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FULL ARTICLE

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Impact of chitosan in combination with potassium sorbate treatment on chilling injury and quality attributes of pomegranate fruit during cold storage

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Abstract

The impact of chitosan (CH) and potassium sorbate (PS) on quality attributes of pomegranate fruits (cv. Malas e Saveh) was evaluated during 90 days of storage at 4°C and 85%–90% relative humidity. The CH as well as the combined treatments (CH + PS) were effective in decreasing the chilling injury (CI) symptoms, electrolyte leakage, and malondialdehyde contents of fruit peel. Furthermore, CH, PS, and CH + PS treatments enhanced the activity of DPPH radical scavenging, and antioxidant enzymes of arils, and preserved the ascorbic acid content at high levels during the storage period. Fruits treated with CH1% + PS10% and CH2% + PS10% exhibited the lowest decay incidence and weight loss. Higher content of arils' phenols, flavonoids, and anthocyanin was observed in treated-fruits as a result of more activity of phenylalanine ammonia-lyase (PAL) and low activity of polyphenol oxidase (PPO). Consequently, CH + PS showed positive effects on the storage life of pomegranate fruits in terms of CI, decay incidence, and also nutritional values.

Practical applications

Enhancement of chilling tolerance of pomegranate fruits during cold storage condition is a crucial issue. Application of CH coating alone or in combination with potassium sorbate could decrease the CI symptoms and preserved peel cell membrane integrity by maintaining electrolyte leakage (EL) and malondialdehyde in lower levels. Also, these treatments prevent weight loss and decay incidence in peel, and increase the activity of phenylpropanoid pathway and antioxidant systems in arils. All in all, usage of CH edible coating (2%) plus PS (10%) illustrated high efficiency in alleviating CI, decay incidence, and preserving nutritional quality of pomegranate fruits.

KEYWORDS

antioxidant enzymes, chilling injury, decay incidence, pomegranate, postharvest, total phenols

1 | INTRODUCTION

High content of dietary and health beneficial compounds such as sugars, ascorbic acid, amino acids, anthocyanin, minerals, pectin, fibers, and above all polyphenolic flavonoids along with antioxidant activity is the most crucial reason of increasing the pomegranate (*Punica granatum* L.) production and consumption around the world (Palou et al., 2007; Pareek et al., 2015). Pomegranate is a non-climacteric fruit that does not ripen after picking up even with ethylene treatment (Elyatem & Kader, 1984). However, physiological and biochemical alterations are continued after harvesting and decreases the quality of harvested fruit. Due to this, the harvested

fruit must be stored in proper conditions, that is, cold storage with appropriate temperature and humidity, though some limitations are brought about by CI, decay, and weight loss during storage time (Palou et al., 2007). The CI occurs in pomegranate when they are stored at the temperatures lower than 5°C for longer than a couple of months (Kader, 2006; Opara et al., 2015; Pareek et al., 2015). Superficial pitting, husk scald, decay, and skin browning are the most critical symptoms of CI. But, in some cases, depending on temperature and storage duration, these symptoms are more intense and affect the internal parts of the fruit, that is, pale color of arils and white segments browning as well (Ehteshami et al., 2019; Pareek et al., 2015). Postharvest decay of pomegranate caused by *Botrytis cinerea*, which infects the fruit via injuries or micro-wounds is located in different parts of the skin. However, the decay of latent blossom infection is more notable. Crown decay is the consequence of young fruit infection that develops from the crown to the whole mature harvested fruit.

Due to mentioned economic value and health benefits of pomegranate fruits, various researchers around the world have tried to extend the storage life of this fruit along with preventing CI and decay with various postharvest treatments such as application of polyamines (Mirdehghan et al., 2007), food additives and antifungal chemicals (Palou et al., 2007), salicyloyl chitosan (Sayyari et al., 2016), argenin (Babalar et al., 2017), CH, and carboxymethyl cellulose in combination with oxalic and malic acids (Ehteshami et al., 2019), etc. Among the mentioned approaches, using CH in combination with potassium sorbate (PS) (E-202) could be a horizon sight into postharvest issues of this fruit, especially fungal decay and CI injury, during the cold storage period. The CH is a natural biopolymer and cationic polysaccharide with high molecular weight which is generated from the acetylation of chitin, and used individually or in combination with other alternative systems in preharvest or postharvest stage of fruits or vegetables (Candir et al., 2018; Ehteshami et al., 2019). The CH-based coating has been proved to control the fungal decay, alleviate weight loss, and preserve postharvest quality of fruits (Munhuweyi et al., 2017). The PS, moreover, is one of the low-toxicity food additives that is widely used as a food preservative. Since 1978, PS has been used alone or incombination with synthetic fungicides, heat, or different waxes, to control fruit decay during storage time (Parra et al., 2014).

Zhang et al. (2015) reported that exogenous CH plus salicylic acid coating of cucumber fruits during cold storage inhibited CI and their quality maintained better than the application of the salicylic acid alone. These treatments also limited weight loss, MDA, and EL increasing, and maintained higher content of phenolic compounds and antioxidant enzyme activities. It has been reported that postharvest application of CH–ascorbic acid combined treatment on plum fruits, significantly reduced weight loss, membrane integrity, MDA content, and polyphenol oxidase enzyme activity, while increased superoxide dismutase (SOD) and catalase (CAT) enzymes activities within cold storage (Liu et al., 2014). In citrus fruits the application of PS in water was more useful to decay control than when it was used in wax, though a considerable increase was observed in weight loss (Parra et al., 2014).

To the best of our knowledge, there is no scientific report dealing with usage of CH coating in combination with PS on postharvest issues of pomegranate fruits. Hence, CH, PS, and their combination treatments were used in the current work in order to evaluate their coating effects on the quality attributes of pomegranate fruits during cold storage period.

