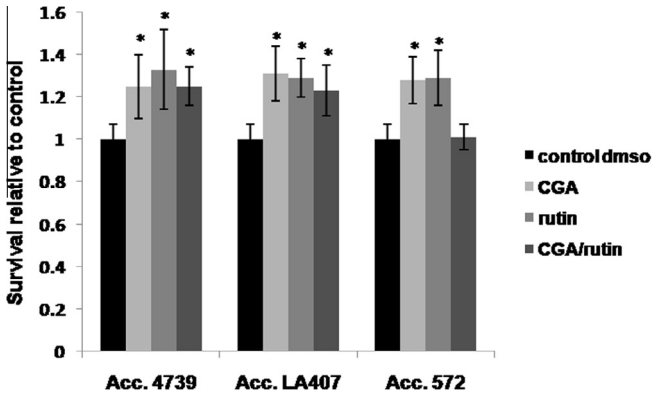

Fig. 6. Survival rate to thermal stress of *C. elegans* worms pre-incubated at different concentrations of chlorogenic acid and rutin standards (chlorogenic acid: 0.33, 0.48 and 0.135 μg/mL of culture media and rutin: 0.105, 0.049 and 0.486 μg/mL of culture media for accessions #4739, LA407 and #572 respectively). These concentrations correspond to the same levels as those found in tomato accessions #4739, LA407 and #572. (*) Means significant differences ($p < 0.05$) with respect to control.

phenolic acids (reviewed by Kaushik et al., 2015) should consider the interactions between individual phenolic acids.

As a conclusion, antioxidant compositions of wild species and landraces were significantly different. These compositions were associated to fruit traits and geographical origin and their altitude, where accessions of Argentine northwest contained the highest levels of most antioxidants. The *in vitro* and *in vivo* antioxidant activities were also significantly different among accessions. However, they were not associated to fruit traits or geographical origin like antioxidant composition. Rather, antioxidant activities were significantly associated to specific antioxidant metabolites, such as caffeoylquinic acids, ferulic acid-*O*-hexoside and rutin and their activities also seemed to be associated with the specific antioxidant ratio and with other antioxidant metabolites.

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