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ARTICLE

Prestorage Oxalic Acid Treatment Maintained Visual Quality, Bioactive Compounds, and Antioxidant Potential of Pomegranate after Long-Term Storage at 2 °C

Mohammad Sayyari,[†,#] Daniel Valero,[†] Mesbah Babalar,[§] Siamak Kalantari,[§] Pedro J. Zapata,[†] and María Serrano*[,‡]

[†]Department of Food Technology, and [‡]Department of Applied Biology, EPSO, University Miguel Hernández, Ctra. Beniel km. 3.2, 03312 Orihuela, Alicante, Spain, [§]Department of Horticulture, College of Agriculture, University of Tehran, Karaj, 31587-77871 Tehran, Iran, and [#]Department of Horticulture, College of Agriculture, University of Ilam, 69315-516 Ilam, Iran

Oxalic acid at three concentrations (2, 4, and 6 mM) was applied by dipping to pomegranate fruits of cv. Mollar de Elche, which were then stored for 84 days at 2 °C. Pomegranate is a chilling-sensitive fruit and, thus, control fruits exhibited chilling injury (CI) symptoms after long-term storage at 2 °C that were accompanied by increased respiration rate, weight loss, and electrolyte leakage (EL). The CI symptoms were significantly reduced by oxalic acid treatment, especially for the 6 mM concentration. In addition, control pomegranates showed significant reduction in the content of total phenolics and ascorbic acid as well as in total antioxidant activity (TAA), in both hydrophilic (H-TAA) and lipophilic (L-TAA) fractions. The application of oxalic acid led to lower losses of total phenolics and significant increase in both ascorbic acid content and H-TAA, whereas L-TAA remained unaffected. Thus, oxalic acid could be a promising postharvest treatment to alleviate CI and increase antioxidant potential.

KEYWORDS: Fruit quality; organic acids; phenolic compounds; antioxidant activity

INTRODUCTION

Pomegranates have been used broadly in the folk medicine of many cultures (*1*), but recently other properties have been claimed, such as antioxidant, anticancer, and antiatherosclerosis activities, among others (*2*,*3*). Pomegranate (*Punica granatum* L.) is considered to be one of the oldest known edible fruits and probably originated in northern Turkey (*1*). The arils are the edible part of the fruit, which contain around 80% juice and 20% seed. The juice contains high concentrations of sugars, organic acids, vitamins, polysaccharides, and essential minerals (*4*).

During postharvest storage of pomegranate important quality loss occurs due to several physiological and enzymatic disorders, the major storage problem being desiccation leading to browning symptoms in both peel and arils. Refrigeration is effective to prolong the storability of pomegranate, but the fruits are susceptible to chilling injury (CI) when exposed to temperatures below 5 °C, and then increased skin browning, surface pitting, and higher susceptibility to decay occur. In most cases these symptoms reach the arils, which depreciate both internal and external fruit quality (*5*). Losses of firmness, aril color, vitamin C, and acidity were reported, which were accompanied by reduction of acceptability in terms of freshness, juiciness, and taste (*6*,*7*). To reduce CI incidence and to extend storability and marketing of pomegranates, good results were obtained with polyamines (*8*) or heat treatments (*9*) prior to cold storage. Other treatments such as salicylic acid were highly effective in reducing CI and electrolyte leakage in the husk of pomegranate, as well as ascorbic acid loss (*10*).

Oxalic acid is a natural organic anion that is found ubiquitously in plant species, including some important crops, and plays different roles in the living organisms. Accordingly, oxalic acid seems to be involved in controlling fruit tissue browning (*11*), inducing systemic resistance (*12*), retarding fruit ripening, and controlling decay (*13*). In addition, the endogenous concentration of oxalic acid in many plant foods has been considered as a natural antioxidant by suppressing in vitro lipid peroxidation (*14*).

However, to the best of our knowledge no information is available about the use of oxalic acid treatment in reducing CI in pomegranate, neither on the organoleptic nor nutritive or bioactive compounds of the arils. Consequently, the aim of this work was to study the effect of prestorage oxalic acid treatment at three concentrations (2, 4, and 6 mM) on pomegranate fruit quality and on the contents of ascorbic acid, total phenolic compounds, total anthocyanin profile, and total antioxidant activity (measured in both hydrophilic and lipophilic fractions) after prolonged postharvest storage.