2 | MATERIALS AND METHODS

2.1 | Plant material and treatments

Pomegranate fruits (cv. Malas e Saveh) were collected at commercial maturity from 15-year-old shrubs of a commercial orchard located at Tarom county (Zanjan province, Iran), and immediately transported to postharvest physiology laboratory in University of Zanjan. Afterward, fruits were separated based on uniform size and maturity, and those with substantial defects such as sunburn, cracks, bruises, and cut in husk were discarded. The selected fruits were randomly divided into 27 groups; each contained 45 fruits at three replications. At each sampling date (30, 60, and 90 days of storage period) five fruits of every replicate were used for analyzing various parameters. CH (medium molecular weight, degree of deacetylation ≥ 70%) and PS were provided from Sigma-Aldrich (USA) and Merck Company (Germany), respectively. The treatments included CH (0%, 1%, and 2%), PS (0%, 5%, and 10%), and their combination, and fruits treated with distilled water were considered as control. Fruits were immersed into different mentioned solutions for 2 min, then set aside to dry at room temperature for 2 hr. Finally, fruits were kept in cold storage for 90 days at 4°C with 85%–90% relative humidity. Sampling for laboratory analysis was conducted at time intervals of 30 days. After moving into the lab, before measuring different physiological and biochemical criteria, fruits were kept at 20°C for 3 days as shelf life.

2.2 | CI, EL, and MDA content

Fruit CI was evaluated by visualizing CI symptoms (browning, scald, and surface pitting) of nine fruits based on 1–3 scale: 0 (no symptom), 1 (1%–25%), 2 (26%–50%), and 3 (≥51%) (Sayyari et al., 2009). The CI index was calculated by the following formula:

$$\mathrm{CI} = \sum \left(\frac{(\text{CI level}) \times (\text{fruit No. at the CI level})}{\text{total fruit No. in the sample} \times 3} \right) \times 100.$$

The EL was determined by the method described by Mirdehghan et al. (2007). For each sample, six disks (10 mm) of the husk tissue (1.5 ± 0.02 g) were randomly cut and incubated in 25 ml of 0.4 M mannitol and the initial EL was measured in incubation following shakes for 4 hr. Then, the mixtures were autoclaved in 121°C for

20 min. After staying for 24 hr at room temperature, the final EL was measured and used for calculating EL (%) according to the following formula:

$$\text{EL}(\%) = \frac{(\text{initial EL} - \text{final EL})}{\text{initial EL}} \times 100.$$

Thiobarbituric acid (TBA) method demonstrated by Hodges et al. (2004), was used for MDA content determination. At first, 25 ml of 5% (v/v) trichloroacetic acid (TCA) was mixed with one gram of fruit husk and centrifuged for 10 min at 10,000*g*. Then, 1.5 ml of the supernatant was separated and 2.5 ml of 0.5% TBA in 15% TCA was added to it. After which, the mentioned mixture was held for 30 min in boiling water and then, cooled swiftly. Have been centrifuged at 12,000*g* for 10 min, the absorbance was measured at 532 nm. Finally, MDA content was represented as μm/kg of fresh weight (FW).

2.3 | Decay incidence and weight loss

Fruit decay was visually evaluated and any fruit with visible mold growth was considered as decay fruit. The percentage of fruit with decay symptoms was expressed as decay incidence (Selcuk & Erkan, 2014). Fruit weight was measured at the end of each storage period and its loss was computed as the percentage of initial weight by the following formula (Nanda et al., 2001):

$$\text{Weight loss}(\%) = \frac{(\text{initial FW} - \text{FW after storage})}{\text{initial FW}} \times 100.$$

2.4 | Antioxidant system activity

The free radical DPPH scavenging method was used for total antioxidant activity determination (Dehghan & Khoshkam, 2012). A volume of 1.9 ml of DPPH solution (0.1 mM in methanol) was mixed with 0.1 ml of samples and was left for 30 min in a dark place and then, the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated with the formula below and was expressed as the percentage of inhibition of DPPH radical.

$$\text{Inhibition of DPPH} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100.$$

Ascorbic acid content was determined by 2.6-dichloroindophenol dye (Terada et al., 1978). For this, 2 g of the flesh tissue of fresh fruit was homogenized with 6 ml of metaphosphoric acid 1% (v/v) and centrifuged at 6,000*g* for 15 min at 4°C. Then, 300 μl of supernatant was mixed with 2 ml of indicator solution (50 mg of 2.6-dichlorophenolindophenol was solved in 200 ml distilled water and 42 mg of sodium bicarbonate was added). Finally, the absorbance was measured at 520 nm and expressed as mg 100 g^{-1} FW.

To analyze the activity of antioxidant enzymes, including SOD, CAT, and ascorbate peroxidase (APX), frozen flesh tissue (5 g) was homogenized in 50 mM of phosphate buffer (pH 7.8) that contained 0.2 mM of EDTA and 2% of polyvinylpolypyrrolidone (PVPP). This homogenate mixture was centrifuged at 12,000*g* for 20 min at 4°C and the supernatant was used as enzyme extract (Zhang et al., 2013). To measure CAT activity, 100 μl of enzyme extract was added to 2.9 ml of the reaction solution (15 mM of H_2O_2 and 50 mM of phosphate buffer, pH 7). One unit of CAT activity was defined as a reduction in absorbance at 240 nm. The APX activity was measured by adding 100 μl of enzyme extract into 2.9 ml of the reaction solution (0.5 mM of ascorbic acid, 1 mM of H_2O_2, and 50 mM of phosphate buffer pH 7). One unit of APX activity was described as an amount of enzyme that oxidizes 1 μmol of ascorbate, and was measured by the decline in absorbance at 290 nm during 1 min. The SOD activity assessment was done by putting 60 μl of enzyme extract together with 2.9 ml of the reaction solution (50 mM of phosphate buffer (pH 7), 5 mM of methionine, 100 μM of EDTA, 65 μM of nitro blue tetrazolium (NBT), and 40 μl of riboflavin (0.15 mM). This mixture was put in fluorescent light (40W) incubator for 10 min and the formation of blue formazan was controlled by recording the absorbance at 560 nm. One unit of SOD activity, expressed as U g^{-1} FW, was defined as the enzyme that caused 50% inhibition of NBT decrement under assay condition.

2.5 | Phenols metabolism

Folin–Ciocalteu reagent was used for total phenols analyzing (Singleton & Rossi, 1965) and methanol/water (70:30 v/v) was used as the extraction solvent. 0.1 ml of 50% (v/v) Folin–Ciocalteu reagent was mixed with 0.1 ml of extract and let to react for 2 min at room temperature; after that, 2 ml of 2% Na_2CO_3 solution was added and put away for 30 min. Finally, the absorbance of the mixture was recorded at 720 nm and total phenols were expressed as mg gallic acid equivalent (GAE) 100 g^{-1} FW.

