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Effect of pectin methyl esterase and Ca^{2+} ions treatment on antioxidant capacity, shelf-life and quality of minimally processed pomegranate (*Punica granatum* L.) arils

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Abstract

Pomegranate fruits are difficult to peel and once peeled, extracted arils have very short shelf-life. Therefore, present investigation was carried out to extend the shelf life of minimally processed pomegranate arils using pectin methyl esterase (PME) and $CaCl_2$ treatment during refrigerated storage. The arils of freshly harvested pomegranate fruits (*Punica granatum* L.) were treated with different concentrations of food-grade PME (50-300 units) and calcium ions (0.5-2.0 % $CaCl_2$) for a period of 5-30 min using response surface methodology. Treated and untreated arils were then packed in low density polyethylene bags (25μ) and maintained under low temperature (5ºC; 90% RH) for evaluating the physical, biochemical and microbial quality of pomegranate arils at four day interval. Physiological loss in weight increased during storage but no food-borne pathogens were found during 28 day of cold storage in treated arils. Color and firmness of both treated and untreated arils decreased during storage but it was better maintained in treated arils. The firmness was found to be 0.630 N in treated samples compared to untreated one (0.511 N) after 20 d of storage. Total antioxidant capacity, ferric reducing antioxidant power, polyphenol oxidase and lipoxygenase activities increased during storage. Treatment with 249.33 units of PME and 1.70 % $CaCl_2$ for an immersion time of 24.93 min was found to be most effective treatment for maintaining the quality of minimally processed arils for longer period. Sensory score was also higher in treated pomegranate arils that were quite acceptable even after 20 day of referigerated storage as against 12 day for untreated ones.

Key words

Minimal processing, Pomegranate arils, Pectin methyl esterase, Response surface methodology, Shelf-life

Introduction

Traditional preservation techniques are known to affect the sensorial and nutritional quality of fruits. Consequently, consumer demand for fresh and healthy fruits/foods are mounting. The changing public perception and mood is driving food industries to apply newer and sustainable preservation techniques, which can satisfy the increasing market demands for fewer preservatives, higher nutritive value and fresh sensory attributes. Arguably, minimal processing is one of the techniques that can replace traditional methods of preservation whilst retaining nutritional and sensory attributes (Ohlsson and Bengtsson, 2002). "Minimally processed" is an equivocal term applied to such different types of products as pre-cut, pre-packaged fresh produce and mildly cooked or pasteurized foods that can be stored under refrigeration for more than one week (Balla and Farkas, 2006). However, minimal processing makes fruits and vegetables relatively more perishable than original raw materials (Arias *et al.*, 2008) and also known to be associated with change in color, flavour and texture (Oms-Oliu *et al.*, 2006).

The greatest hurdle in marketing of minimally processed produce is its relatively shorter shelf-life that is attributed to tissue softening and browning (Vilas-Boas and

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Kader, 2006). Pectins are present in considerable amount in fruits and vegetables, thus, contributing to their firmness (Micheli, 2001; Kumar *et al.*, 2012; Kumar *et al.*, 2014). The structure of pectin "gel" in the middle lamella is generally explained by the "egg box" model in which pectins have few "hairy regions" and low methoxyl rate. For calcium to be an effective firming agent, a low degree of methoxylation must be present (Suutarinen and Autio, 2004). It may be added here that partial hydrolytic demethylation of pectin to pectic acid by pectin methyl esterase (PME) in the presence of Ca^{2+} can result in texture firming due to formation of cross-bridges between Ca^{2+} and carboxyl groups of the pectic acids and can produce minimally processed /fresh-cut fruits having comparatively longer shelf-life (Micheli, 2001). Martin-Belloso *et al.* (2006) reported that hydrolysis of pectin to pectic acid in the presence of Ca^{2+} results in texture firming due to formation of cross-bridges between Ca^{2+} and carboxyl groups of pectic acids.