MATERIALS AND METHODS

Plant Material and Experimental Design. Pomegranates (*P. granatum* L. cv. Mollar de Elche) were picked on October 2008 in a commercial orchard in Elche (Alicante). This is a late-ripening cultivar with delicious sweet arils and soft stones. Fruits were harvested when fully mature according to commercial practice and immediately transported to the

*Corresponding author (telephone 34-96-6749616; fax 34-96-6749678; e-mail m.serrano@umh.es).

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laboratory. Pomegranates with defects (sunburn, crack, bruises and cuts in the husk) were discarded, and 75 fruits homogeneous in size and color were selected; they were sorted at random in lots of 5 fruits. Three lots were used to analyze fruit properties at harvest (day 0), and the remaining lots were randomized and divided into four groups for the following treatments in triplicate (each replicate contained five individual fruits): control (distilled water at 25 °C) and oxalic acid treatment at three concentrations (2, 4, and 6 mM). Oxalic acid was purchased from Sigma-Aldrich (Madrid, Spain; 97% purity). Fruits were dipped in 20 L of solution for 10 min. Following treatments, fruits were placed on kraft paper and allowed to dry for 20 h at 20 °C. Then, they were transferred to a temperature-controlled chamber at 2 °C, in permanent darkness and with relative humidity of 90%. After 84 days, the fruits were sampled for nondestructive quality assessment. This period was set according to previous papers in which severe CI symptoms occurred after 2−3 months of cold storage (*8*, *9*). Then, each husk was carefully cut at the equatorial zone with a sharpened knive, and then arils were manually extracted. The arils of each replicate (composed by five fruits) were combined and frozen in liquid N_2, milled to obtain homogeneous subsamples, and stored at −20 °C until analytical determinations. The skin was used for electrolyte leakage analysis.

Assessment of Fruit Quality. Total soluble solids (TSS) was determined in duplicate from the juice obtained for each subsample with an Atago PR-101 (Atago Co. Ltd., Tokyo, Japan) digital refractometer at 20 °C and expressed as percent (°Brix). Total acidity (TA) was determined in duplicate by automatic titration (785 DMP Titrino, Metrohm) with 0.1 M NaOH up to pH 8.1, using 1 mL of diluted juice from each subsample in 25 mL of distilled H_2O, and results were expressed as grams of malic acid equivalent per 100 g of fresh weight. Results were expressed as mean ± SE.

Fruit firmness was individually measured on each fruit using a flat steel plate coupled on a texturometer (TX-XT2i Texture Analyzer, Stable Microsystems, Godalming, U.K.) interfaced to a personal computer. A beveled holder prevented bruising of the opposite side. For each fruit, the diameter was measured and then a force that achieved a 3% deformation of the fruit diameter was applied. Results were expressed as the force−deformation (N mm^{-1}) and were the mean ± SE. Weights of individual fruit were recorded on the day of harvesting and after the sampling date. Cumulative weight losses were expressed as percentage loss of original weight.

Respiration rate was measured by placing each fruit in a 2 L glass jar hermetically sealed with a rubber stopper for 1 h. One milliliter of the holder atmosphere was withdrawn with a gas syringe, and CO_2 was quantified using a Shimadzu 14A gas chromatograph (Kyoto, Japan), with a thermal conductivity detector and a molecular sieve 5A column, 80−100 mesh (Carbosieve SII, Supelco Inc., Bellefonte, PA) of 2 m length and 3 mm i.d. Oven and injector temperatures were 50 and 110 °C, respectively. Helium was used as carrier gas at a flow rate of 50 mL min^{-1}. Results were the mean ± SE and expressed as milligrams of CO_2 per kilogram per hour.