For total flavonoids content determination, spectrophotometric method of Zhishen et al. (1999) was used. In this method, 75 μl of aqueous $NaNO_2$ (5%) was added to 0.25 ml of sample and left for 5 min at room temperature. The solution was mixed with 0.15 ml of $AlCl_3$ (10%) and vortexed. After 6 min, 0.5 ml of 1 mol/L NaOH, and then distilled water was added to reach 2.5 ml as final volume and the absorbance was measured at 510 nm. Total flavonoids were expressed as mg quercetin equivalent (QE) per 100 g^{-1} FW.

Anthocyanin content was approved by the pH-differential method disseminated by Giusti and Wrolstad (2001). In this method, one gram of fruit flesh tissue was mixed with 10 ml of methanol solution, which contained HCl (1% v/v) and stayed for 10 min at 0°C. The mixture was centrifuged at 17,000 ***g*** for 15 min at 4°C, and then the supernatant was added separately to two buffers with different pHs (pH 1 buffer that contains hydrochloric acid–potassium chloride, 0.2 M, and pH 4.5 buffer that contains acetate acid–sodium acetate, 1 M). The absorbance was measured at 510 and 700 nm and the anthocyanin content (mg cyaniding 3-glucosides 100 g^{-1} FW) was calculated based on the following formula:

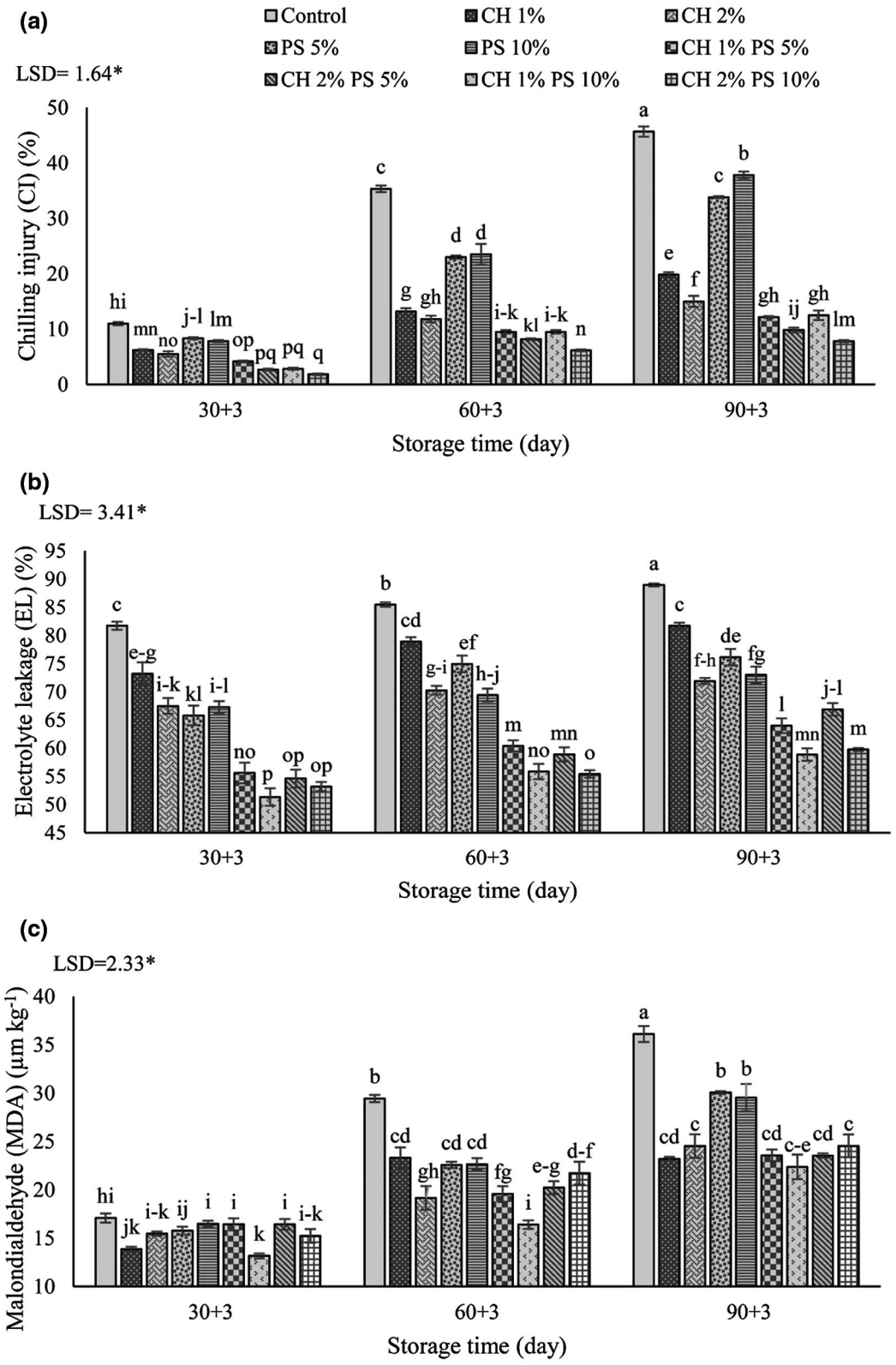

FIGURE 1 Chilling injury (CI) (a), Electrolyte leakage (EL) (b), and Malondialdehyde (MDA) (c) variation in pomegranates peel in response to postharvest application of Chitosan (CH), Potassium Sorbate (PS), and their combination treatments during storage at 4°C for 90 days plus 3 days at 20°C. *showing a significant level at $p \leq .05$. Values are the mean ± *SE*

$$\text{Absorbance}\,(A) = (A520 - A700)\,\text{pH1} - (A520 - A700)\,\text{pH4.5}$$

The activity of the PAL enzyme was determined according to Nguyen et al. (2003). The frozen flesh tissue (one gram) homogenized in 20 ml of 50 mM borate buffer (pH 8.5) containing 5 mM of 2-mercaptoethanol, 2 mM of EDTA, and 0.5 g PVPP. Then, the homogenized mixtures were centrifuged at 15,000*g* for 20 min at 4°C. The enzyme extract (0.3 ml) along with 0.7 ml of L-phenylalanine

(100 mM) and 3 ml of borate buffer (50 mM) was used as reaction mixture and incubated at 37°C in water-bath for 6 min. After that, 0.1 ml of HCl (2 mM) was added to stop the reaction. The enzyme activity, based on U g^{-1} FW, was determined by measuring the absorbance at 290 nm. To evaluate the activity of PPO one gramof flesh tissue homogenized with 10 ml of phosphate buffer 100 mM (pH 7.8) which contained 2% of PVP and centrifuged in 14,000*g* for 15 min at 4°C. Then, 100 μl of enzyme extract was added to reaction mixture (100 mM of catechol, 50 mM of phosphate buffer (pH 6)) and was used for the determination of PPO activity by measuring the absorbance at 410 nm during 1 min. One unit of PPO activity was expressed as U g^{-1} FW min^{-1} (Nguyen et al., 2003).