Pomegranate (*Punica granatum* L.) is one of the most valuable commercially grown fruit in India. The fruits are good source of protein, carbohydrate, minerals, antioxidants and vitamins (A, B and C). Its juice is used for controlling diarrhoea, hyperacidity, tuberculosis, leprosy, abdominal pain and fever (Aseri *et al.*, 2008). However, its consumption is very limited due to the difficulty encountered in extraction of arils which is the main edible portion of the fruit. Arils stored at temperatures between 1º and 5°C have shelf-life of 7-14 days, depending on postharvest treatments like packaging and storage temperature (Lopez-Rubira *et al.*, 2005; Ergun and Ergun, 2009). Pomegranate fruit is sensitive to sunburn and cracking and the external defects usually make the injured fruit unsuitable for fresh marketing and consumption despite their excellent internal quality (Artés *et al.*, 2000; Nabigol and Asghari, 2013). Thus, the present investigation was carried out to optimize PME and $CaCl_2$ concentration and their treatment time on minimally processed pomegranate arils for prolonging their shelf-life and maintaining post-harvest quality, and establishing their role in controlling microbial spoilage and decay during refrigerated storage.

Materials and Methods

The experiment was performed at research laboratory of Horticultural Crop Processing Division of the institute. Physiologically mature pomegranate fruits with TSS of 11-12°B, defect free, healthy and uniform in size and appearance having approximate fruit weight of 350-400 g were selected. Extracted arils were collected in trays and mixed thoroughly to assure uniformity.

Shelf-life experiment : The containers, utensils and probable surfaces likely to come in contact with the fruits/arils during primary processing were thoroughly washed and sanitized with 0.1 % NaOCl. The operations of aril extraction and initial sanitizations were carried out in an air conditioned room. Manually extracted arils were first washed with distilled water and then dipped in 0.1 % NaOCl (5 ºC) for 1 min. After preliminary standardization, a Central composite rotatable design of Response surface methodology (CCRD) based on five levels and three independent variables, was used to study the composite influence of independent variables (concentration of PME, $CaCl_2$ and their time of treatment) on shelf-life of pomegranate arils. The design consisted of 20 experiments with 8 equatorial points, 8 axial points and 10 central points for replication (Table 1). For experimental treatment, arils were dipped in solution(s) containing food-grade PME and $CaCl_2$ (concentrations as per Table 1) and 0.07% sodium benzoate prepared in 0.05 M acetate buffer (pH 4.0). The dip treatment was performed in water bath maintained at 35 ºC temperature for specific period of time as per matrix of Table 1. The control arils were simply washed with chlorinated water. After treatment, the pomegranate arils (treated as well as control) were drained and dried under shade with forced air. Treated and untreated arils were packed in 25 µ LDPE perforated bags. Perforation was provided by piercing 8 uniform pin holes (0.85 mm each) to avoid anaerobic growth during storage. Samples were maintained under low temperature (5 ºC; relative humidity 90%) and appropriate physico-chemical and microbiological parameters were determined at 4 day interval during storage.

Physico-chemical and biochemical parameters : Fruit color was measured with hunter color meter (Model: NR-3000, Make: Nippon Denshoku, Japan). Fruit firmness was estimated with the help of hand held penetrometer (Model: Erma, Italy) using 1 mm probe and results were expressed in terms of N (newton). The physiological loss in weight (PLW) of stored arils was calculated and expressed in per cent. Sensory evaluation was done on a 9-point hedonic scale. Scores were given manually to appearance/color, texture, flavor, taste and overall quality. For total antioxidant capacity (TAC) and ferric reducing antioxidant power (FRAP), 5.0 g pomegranate arils were ground with 25 ml of 60 % methanolic extract containing 0.1 % HCl and incubated for 4 hrs at 30 ºC with constant agitation of 150 rpm. The resulting extract was filtered, centrifuged (7000 rpm; 20 min) and further clarified through grade-4 filter paper. Total antioxidant capacity (TAC) was determined by evaluating the free radical scavenging effect on 2, 2'-diphenyl-2-picrylhydrazyl (DPPH) following the procedure of Yu *et al.* (2002). The reaction mixture consisted of 1.8 ml of phosphate buffer (0.05 M; pH 7.0), 1.0 ml of DPPH reagent (0.1 mM in methanol) and 0.2 ml of methanolic extract. The blank (serving as 0) contained 2.0 ml of respective buffer and 1.0 ml