Determination of CI. CI was individually evaluated in each fruit with a 4-point hedonic scale based on the percentage of husk surface affected by CI symptoms (dehydration, browning, and pitting): 0 (no symptoms), 1 (1−25%), 2 (26−50%), and 3 (>51%). Results were expressed as the mean ± SE (n = 15) of CI, which was calculated using the following formula:

$$\text{CI} = \Sigma(\text{value of hedonic scale}) \times (\text{no. of fruit with corresponding scale number}) / (\text{total no. of fruit in sample})$$

The rate of electrolyte leakage (EL) was determined as described by Mirdehghan et al. (*9*) in duplicate for each replicate, using six disks (10 mm) of peel tissue (1.50 ± 0.02 g), which were cut with a cork borer. Conductivity was measured after 4 h of incubation in 25 mL of 0.4 M mannitol under constant shaking, using a Crison conductivity meter (Metrohm 664). After readings were taken, the vials were autoclaved at 121 °C for 20 min and held for 24 h, and then conductivity was measured again for total electrolytes. The rate of EL was expressed as a percentage of total, (initial/total) × 100, and results were the mean ± SE (n = 6).

Total Antioxidant Activity (TAA), Total Phenolics, Total Anthocyanin, and Ascorbic Acid Determination. TAA was quantified according to the method of Arnao et al. (*15*), which enables one to determine TAA due to both hydrophilic and lipophilic compounds in the same extraction. Briefly, for each subsample, 5 g of tissue was homogenized in 5 mL of 50 mM phosphate buffer, pH 7.8, and 3 mL of ethyl acetate and then centrifuged at 10000*g* for 15 min at 4 °C. The upper fraction was used for TAA due to lipophilic compounds (L-TAA) and the lower for TAA due to hydrophilic compounds (H-TAA). In both cases, TAA was determined using the enzymatic system composed of the chromophore 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), the horseradish peroxidase enzyme (HRP), and its oxidant substrate (hydrogen peroxide), in which $ABTS^{\bullet+}$ radicals are generated and monitored at 730 nm. The decrease in absorbance after adding the extract was proportional to TAA of the sample. A calibration curve was performed with Trolox ((*R*)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (0−20 nmol) from Sigma (Madrid, Spain), and results are expressed as milligrams of Trolox equivalent per 100 g.

Total phenolics were extracted according to the Tomás-Barberán et al. protocol (*16*) using water/methanol (2:8) containing 2 mM NaF and quantified using the Folin−Ciocalteu reagent (*17*), and results (mean ± SE) were expressed as milligrams of pyrogallol equivalent per 100 g of fresh weight. Total anthocyanins were determined according to the method of García-Viguera et al. (*18*) adapted as previously reported (*19*) and calculated as cyanidin 3-glucoside equivalent (molar absorption coefficient of 23900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}), and results were expressed as milligrams per 100 g of fresh weight and were the mean ± SE.

For ascorbic acid, 5 g of arils were homogenized in 10 mL of 50 mM phosphate buffer, pH 7.8, and then centrifuged at 10000*g* for 15 min at 4 °C. One milliliter of the extract was filtered through a 0.45 μm Millipore filter and then injected into a Hewlett-Packard HPLC series 1100. The elution system consisted of 0.1% phosphoric acid running isocratically with a flow rate of 0.5 mL min^{-1}. Ascorbic acid was eluted through a Supelco column (Supelcogel C-610H, 30 cm × 7.8 mm, Supelco Park, Bellefonte, PA) and detected by absorbance at 210 nm (*19*). A standard curve of pure L-ascorbic acid (Sigma, Madrid, Spain) was used for quantification. Results were expressed as milligrams of ascorbic acid per 100 g.

Anthocyanins. Anthocyanins were assayed by high-performance liquid chromatography coupled with diode array detector (HPLC-DAD) as previously described (*18*). One milliliter from the extracts obtained for total anthocyanin quantification was filtered through a 0.45 μm Millipore filter and then injected into a Hewlett-Packard HPLC series 1100 equipped with a C18 column (Mediterranea Sea18, 25 cm × 0.4 mm i.d., particle size = 5 μm, TR010013, Teknokroma, Barcelona, Spain) and detected by absorbance at 510 nm. The peaks were eluted by a gradient using the following mobile phases: 95% water + 5% methanol (A); 88% water + 12% MeOH (B); 20% water + 80% MeOH (C); and MeOH (D), at a rate of 1 mL min^{-1}. Elution started with 100% A, which remained isocratic until 5 min. A gradient was then used to reach 100% B at 10 min, held isocratic for am additional 3 min. From 13 to 35 min a linear gradient was used to reach 75% B and 25% C, then 50% B and 50% C at 50 min, and then 100% C at 52 min, which was maintained isocratic until 57 min. The column was then washed with 100% D at 60 min. Peaks were identified using authentic standards by comparison of the retention times and peak spectral analysis. The anthocyanin standards were provided by Dr. García-Viguera.