2.6 | Statistical analysis

This experimental design was a factorial based on completely randomized design. Data were analyzed using the ANOVA procedure using SAS software v.9.2. The mean values of treatments were compared using the least significant difference test (LSD) at $p \leq .05$.

3 | RESULTS AND DISCUSSION

3.1 | CI, EL, and MDA

The CI symptoms (browning, scald, and surface pitting) appeared in fruit during storage period and tended to increase by the extension of the storage duration. At the end of 90 days of cold storage plus 3 days at 20°C as shelf life, the lowest and highest levels of CI were observed in fruits of combined (CH2% + PS10%) and control treatments, respectively. Furthermore, PS-treated fruits showed high levels of CI compared to the combined treatments and CH alone (Figure 1a). According to Figure 1b, EL amount increased during cold storage in both treated and control fruits. However, administration of CH, PS, and CH + PS treatments, controlled the enhancement of the EL amounts. At the end of storage time, the highest (88.96%) and moderate (58.78%) amount of fruit's EL was acieved through control and combined (CH2% + PS5%) treatments, respectively. As shown in Figure 1c, accumulation of MDA increased by passing time. After passage of the storage period, the MDA accumulation hit a peak with the amount of 36.11 μm/kg in control fruits but, the least value (22.36 μm/kg) of this trait was recorded through the application of CH2% + PS5%. At this time, the MDA accumulation in fruits just treated with PS (5% and 10%) showed high MDA accumulation compared with the CH + PS and or CH alone treatments.

Based on the results, in comparison to PS, the CH treatment was more effective in decreasing CI symptoms, EL, and MDA content. However, the combined treatments of CH + PS had high influence in terms of declining CI in pomegranate fruits during cold storage. In this respect, CH2% + PS10% and CH2% + PS5% showed the lowest CI, which was almost five times lower than the control fruits. The manifestation of CI symptoms mostly has been associated with some problems such as cell membrane alterations, physiological disorders, and elevating of ROS levels (Hashim et al., 2017). According to these issues, CH may act as an exogenous agent to induce several defense mechanisms, such as antioxidant metabolisms and the accumulation of antifungal compounds (Bautista-Banos et al., 2006). Also, edible coating like CH creates a protective barrier against gasses movement, moisture, and other substances (Baldwin et al., 1999).

Refering to PS treatment, it should be noted that PS is a safe food preservative, has high efficiency in ++ (Liu et al., 2014), and applying this substance in combination with CH resulted in reduction of CI symptoms. This may be due to the inhibitory effect of PS on pectin degrading enzymes activity (one of the PS mode of actions). Under chilling stress, the activity of cell walls hydrolysis enzymes such as polygalacturonase and pectin methyl esterase lead to depolymerization of pectin in cell wall structure and cause softening of fruits tissues (Khan et al., 2018). Some evidence depict that PS can decrease the severity of disorders by limiting the activity of polygalacturonase enzymes, so preventing depolymerization and solubilization of pectin and preserving the firmness in higher levels in postharvest stage of different fruits and vegetables (Gregori et al., 2008) and, therefore, results in declining of CI.

It has been documented that chilling temperature as an oxidative stress, changes the cell membrane phase from fluid liquid-crystalline to rigid solid-gel, which its severity can be assayed by EL and MDA. Exposure to chilling stress leads to the accumulation of ROS as result of oxidative destruction of cellular structures such as the peroxidation of membrane lipids (Wang et al., 2016; Yabuta et al., 2002). Peroxidation of membrane lipids leads to decrease of cell membrane performance and fluidity as a first part of cell, which separates cell components from the environment (Wang et al., 2019; Zhang & Tian, 2010). Under long time low temperature conditions, the manifestation of CI symptoms such as husk browning, surface pitting, and scald results from the loss of the cell membrane integrity, which concurrent with destroying the structure of cell membrane and increasing the leakage of intracellular metabolites, ions, and water. So, EL can be an effective indicator of cell membrane integrity. MDA as the final product of the peroxidation of cell membrane lipids, is another marker of cell membrane integrity and the level of this substance can indicate the severity of damage on cell membrane (Aghdam & Bodbodak, 2013; Wongsheree et al., 2009). A lower accumulation of EL and MDA indicated less damage to the cell membrane integrity, which led to lower CI symptoms in CH + PS-treated fruits. In this regard, current results revealed that despite increasing the accumulation of EL and MDA by extending the storage time, the combined treatments (CH + PS) showed lower accumulation of EL and MDA and hence higher cell membrane integrity in comparison to control ones. CH as a mechanical barrier to oxygen prevented from more peroxidation of cell membrane lipids, so it maintained cell membrane integrity in greater levels. Our findings are in coordination with those of Ehteshami et al. (2019), who reported that EL and MDA accumulation in treated pomegranate fruits with CH + Malic and CH + Oxalic acid is lower than the control fruits. Also, Zhang et al. (2015) indicated that using combined treatments of CH + Salicylic acid, in

cucumber fruits during the cold storage, was more effective in declining of EL and MDA than either CH or salicylic acid alone. Besides, the results of Liu et al. (2014) on plum fruits illustrated that applying CH + Ascorbic acid had positive effects on MDA accumulation that lead to the lower level of CI over 20 days of storage.

3.2 | Weight loss and decay incidence

Weight loss was affected by CH and PS treatment over the cold storage (Figure 2a). After 90 days at 4°C and 3 days at 20°C as shelf life, the weight loss was reached at 13.7% in control fruits. Fruits treated with CH2% + PS10% showed the least weight loss (4.25%), and the next low levels were owned by CH2% + PS5% (4.77%) and CH2% (5.98%), respectively. PS-treated fruits showed high levels of weight loss during the cold storage period. According to Figure 2b, the application of both CH and PS treatments alone or in combination with each other declined decay incidence significantly during the cold storage. The incidence of decay in control fruits (38.66%) at the end of storage time, was much higher than which of combined treatment of CH1% + PS10 (11.6%) and CH1% + CH5% (12.33%). In terms of weight loss, the results indicated that the PS treatment alone has nearly no effect on the prevention of weight loss. Nonetheless, CH, either alone or in combination with PS, declined this criterion considerably.