of 60 % methanol while control contained 2.0 ml buffer with 1.0 ml of 0.1 mM DPPH reagent. Discoloration of DPPH by methanolic extract was measured against blank at 517 nm by UV-Visible spectrophotometer (Shimadzu, Model 2550, Japan). Percentage inhibition of DPPH discoloration by methanolic extract was calculated and TAC was expressed in terms of nmols of ascorbic acid equivalents g^{-1} fresh weight (f.wt.) of pomegranate arils. Ferric reducing antioxidant power (FRAP) was measured by the method of Benzie and Strain (1996). The reaction mixture contained 2.5 ml of FRAP reagent, 0.9 ml of acetate buffer and 0.1 ml of methanolic extract. The blank contained 2.5 ml of FRAP reagent and 1.0 ml of acetate buffer. The absorbance was recorded at 593 nm and activity was expressed in terms of nmols of $FeSO_4$ (Fe^{2+}) equivalents g^{-1} f.wt. Enzymes lipoxygenase (LOX) and polyphenol oxidase (PPO) were extracted by macerating 5.0 g pomegranate arils with 15 ml of ice cold 0.1 M potassium phosphate buffer (pH 6.8) containing 3 % (*w/v*) polyvinylpyrrolidone and 1 mM EDTA, in a pre-chilled pestle and mortar using acid washed sand as abrasive. The homogenate was filtered, centrifuged and supernatant was used as crude enzyme preparation. Polyphenol oxidase (PPO) activity was estimated by the method of Kar and Mishra (1976). The reaction mixture contained 1.5 ml of 0.05 M phosphate buffer (pH 6.8), 1.0 ml of 50 mM pyrogallol (made in above buffer) and 0.5 ml of buffer extract. Activity was calculated using molar extinction coefficient (2.47 mM^{-1} cm^{-1}) for purpurogallin and expressed as nmols of purpurogallin produced min^{-1} g^{-1} f.wt. Lipoxygenase (LOX) was assayed spectrophotometrically at 234 nm by the method of Suurmeijer *et al.* (1998). The reaction mixture (3.0 ml) contained 2.91 ml of potassium phosphate buffer (0.1 M, pH 6.2), 40 µl of 30 mM linoleic acid in ethanol and 50 µl of enzyme extract. Increase in absorbance was recorded at 234 nm for 3 min against buffer blank. The LOX activity was calculated using molar extinction coefficient of 2.74×10^4 M^{-1} cm^{-1} (Chen and Whitaker, 1986) and expressed as nmols of conjugated dienes min^{-1} g^{-1} f. wt.

Microbial analysis : For microbiological parameters, 5.0 g of arils were ground in a chlorinated and sanitized pestle and mortar and transferred to an aseptic plastic vial. Serial dilutions up to 10^{-3} were prepared for determining different microbiological parameters. For total plate count, 100 µl of 10^{-3} dilution of microbiological extract while, for coliform and *Staphylococcus aureus* count, 100 µl of 10^{-2} dilution each was used respectively, on plate count agar, coliform and Chapman stone media. However, for *Salmonella* count, 100 µl of 10^{-1} dilution of microbiological extract was used on Bismuth sulphite medium. The plates were incubated at 30 °C for 24 hr and the colonies developed were counted manually.

Data analysis : Design expert 7.1.4 (Stat-Ease, Inc., Minneapolis, USA) was used for regression analysis of the experimental data. Quality of fit of polynomial model equation was expressed by coefficient of determination (R^2) and its statistical significance was checked by Fisher's F test for analysis of variance. The level of significance was given as 'p' value. For dependent variables, standard error of mean (SEm) was calculated.

Results and Discussion

The experimental results were fitted in a quadratic model that enabled predictions of the output response (shelf-life) under any given condition of enzyme and $CaCl_2$ concentration applied for a defined time. Application of RSM yielded the following regression equation, which is an empirical relationship between shelf-life and the test variables in actual units:

$$\text{Shelf-life} = +11.84 + 0.042\,EC + 0.452\,Time - 0.597\,CaCl_2 + 6.788\,EC.Time + 3.771\,EC.CaCl_2 + 0.113\,Time.CaCl_2 - 7.364\,EC^2 - 0.017\,Time^2 - 0.268\,CaCl_2^2$$