Statistical Analysis. Data for the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were storage and treatment. Mean comparisons were performed using HSD, the Tukey test to examine if differences were significant at $P < 0.05$. All analyses were performed with SPSS software package v. 12.0 for Windows (*20*). A summary of the statistical results is shown in **Table 1**.

RESULTS AND DISCUSSION

CI and Quality Parameters. Control pomegranates exhibited CI symptoms manifested as browning, pitting, and dehydration of the husk surface, reaching a CI index value of 1.64 ± 0.24; that is, CI symptoms affected from 25 to 50% of the fruit surface (**Table 2**). These CI symptoms were similar to those observed in other pomegranate cultivars, such as 'Wonderful' (*21*) or 'Malas Saveh' (*10*), although significant differences were found between

cultivars. Thus, 'Wonderful' held at 5 °C for 8 weeks showed only slight CI symptoms manifested as brown discoloration of the locular septa (*22*). Moreover, differences exist also between harvest dates, with late-season fruit being more sensitive to CI than mid-season, indicating that this disorder may be associated with senescence (*21*). In addition, increases in respiration rate, weight loss, and EL were observed after 84 days of cold storage (**Table 2**). However, these changes were significantly ($P < 0.05$) reduced in oxalic acid treated fruits, the efficacy being higher for the 6 mM oxalic acid applied dose, especially by reducing respiration rate, CI, and EL. It is known that CI is characterized by membrane disruption resulting in cellular decompartmentation and loss of tissue integrity accompanied by skin browning, which was reduced by the oxalic acid treatments. This effect could be attributed to the inhibition of polyphenol oxidase and peroxidase activities, as has been reported in litchi fruits (*23*). In addition, oxalic acid could act as an antisenescence agent, because mangoes treated with oxalic acid at 5 nmol L^{-1} enhanced their tolerance to low-temperature stress, the effect being attributed to the ability of oxalic acid to inhibit O_2^- accumulation, delay H_2O_2 decrease, and induce higher reducing status of ascorbate−gluthatione (*24*). The 5 nmol L^{-1} oxalic acid showed also a reduction in respiration rate of jujube fruits (*25*), which was related to a delay of the senescence process. With respect to quality parameters, TSS was not affected by either oxalic treatments or storage, because no significant changes were observed from day 0 to the end of storage independently of the applied treatment. However, TA decreased in arils from control fruits during storage, although this decrease was lower with the three oxalic acid concentrations used, because TA was significantly higher in arils from treated fruits than in those from control ones, at the end of storage time. The diminution in fruit firmness was similar in both control and treated pomegranates after 84 days of storage (**Table 2**). However, in mango and peach, oxalic acid at 5 mM showed a reduced softening rate and TA retention (*13*, *26*), but fruits were stored at room temperature (no CI conditions).

Table 1. ANOVA for Dependent Variables for Oxalic Acid Treatment Applied, Storage Time, and Their Interactions for Pomegranate Cv. Mollar[a]

	treatment	time	time × treatment
weight loss	**	**	**
total soluble solids (TSS)	ns	**	ns
total acidity (TA)	**	**	**
fruit texture	ns	**	ns
chilling injury index (CI)	*	**	*
electrolyte leakage (EL)	*	**	*
respiration rate	*	**	ns
total polyphenols	*	**	*
hydrophilic antioxidant activity (H-TAA)	**	**	**
lipophilic antioxidant activity (L-TAA)	*	ns	*
total anthocyanins	**	**	**

[a] ** and * represent significance at the 0.01 and 0.05 levels, respectively, and ns represents nonsignificance at $P < 0.05$.

Bioactive Compounds and TAA. Along prolonged storage, control pomegranates showed significant reduction in the content of total phenolics and ascorbic acid, as well as in TAA, in both hydrophilic (H-TAA) and lipophilic (L-TAA) fractions (**Table 3**). The application of oxalic acid led to lower losses of total phenolics and to a significant increase in ascorbic acid after 84 days of cold storage. H-TAA was also enhanced after oxalic acid treatment, whereas L-TAA remained unaffected from the initial values. With respect to total anthocyanins, a significant increase was observed during storage, although final concentrations were affected by treatment, because those fruits treated with oxalic acid showed significantly higher total anthocyanins at the end of the experiment, especially for the 6 mM dose (**Table 3**). The increase in anthocyanin concentration in control fruits is in agreement with previous results (*27*, *28*) and was associated with the advancement of the ripening process during postharvest storage. Although no information is available on the role of oxalic acid in anthocyanin biosynthesis, exogenous oxalic acid could act as elicitor of anthocyanin synthesis.