Water loss and loss of carbon reserves due to transpiration and respiration, respectively, are two main reasons of weight loss in fruits, which are mainly related to the high porosity of peel that permits free movement of water vapor (Fawole & Opara, 2013; Sogvar et al., 2016). Thus, coating the fruit surface with CH, would apply a barrier to water vapor and prevent fruit weight loss. The effectiveness of CH application on controlling weight loss has been noticed previously in plum (Liu et al., 2014) and strawberry (Eshghi et al., 2014). As for PS, similar to our results, Parra et al. (2014) showed that the PS-treated citrus fruits showed statistically the same weight loss as control.

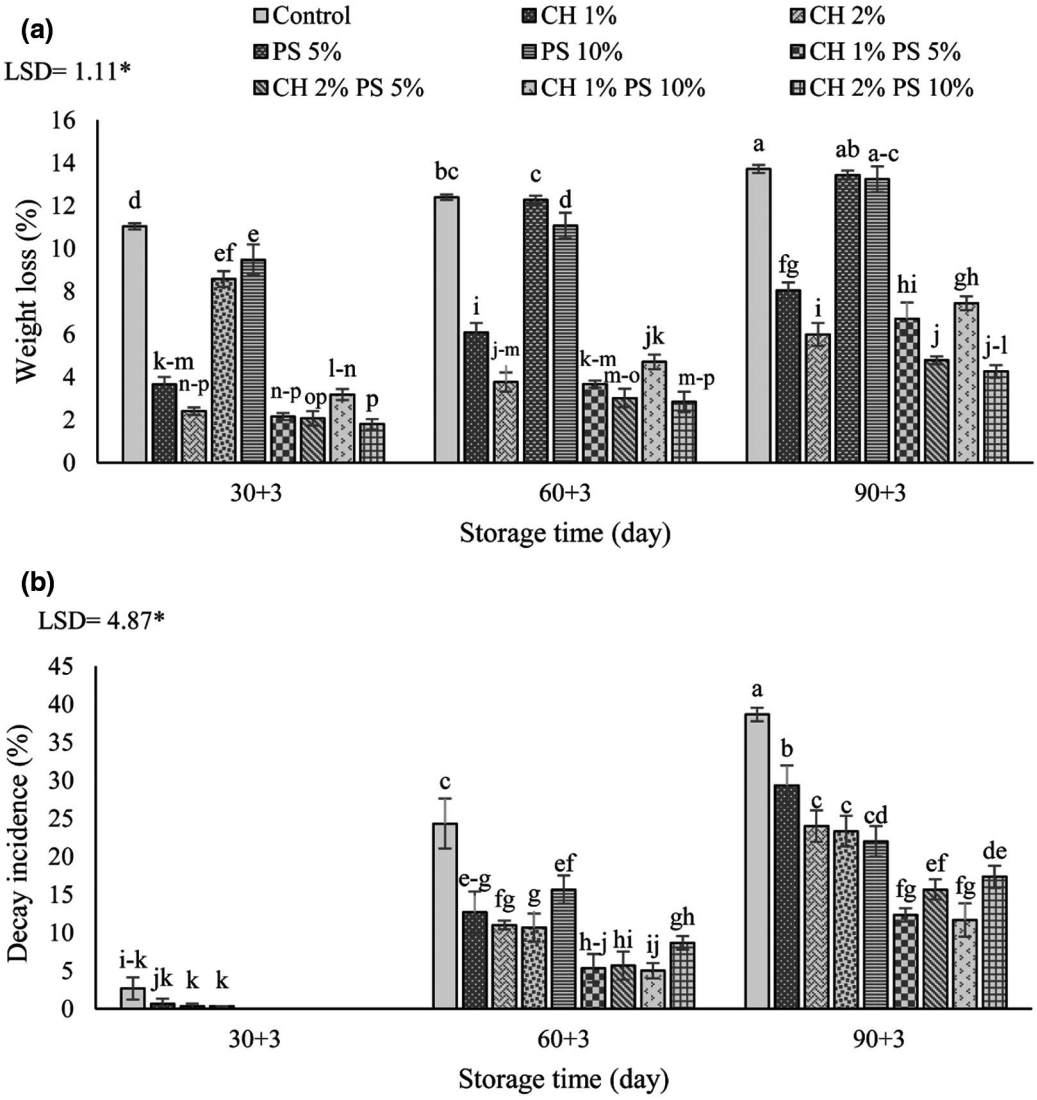

FIGURE 2 Pomegranate weight loss (a) and decay incidence (b) in response to postharvest application of Chitosan (CH), Potassium Sorbate (PS), and their combination treatments during storage at 4°C for 90 days plus 3 days at 20°C. *showing a significant level at $p \leq .05$. Values are the mean ± *SE*