High significance of these terms indicated that they can act as limiting factors and even small variation in their values could alter shelf-life to a considerable extent. The sign and magnitude of coefficients indicated the effect of response variable. Negative sign of the coefficient indicated decrease in response value with increase in level of variable. The predicted shelf-life was compared with the experimental (observed) values (Table 1). Statistical testing of the model was carried out by Fisher's statistical (F) test for analysis of variance. If model is a good predictor of experimental results, the calculated F values should be fairly greater than the tabulated F value. In the present case, the calculated F value of 10.76 was greater than the tabulated [$F_{9,10}(1\%) = 3.02$] one, which implied that model was significant and there was a quadratic relationship between the independent variables and response variables. The coefficient of determination (R^2) calculated as 0.897 implied that the fitted model explained nearly 89.7% of total variation in the shelf-life. The value of R^2 suggested fair agreement between the experimental and predicted values obtained from the model. It was deduced from the results that treatment combination of 249.33 units of enzyme and 1.70 % $CaCl_2$ applied for 24.93 min was found to be optimum for maximum shelf-life of pomegranate arils (28 day) at low temperature (5 ºC and 90 % RH) as compared to control (20 days) ran under similar conditions (Table 1). The developed technology is safe, environment friendly and has generally regarded as safe (GRAS) ingredients within permissible limits with no side effects. The response surface methodology has been instrumental within a variety of research activities like enzyme characterization, recovery of oil/active ingredient, shelf-life optimization *etc.* using software mediated speculations and minimizing the workload (Ghafoor *et al.*, 2010; Kumar *et al.*, 2011a; Kumar *et al.*, 2014; Kumar *et al.*, 2015). The

Table 1 : The experimental domain and response in terms of shelf-life for minimally processed pomegranate arils

Standard	Run number	Actual value (coded level)			Response (shelf-life in days)		Residual error
		Factor 1 (A): PME units (IU) (x_1)	Factor 2 (B): Time of treatment (Min) (x_2)	Factor 3 (C): $CaCl_2$ concentration (%) (x_3)	Observed	Predicted	
4	1	249.33 (+1)	24.93 (+1)	0.8 (-1)	20	19.46	0.54
1	2	100.67 (-1)	10.07 (-1)	0.8 (-1)	24	23.41	0.59
17	3	175 (0)	17.5 (0)	1.25 (0)	21	19.73	1.27
10	4	300 (+α)	17.5 (0)	1.25 (0)	25	25.17	-0.17
13	5	175 (0)	17.5 (0)	0.5 (-α)	21	19.69	1.31
3	6	100.67 (-1)	24.93 (+1)	0.8 (-1)	24	24.13	-0.13
16	7	175 (0)	17.5 (0)	1.25 (0)	22	21.45	0.55
8	8	249.33 (+1)	24.93 (+1)	1.7 (+1)	28	27.40	0.60
18	9	175 (0)	17.5 (0)	1.25 (0)	17	18.63	-1.63
15	10	175 (0)	17.5 (0)	1.25 (0)	27	26.96	0.04
5	11	100.67 (-1)	10.07 (-1)	1.7 (+1)	19	19.82	-0.82
6	12	249.33 (+1)	10.07 (-1)	1.7 (+1)	22	22.78	-0.78
12	13	175 (0)	30 (+α)	1.25 (0)	22	22.77	-0.77
11	14	175 (0)	5 (-α)	1.25 (0)	24	24.83	-0.83
9	15	50 (-α)	17.5 (0)	1.25 (0)	23	23.95	-0.95
19	16	175 (0)	17.5 (0)	1.25 (0)	24	23.95	0.05
7	17	100.67 (-1)	24.93 (+1)	1.7 (+1)	24	23.95	0.05
20	18	175 (0)	17.5 (0)	1.25 (0)	24	23.95	0.05
14	19	175 (0)	17.5 (0)	2.0 (+α)	25	23.95	1.05
2	20	249.33 (+1)	10.07 (-1)	0.8 (-1)	24	23.95	0.05

Values in brackets are coded values of five levels (-α, -1, 0, +1, +α)

Table 2 : Changes in color scale (L, a, b values) of pomegranate arils during low temperature (5 ^{O}C and 90 % RH) storage

Day(s) after storage	Color scale					
	'L'		'a'		'b'	
	C	T	C	T	C	T
0	40.39±3.165	40.39±3.165	40.43±5.284	40.43±5.284	6.31±0.978	6.31±0.978
4th	37.19±4.796	38.46±3.631	41.03±1.311	41.82±2.282	15.73±5.550	16.55±2.510
8th	33.50±4.181	37.81±1.209	42.08±14.364	43.15±3.585	17.58±3.924	17.77±2.591
12th	32.48±1.815	37.31±4.015	40.89±3.069	42.32±11.310	14.05±1.242	15.39±1.605
16th	31.64±2.419	37.63±8.879	35.98±3.247	36.12±1.111	12.59±4.215	14.25±0.514
20th	31.07±1.764	37.90±1.684	35.04±8.799	35.70±7.386	12.46±4.635	13.17±3.390
24th	32.10±0.605	36.91±2.165	28.74±2.559	31.70±0.779	13.66±2.789	13.20±1.068
28th	-	29.69±4.182	-	31.85±8.693	-	11.28±4.922
32nd	-	26.34±1.828	-	29.86±6.394	-	6.63±1.448