The HPLC-DAD analysis of individual anthocyanins revealed that in pomegranate arils five different anthocyanins were identified: cyanidin-3-glucoside (Cy-3-Gluc), delphinidin-3-glucoside

Table 2. Respiration Rate and Quality Parameters (Weight Loss, CI, TSS, TA, Firmness, and EL) of Pomegranate Fruits at Harvest (Day 0) and after 84 Days of Storage in Control and Oxalic Acid Treated Fruits[a]

		day 84			
parameter	day 0	control	2 mM oxalic	4 mM oxalic	6 mM oxalic
respiration rate (mg kg^{-1} h^{-1})	23.80 ± 0.63 a	32.20 ± 0.98 b	28.48 ± 1.54 c	24.59 ± 1.19 a	23.01 ± 0.78 a
weight loss (%)		19.64 ± 0.65 a	17.72 ± 0.88 b	18.04 ± 0.91 ab	17.68 ± 0.59 b
CI		1.64 ± 0.24 a	0.21 ± 0.11 b	0.27 ± 0.12 b	0.22 ± 0.11 b
TSS (%)	16.08 ± 0.15 a	17.17 ± 0.05 a	17.28 ± 0.06 a	17.84 ± 0.04 a	17.57 ± 0.09 a
TA (g 100 g^{-1})	0.30 ± 0.006 a	0.24 ± 0.006 b	0.26 ± 0.004 c	0.26 ± 0.004 c	0.28 ± 0.07 d
firmness (N mm^{-1})	20.36 ± 1.22 a	16.08 ± 1.65 b	13.73 ± 1.44 b	13.30 ± 0.69 b	12.45 ± 1.03 b
EL (%)	22.34 ± 0.78 a	68.18 ± 2.08 b	61.21 ± 0.78 c	60.08 ± 4.13 c	58.04 ± 2.01 c

[a] For each parameter means followed by a different letter are significantly different at $P < 0.05$ from day 0 to day 84 and among treatments.

Table 3. Bioactive Compounds (Total Phenolics, Total Anthocyanins, and Ascorbic Acid) and Antioxidant Activity (Hydrophilic H-TAA and Lipophilic L-TAA) in Pomegranate Arils at Harvest (Day o) and after 84 Days of Storage in Control and Oxalic Acid Treated Fruits[a]

		day 84			
parameter	day 0	control	2 mM oxalic	4 mM oxalic	6 mM oxalic
total phenolics (mg 100 g^{-1})	252.47 ± 17.09 a	167.69 ± 2.49 b	192.88 ± 5.95 c	212.07 ± 3.55 d	182.13 ± 6.96 c
total anthocyanins (mg 100 g^{-1})	59.03 ± 7.97 a	109.67 ± 1.15 b	107.19 ± 1.39 b	122.56 ± 2.94 c	135.31 ± 3.09 d
ascorbic acid (mg 100 g^{-1})	36.66 ± 0.05 a	33.06 ± 0.08 b	39.96 ± 0.04 c	40.90 ± 0.14 c	44.34 ± 0.06 d
H-TAA (mg 100 g^{-1})	81.96 ± 3.84 a	73.32 ± 4.45 b	122.25 ± 7.38 c	116.54 ± 4.64 c	112.96 ± 4.22 c
L-TAA (mg 100 g^{-1})	15.98 ± 0.38 a	12.65 ± 0.87 b	16.68 ± 0.71 a	17.61 ± 0.42 a	16.99 ± 0.36 a

[a] For each parameter means followed by different letter are significantly different at $P < 0.05$ from day 0 to day 84 and among treatments.