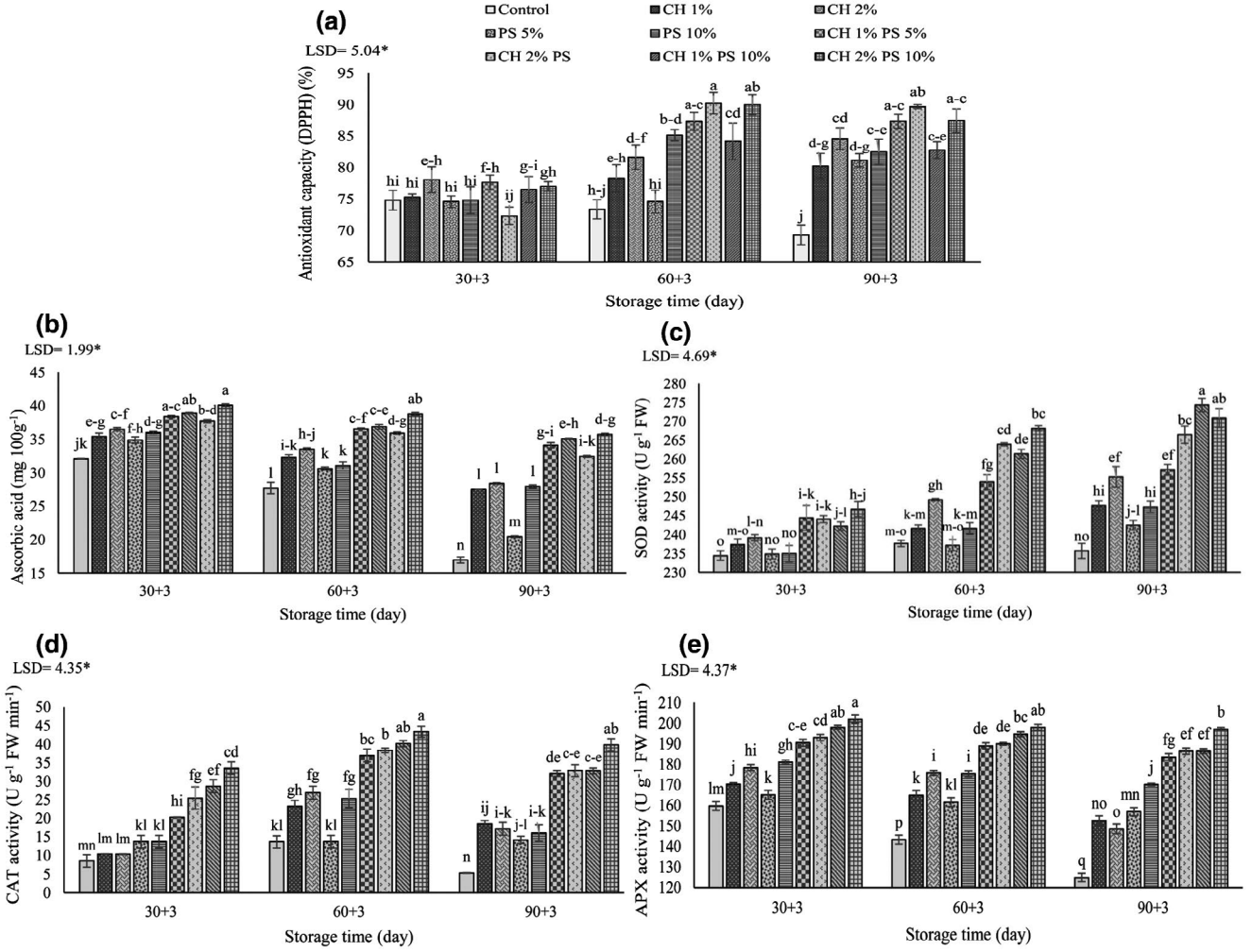

FIGURE 3 Pomegranate antioxidant capacity (DPPH scavenging capacity) (a), ascorbic acid (b), Superoxide dismutase (SOD) (c), Catalase (CAT) (d), and Ascorbate peroxidase (APX) (e) in response to postharvest application of Chitosan (CH), Potassium Sorbate (PS), and their combination treatments during storage at 4°C for 90 days plus 3 days at 20°C. *showing a significant level at $p \leq .05$. Values are the mean ± *SE*

Decay incidence was also prevented more efficiently by combined treatments. The lowest point of this index, for instance, was recorded with CH1% + (PS5% or PS10%) after 90 + 3 days. Superficial skin wound and crown decay, which caused more likely by *Penicillium Sp.* and *B. cinera*, in turn (Candir et al., 2018), were witnessed in stored fruits. The mechanism by which CH prevents pathogens main infection is partly related to the mechanical barrier on fruit surface that is created by this compound. Also, different studies previously have shown that CH could directly inhibit germ tube elongation, mycelial growth, and spore germination of many pathogens, which lead to drop decay incidence and prolong storage time of fruits (Xu et al., 2015). Referring to the PS in combination treatment, it is a food additive that has inhibitory action against fruit postharvest pathogens such as fungi, yeast, and aerobic bacteria. The antimicrobial activity of this organic acid salt is primarily due to the declining pH value of the intercellular part, after ionization of acid molecules. Also, inhibition of polygalacturonase activity is another mode of PS action, which was already mentioned (Gregori et al., 2008; Palou et al., 2007). Similar results have been mentioned previously, such as Ghasemnezhad et al. (2013) which exhibit that bacterial and fungal growth on pomegranate fruits surface inhibited by CH coating. Also Candir et al. (2018) reported that decay incidence in pomegranate fruits was treated with CH and modified atmosphere packaging (MPA) was lower than the control fruits. Refereeing to PS treatment, our results are in agreement with some previous studies. Parra et al. (2014), for instance, reported that fungal decay decreased in citrus fruits by the application of PS. In contrast with the positive influence of PS + CH in our results, Palou et al. (2007) showed that applying the PS in combination with other food additives like sodium carbonate did not provide significant benefits on pomegranate fruits in comparison to PS alone.

3.3 | Antioxidant system activity

DPPH scavenging activity increased in treated-fruits during the first 60 days of the cold storage and then, decreased slightly over the last 30 days of storage (Figure 3a). However, a downward trend was observed in control fruits during the whole storage duration. At the end

of the storage period, the highest (89.71%) and the lowest (69.3%) amount of DPPH scavenging activity were observed with CH2% + PS5% and control, respectively. During the cold storage, ascorbic acid content declined in both treated and control fruits. However, at the end of the storage time, treated-fruits showed higher amount of ascorbic acid compared with control ones, and the highest level of ascorbic acid content (35.74 mg 100 g^{-1} FW) belonged to CH2% + PS10% treated-fruits (Figure 3b).

The activity of SOD enhanced during storage time, and reached to its highest level at the end of 90 days (Figure 3c). Fruits treated with CH, PS, and CH + PS displayed higher activity of SOD than control ones, and the maximum activity (274.35 U g^{-1} FW) was achieved by CH1% + PS10% treatment. Considering Figure 3d, the activity of CAT rose over the first 60 days of storage and after which, decreased slightly during the last 30 days of storage. At the end of the storage time, CH2% + PS10% exhibited the highest (39.78 U g^{-1} FW min^{-1}) and the control showed the lowest (5.28 U g^{-1} FW min^{-1}) levels of CAT activity. According to Figure 3e, the activity of APX declined during cold storage period however, treated-fruits exhibited higher APX activity than the control ones. After 90 days, the maximum (197.04 U g^{-1} FW min^{-1}) and minimum (124.84 U g^{-1} FW min^{-1}) level of APX belonged to CH2% + PS10% and control, respectively.