C= Control; T= Treated arils; Scale value±SEm; n = 3; L= Lightness 0 (pure black) to 100 (pure white), a= -a (green) to +a (red), b= -b (blue) to +b (yellow)

chlorinated and UV-C treated pomegranate arils stored under modified atmospheric packaging (MAP) in polypropylene baskets at 5 ºC were found to have a maximum shelf-life of 15 day (López-Rubira *et al.*, 2005). Ergun and Ergun (2009) observed a shelf-life of 10 day of pomegranate arils treated with 10 to 20 % honey on subsequent storage at 4 ºC.

Changes in physico-chemical parameters were assessed during low temperature storage of pomegranate arils. Brightness (L value) of fruit decreased with increase in storage period, although this decrease was more in treated arils as compared to its control towards the end of storage period (Table 2). Similarly 'a' value which represents red color of the fruit decreased during storage (Table 2). The 'a' value decreased to 28.74 after 24th day of storage while almost same decrease reached in case of treated ones (29.86) after 32nd day of storage from their initial value of 40.43. Increase in 'b' value is indicative of increase in yellowness while decreasing value indicates bluishness. The 'b' value, however, first increased and then decreased during storage (Table 2). The firmness of pomegranate arils decreased with

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the advancement of storage period (Fig. 1a). The firmness of low tempaerature stored arils was found to be 0.511 N on 20th d for control, while the corresponding value for treated arils was 0.630 on 20th day and reached the stage of control sample after 28th day (0.519) of storage (Fig. 1a). Sensory evaluation of the control and treated samples showed a sensory score of 7.5 and 8.0, respectively, for control and treated on 12th day of storage, while the respective corresponding values were 5.5 and 7.5 on 20th day of storage. The value decreased to 5.25 on 28th day of storage for the enzyme treated samples. So, on the basis of sensory score at cold storage, pomegranate arils could be said acceptable up to 20th day while control upto 12th day. Calcium chloride pre-treatment with PME in vacuum (Suutarinen *et al.*, 2002) enhanced firmness independent of the species. The treatments did not affect the sensory quality of fruits (Suutarinen and Autio, 2004). Kumar *et al.* (2014) reported a shelf-life of 10 day when strawberry fruits were treated with PME and calcium chloride and stored at 7^{0}C and 90% RH. Activation of PME increased the number of calcium-binding sites in pectins that in turn facilitated increased calcium cross-linking and better texture. It was found that adding PME of microbial source to diced tomatoes enhanced the ability of calcium to improve firmness (Grassin, 2002; Anthon *et al.*, 2005). Increase in tissue firmness with elevation of tissue calcium is due to interaction of calcium ions with pectin polysaccharides both in the middle lamellae and parenchyma cell walls (Balla and Farkas, 2006). The results of the present study is in confirmation with that of Kumar *et al.* (2014) and Anthon *et al.* (2005). Changes in PLW were slightly less for treated (1.20 %) arils as compared to control (1.53 %) (Fig. 1b). Thus, the physico-chemical data of low temperature stored pomegranate arils suggested that simultaneous application of PME, and calcium chloride was capable of making cell wall/ pomegranate arils tissue firm enough to prevent weight loss for longer time and extended the biochemical, as well as, sensorial shlef-life by 8 day as compared to control under similar conditions (Micheli, 2001; Balla and Farkas, 2006).

No growth of *Salmonella*, *Staphylococcus* and coliforms was seen in fresh as well stored pomegranate aril samples during storage. However, total plate count increased sharply for control samples (from initial TPC of 13x10^{3} to 63x10^{3} cfu g^{-1} at 24th d) while it reached the corresponding value (62x10^{3} cfu g^{-1}) to control for treated samples on 32nd day under low temperature storage (Fig. 1c). This may be attributed to treatment effect resulting in firmer cell wall, low storage temperature, low pH of pomegranate arils and decreased spoilage (Anthon *et al.*, 2005).