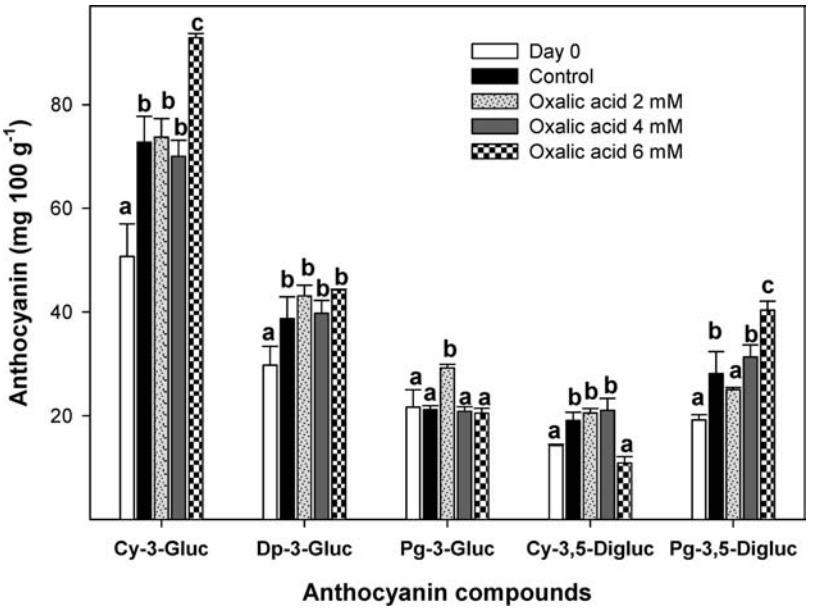

Figure 1. Levels of individual anthocyanins at harvest (day 0) and after 84 days of cold storage in control and oxalic acid treated pomegranate arils. Data are the mean ± SE. For each anthocyanin compound bars with different letters are significantly different at $P < 0.05$ from day 0 to day 84 and among treatments.

(Dp-3-Gluc), pelargonidin-3-glucoside (Pg-3-Gluc), cyanidin-3,5-diglucoside (Cy-3,5-Digluc), and pelargonidin-3,5-diglucoside (Pg-3,5-Digluc), the predominant one being Cy-3-Gluc (**Figure 1**), for which a significant increase ($P < 0.05$) was obtained in 6 mM oxalic treated fruits. In addition, the anthocyanin profile obtained for 'Mollar de Elche' cultivar was similar to that obtained for other sweet pomegranate cultivars, such as 'Assaria' (*29*) and 'Primosole' (*30*). Contrarily, in the sour cultivars, total anthocyanins decreased during cold storage and the predominant anthocyanin was Cy-3,5-Digluc, followed by Dp-3,5-Digluc and Cy-3-Gluc (*31*).

During postharvest storage of fruits and vegetables, loss of health-beneficial compounds has been reported, such as in table grape (*32*) and broccoli (*33*). In pomegranate, loss of ascorbic acid (vitamin C) also occurred either during cold storage or at ambient temperatures (*7*). Several methods have been used to maintain or increase the functional properties of pomegranates during postharvest storage, such as heat treatments (*28*), polyamine applications (*34*), and film wrapping (*7*). All of these treatments have shown also effectiveness in extending shelf life by reducing rates of fruit deterioration and CI.

In this work, the application of oxalic acid treatments to pomegranate fruits induced lower losses of total phenolic compounds and higher increases in ascorbic acid, total anthocyanins, and TAA, especially in H-TAA compared with control arils. As far as we know, this is the first study in which the application of oxalic acid induced beneficial effects in terms of maintaining or increasing the pomegranate potential antioxidant activity during postharvest storage. The mechanism by which oxalic acid increased the bioactive compounds and antioxidant properties is not well-known, although oxalic acid has been reported as a natural antioxidant by suppressing lipid peroxidation in vitro in a concentration-dependent manner and reducing the ascorbic acid oxidation (*14*). In this sense, in mango fruit oxalic acid treatment led to higher reduction states of ascorbate and glutathione, because treated fruits showed higher activities of superoxide dismutase, catalase, guaiacol peroxidase, ascorbate peroxidase, and glutathione reductase (*24*). In summary, the application of oxalic acid treatments to pomegranate could be considered as a natural postharvest tool with good results in terms of reducing CI damage and increasing the antioxidant potential by enhancing or maintaining the bioactive compounds. In the future, the CI-alleviating effect of oxalic acid should be assayed in other CI-sensitive fruits as well as its effect on quality and functional properties.

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