According to obtained results CH, PS, and CH + PS treatments led to the enhancement of DPPH radical scavenging and antioxidant enzymes (SOD, CAT, and APX) activities, as well as inhibiting more declining of ascorbic acid content during the storage period. Antioxidant capacity of fruit is mainly related to the presence of pigments, phenolic compounds, and vitamins (Barman et al., 2014). Ascorbic acid is a part of nonenzymatic antioxidant systems, involving in ROS scavenging in plant cells (Aghdam et al., 2018). High content of ascorbic acid in treated pomegranate fruits may be related to various reasons, such as higher GR/APX (glutathione reductase) system activity (Sayyari et al., 2016). Low oxygen permeability, as a result of CH application, would also lead to reduction of ascorbic acid oxidase activity (Petriccione et al., 2015). A considerable amount of total phenol, flavonoids, anthocyanin, and ascorbic acid contents, which was resulted from high PAL enzyme activity in those fruits treated with combined treatments, is the main nutritional and qualitative parameter of pomegranate fruits. Supporting these results, the positive effects of CH coating alone or in combination with salicylic acid on increasing antioxidant capacity and ascorbic accumulation in pomegranate (Sayyari et al., 2016) and plum (Liu et al., 2014) fruits, have been reported previously. The lower content of EL and MDA in treated-fruits, represented a slight peroxidation of cells' membrane unsaturated fatty acids (unSFA) owing to higher antioxidant capacity.

The activity of SOD, CAT, and APX in pomegranate peel tissue was increased in response to CH, PS, and their combination treatments in particular. Oxidative damage and resulted ROS accumulation throughout the plants' cell are common cross talk between different unfavorable abiotic conditions such as chilling stress. In this regard, plants have efficient enzymatic and nonenzymatic strategies for ROS scavenging (Babalar et al., 2017). SOD, the most potent antioxidant enzyme in the plant cell, acts as a component of the first line defense system against ROS. Accordingly, its activity was at the top of all three evaluated antioxidant enzymes at the present work. Moreover, its activity increased by extending storage time. One probable reason for this would be the existence of various isozymes of SOD within cells (Babalar et al., 2017), which were encoded by different genes in coordination with storage time and the conditions.

CAT is a usual antioxidant enzyme present in all living tissues that utilizes oxygen. This enzyme catalyzes the degradation or reduction of hydrogen peroxide to water and molecular oxygen, consequently completing the detoxification process imitated by SOD (Mittler, 2002). Combined treatments of CH and PS, both especially at the highest used amounts, caused a soaring in CAT activity. However, it seems that cells lost their ability to synthesize and use this enzyme, as its activity experienced a dropping at the third month of storage, this trend has also been observed in the case of APX.

APX is responsible for decomposing of H_2O_2 through receiving the electron from ascorbic acid by ascorbic acid/glutathione cycle (Wang et al., 2013). In the current study, during the last 30 days of storage, both APX activity and ascorbic acid content showed a slight reduction, which demonstrated the influence of substrate declining on the reduction of APX enzyme activity. The positive impacts of CH in combination with other substances such as oxalic and malic acid in pomegranate fruits (Ehteshami et al., 2019), salicylic acid in cucumber fruits (Zhang et al., 2015), and CH alone in sweet cherry fruits (Dang et al., 2010) have been reported previously, in terms of antioxidant enzyme activities.

3.4 | Phenols metabolism

The content of total phenols is shown in Figure 4a, an upward trend of this criterion was observed in treated-fruits up to end of 60 days then, declined slightly at the end of storage time. However, at the end of storage period, fruits treated with CH, PS, and CH + PS had higher level of total phenols than control fruits. The highest (239.89) and lowest (125.42) (mg GAE 100 g^{-1} FW) levels were accompanied with CH2% + PS10% and control, respectively. Figure 4b illustrates data about the total flavonoids content. The amount increased during the first 60 days of storage period and then, slightly decreased. After 90 days, fruits treated with CH1% + PS10% showed almost two times higher flavonoids content (91.31 mg QE 100 g^{-1} FW) than control (47.07 mg QE 100 g^{-1} FW). Figure 4c shows the fluctuations and an increasing trend of anthocyanin content during the storage period. At the end of 90 days, the highest amounts of total anthocyanin (13.78 mg 100 g^{-1} FW) belonged to fruits that were treated with CH1% + PS10% and the lowest amount (11.25 mg 100 g^{-1} FW) was observed in control fruits.

The activity of PAL enzyme in fruit peel tissue increased by the storage time, with more pronounced in treated-fruits (Figure 4d). The maximum PAL activity (371.83 U g^{-1} FW) was brought about by CH2% + PS10% treatment, and the control fruits showed the