Total antioxidant capacity (TAC) is an indicator of the capacity of total antioxidants to counter oxidative stress mediated by biotic and abiotic factors. Overall TAC increased during low temperature storage for control as well as treated samples (Fig. 2a). TAC protected the control samples up to 20th day, but treated samples survived up to 28th day of storage, though the respective increase in TAC from initial value (3929.59 nmols of ascorbic acid equivalents g^{-1} f.wt.) was 4518.91 (control) and 4918.87 (treated) nmols of ascorbic acid equivalents g^{-1} f.wt. (Fig. 2a). Ferric reducing antioxidant power (FRAP) also corroborated with TAC and increased while storage, though increase was more in treated samples as compared to control, thus, suggesting less shelf-life of control fruits in comparison to treated ones (Fig. 2b). Alothman *et al.* (2010) observed concomitant increase in TAC and FRAP by ozone treatment in pineapple and banana while corresponding decrease in guava. Increase in TAC and FRAP may be attributed to increase in phenolic content (Alothman *et al.*, 2010). However, Kumar *et al.* (2011b), in

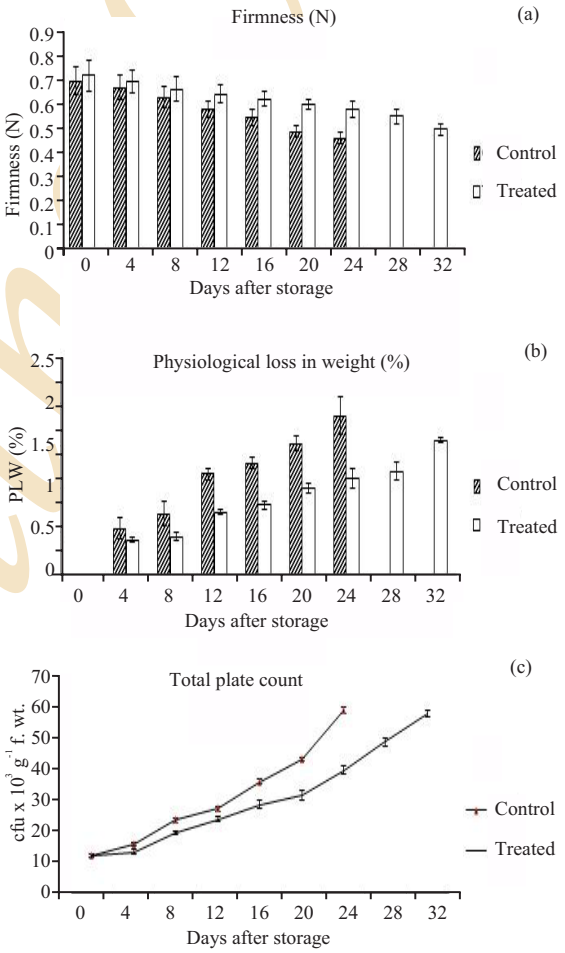

Fig. 1 : Changes in firmness (a) physiological loss in weight (b) and total plate count (c) of pomegranate arils during low temperature (5ºC and 90% RH) storage

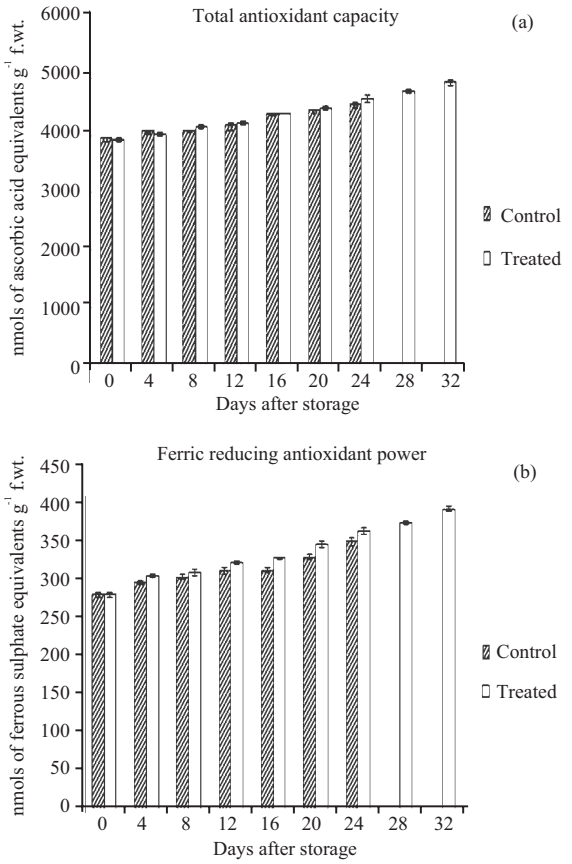

Fig. 2 : Total antioxidant capacity (a) and ferric reducing antioxidant power (b) of pomegranate arils during low temperature (5ºC and 90% RH) storage