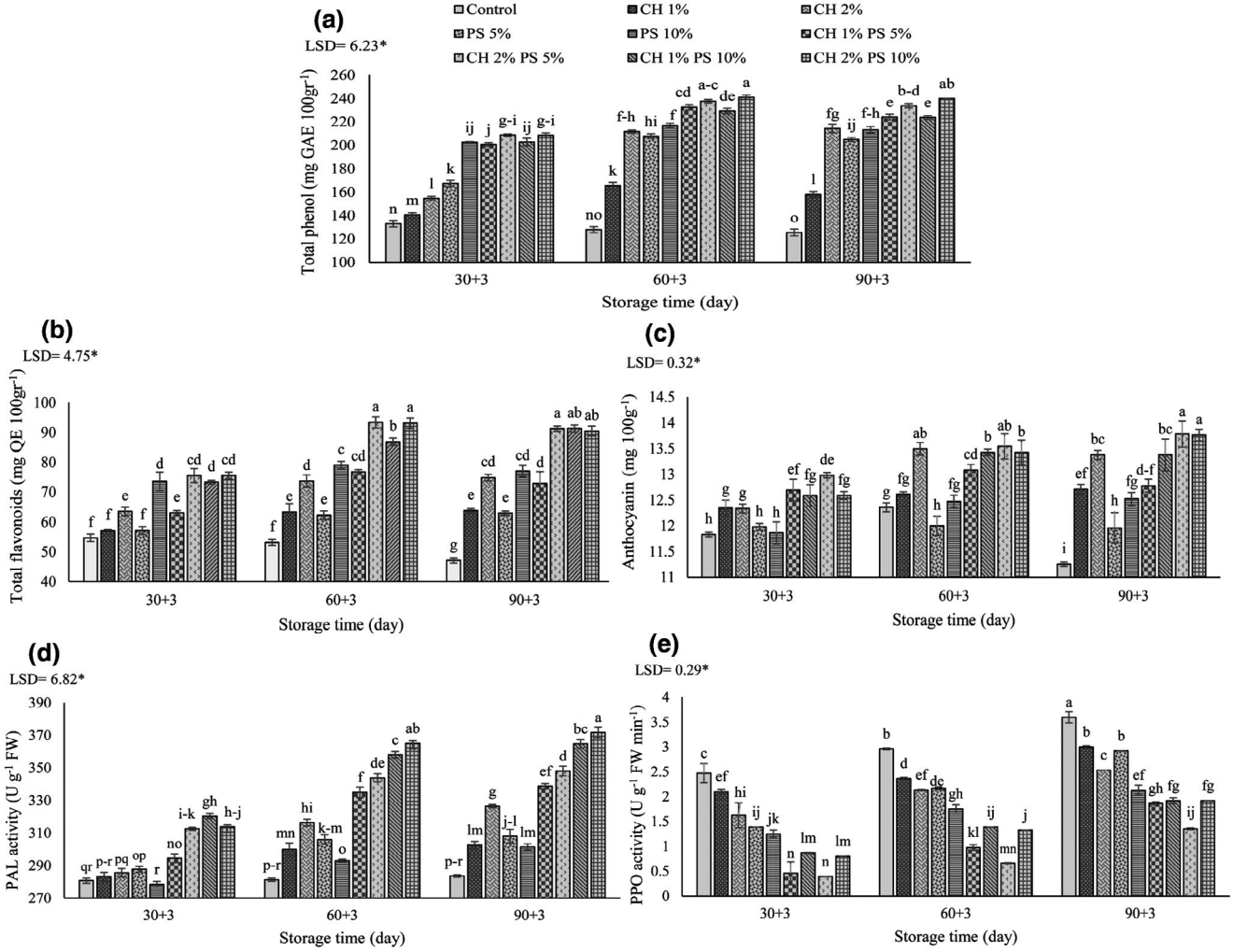

FIGURE 4 Pomegranate total phenol (a), total flavonoids (b), anthocyanin content (c) phenylalanine ammonia-lyase (PAL) (d), and polyphenol oxidase (PPO) (e) activity in response to postharvest application of Chitosan (CH), Potassium Sorbate (PS), and their combination treatments during storage at 4°C for 90 days plus 3 days at 20°C. *showing a significant level at $p \leq .05$. Values are the mean ± *SE*

minimum level (283.56 U g^{-1} FW) after 90 days. Considering Figure 4e, PPO activity increased during storage time. Its activity in fruits treated with CH, PS, and CH + PS treatments was also lower than the control. Fruits treated with CH1% + PS10% experienced the least PPO activity (1.35 U g^{-1} FW min^{-1}) after 90 days at 4°C plus 3 days at 20°C as shelf life.

At the end of the cold storage period and in comparison to control, fruits treated by either CH or PS, and especially by CH + PS, exhibited higher phenylpropanoid pathway activity, illustrating by higher activity of PAL, with lower PPO presence. The alterations of PAL enzyme activity are responsible for the changes in the total phenols during storage time and involved directly in phenolic compound biosynthesis. The total phenols and some of their ingredients such as anthocyanin and total flavonoids increased by storage time and pointed peak at 60 days however, CH + PS treatments raised them much more substantially. After that, they showed decline trends by extending storage time to 90 days.

The influence of handling system, storage conditions, and kinds of treatments on fruit phenolic compounds has been noticed at earlier studies. In pomegranate fruits, for example, Sayyari et al. (2016) reported that fruits treated with salicyloyl chitosan hit a peak, in terms of total phenols and anthocyanin contents. Meighani et al. (2015) also noticed an increased amount of total flavonoids in coated pomegranate fruits by CH just during the first 40 days of cold storage after which, these compounds plunged in both coated and control fruits. During the cold storage, ROS accumulation lead to peroxidation of membrane unSFA, which resulted in losing membrane integrity. This event would exposure the phenols, flavonoids, and anthocyanin to PPO enzyme , which its raised activity during storage period has been revealed in control fruit at the current work. The PPO oxide phenolic reagents, leads to browning (one of the CI symptoms) of plants tissues. Nonetheless, in verse condition, that is, when fruit survive intact with higher membrane integrity because of the treatments like CH + PS, high phenolic compounds are not the only one of the nutritional pros due to their ROS scavenging capacity, but also would participate in defense response by inhibiting oxidation of membrane fatty acids (Kay et al., 2017). The greater content of phenolic compounds and the deterrent impacts on the activity of PPO

in CH + PS-treated pomegranate fruits may be due to the creation of mechanical barrier to oxygen and low levels of respiration in fruits tissues. In plum fruits, for instance, Liu et al. (2014) reported that treated with CH+ ascorbic acid exhibited lower PPO enzyme activity throughout the cold storage. It was revealed that CH could induce PAL activity and decrease tissue browning by inhibiting the activity of PPO in sweet cherry fruits (Dang et al., 2010). In pomegranate fruits treated with CH plus malic and oxalic acid, low browning signs as result of low activity of PPO were observed during the 120 days of cold storage (Ehteshami et al., 2020).

4 | CONCLUSION

To sum up, the application of CH, PS, and their combination in particular, declined decay, weight loss, and CI of pomegranate fruits during cold storage at 4°C. While, CH acts dominantly as an organic barrier against gasses movement, moisture, pathogen agents, etc. on fruit' peel, PS fulfills its role mainly through pathogens growth prevention in vulnerable tissues in long storage period and cold atmospheric conditions. Nonetheless, CH, in combination with PS, showed the boosting performance on fruit storage life in terms of increasing fruit antioxidant capacity and relevant parameters, namely phenolic compounds, the activity of CAT, SOD, APX, PAL, and PPO. These mentioned criteria were cross talk areas that have been influenced by both CH and PS commonly and synergistically. From the applied point of view, using the mixture of CH and PS was much more fascinating in alleviating adverse effects of prolonged and cold storage conditions in pomegranate fruits than either CH or PS alone application, though choosing the best concentration should be taken into account based on genotypes, storage time as well as storage conditions.

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CONFLICT OF INTEREST

The authors have no conflict of interest in this study.

AUTHOR CONTRIBUTION

Sanaz Molaei: Investigation; Writing-original draft. **Ali Soleimani:** Conceptualization; Common Supervisor; Writing; Editing. **Vali Rabiei:** Common Supervisor; Review; Editing. **Farhang Razavi:** Advisor; Data curation; Methodology

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