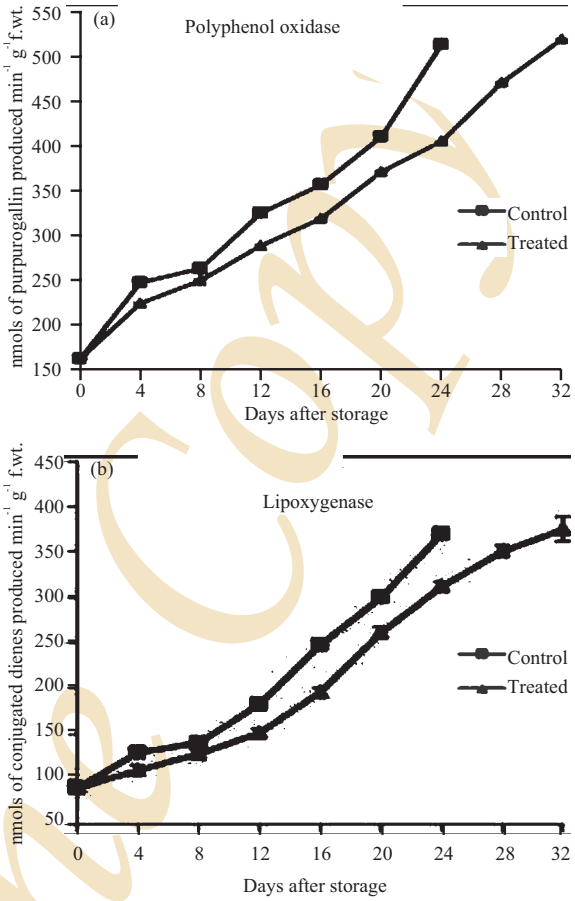

Fig. 3 : Changes in polyphenol oxidase (a) and lipoxygenase (b) activities of pomegranate arils during low temperature (5ºC and 90% RH) storage

their oxidative stress and antioxidant system studies during storage in two ber varieties, found that while oxidative stress increased but overall antioxidant capacity decreased during storage. Similar observations, under storage conditions in tomatoes, have been reported by Mondal *et al.* (2006).

The surface browning of fruits is invariably attributed to enzymatic oxidation of polyphenols by PPO's in the presence of atmospheric oxygen (Oms-Oliu *et al.*, 2010). The activity of PPO increased continuously during storage both in control as well as treated arils, though the increase was more pronounced in control. The PPO activity increased from an initial value of 162.25 to 512.04 nmols of purpurogallin produced min^{-1} g^{-1} f.wt. on 24th day in control fruits, while the corresponding values for treated arils was 404.59 nmols (Fig. 3a). Treated samples reached the stage of control sample on 32nd day of storage (518.17 nmols of purpurogallin produced min^{-1} g^{-1} f.wt.), respectively (Fig. 3a). The LOX activity also showed similar trend during storage period (Fig. 3b). It increased from a minimum value of 89.92 to 370.11 and 374.49 nmols of conjugated dienes produced min^{-1} g^{-1} f.wt., respectively, for control and treated pomegranate arils at the end of their storage period. Ca^{2+} ions in plant tissues is reported to be involved in delaying senescence, reducing respiration, decreasing ethylene production, increasing tissue firmness and preventing enzymatic browning (Balla and Farkas, 2006). From these results, it may be suggested that simultaneous effect of PME and calcium ions might be responsible for decreased and delayed LOX and polygalacturonase (due to improved tissue firmness) and PPO (due to prevention of enzymatic browning) enzyme activities in treated pomegranate arils as compared to untreated ones as advocated by Balla and Farkas (2006).

It may be inferred from the ongoing results that treatment combination of 249.33 units PME and 1.70 % $CaCl_2$ for 24.93 min could extend the shelf-life of pomegranate arils by 8 day over its untreated control samples under low temperature (5°C; 90 % RH) storage conditions.

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