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Efficacy of active modified atmosphere packaging containing thymol on fortification of antioxidant capacity and reducing the microbial contamination of pomegranate fresh arils

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Abstract: Pomegranate arils pose a significant challenge when it comes to preserving their nutritional value and preventing microbial contamination. This study aimed to explore the impact of thymol fumigation and active modified atmosphere packaging (MAP) on enzymatic activity and microbial contamination prevention in pomegranate arils. The results indicated that arils stored in a high O_2 atmosphere (HO_2A) with thymol had notably different catalase (CAT) and peroxidase (POD) activity levels compared to other treatments. These arils exhibited the highest CAT activity and the lowest POD activity. The highest phenylalanine ammonia-lyase (PAL) activity was observed in arils stored in HO_2A with thymol, although it was not significantly different from those stored in a high CO_2 atmosphere (HCO_2A) with thymol ($P<0.05$). Arils stored in a low oxygen atmosphere (LO_2A) and HCO_2A with thymol showed the highest polyphenol oxidase (PPO) activity levels, while arils in HO_2A with thymol had the lowest. The HO_2A with thymol treatment resulted in the lowest presence of psychrophilic bacteria, although it was not significantly different from arils stored in LO_2A with thymol ($P<0.01$). Based on cluster analysis results, HO_2A with thymol, LO_2A with thymol, and HCO_2A with thymol could be considered the most effective treatments for extending the storage life of packaged pomegranate arils.

1. Introduction

To increase the shelf life of fresh-cut fruits, it is essential to slow down biochemical changes, enzymatic degradation, and microbiological deterioration (Kumar *et al.*, 2020). Fruit respiration, which affects metabolic processes, is the main cause of the majority of the physiological changes (Saltveit, 2019), and decreased respiration indirectly slows down ATP-dependent metabolic activities (Wang *et al.*, 2019).

Stress produced after cutting or processing fruit activates numerous defense mechanisms involved in the production and/or degradation of antioxidant compounds in the fruits (Belay *et al.*, 2019 a). Damage by reactive oxygen species (ROS) or phenolic monomer polymerization during storage could be a factor for the decrease in antioxidant activity (Piretti *et al.*, 1996). It is typical to correlate phenolic compounds' nutritive advantages to their antioxidant activity (Karaat and Serce, 2020). Phenolic molecules have a critical function in minimizing or preventing lipid oxidation as well as scavenging oxygen free radicals and they are extremely sensitive to environmental and biological stresses (Gang *et al.*, 2007). The antioxidant activity is affected by redox characteristics of phenolic compounds as well as their capacity as reducing agents, hydrogen ion donors, singlet oxygen quenchers, or metal ion scavengers (Romadanova *et al.*, 2021).

Due to their nature or environmental factors, pathogenic microbes can survive during the food's shelf life (Caleb *et al.*, 2012). So far, few studies on the impact of modified atmospheres containing essential oil on the quality of pomegranate arils (Banda *et al.*, 2015). According to the researchers' results, passive modified atmosphere packaging (MAP) of pomegranate arils cv. Wonderful effectively preserved overall acceptability (El-Eryan *et al.*, 2020). MAP is effective in preserving bioactive compounds while inhibiting the growth of aerobic microorganisms (Ranjbar and Ramezanian, 2022). In a different study, active MAP increased the quality characteristics such as anthocyanin, vitamin C, and the shelf life of the pomegranate arils (Moradinezhad *et al.*, 2020). When pomegranate arils are stored, their chemical, sensory, and quality characteristics can be impacted by the high non-reducing sugars provided by the active modified atmosphere (Patanè *et al.*, 2019). A gaseous mixture of 2-5% O_2 and 10-20% CO_2 is advisable during storage pomegranates (Irtwange, 2006). Decreased oxygen slows senescence, ethylene synthesis, and respiration (Pareek *et al.*, 2015). On the other hand, fresh-cut pomegranate quality has been preserved by the application of high O_2 concentrations (Guo *et al.*, 2019). Super atmospheric O_2 concentration effectively inhibited microbial growth by preventing anaerobic respiration on minimally processed pomegranate arils (cv. Wonderful) (Belay *et al.*, 2017). According to Belay *et al.* (2019 b), O_2 had the biggest impact on color, organic acid, the development of decay, and alcoholic volatile organic compounds. In addition to antimicrobial effects, it has been shown that high concentrations of CO_2 also control overall quality, such as color, texture firmness and volatile organic compounds (aldehydes, ketones, monoterpenes) of the cv. Wonderful. Since the products are free of chemical residues, they are regarded as organic products and have increased their commercial value (Li *et al.*, 2018; Li *et al.*, 2020). However, in certain circumstances, the modified atmosphere is insufficient to assure product quality and safety (Adiletta *et al.*, 2017). The active packaging was used for this purpose (Serrano *et al.*, 2008). Essential oils are an interesting selection of active components used in antimicrobial packaging (Almenar *et al.*, 2006). On the other hand, the lipophilic characteristics of essential oil slow down oxidative reactions by limiting gas release and respiration rate (Ranjbar *et al.*, 2024).

Carvacrol, cinnamaldehyde, citral, p-cymen, eugenol, limonene, menthol, and thymol, are a few active compounds with antibacterial functions that the United States has registered as food flavorings (Mari *et al.*, 2016). Thymol, also known by its chemical names 2-isopropyl-5-methylphenol and 5-methyl-2-isopropyl-5-methylphenol, is a non-toxic food additive (FDA, 2020) and has antifungal and antimicrobial properties (Reyes-Jurado *et al.*, 2020; Ranjbar *et al.*, 2022). The majority of bio-active additives, especially phenolics such as thymol, carvacrol, and tocopherol, function as antioxidants (Maqsoudlou *et al.*, 2020). The increase in antioxidant capacity caused by the components essential oil has a significant impact on the resistance to pathogens and slows down physiological deterioration. *Aspergillus flavus*, *Candida albicans*, and *Botrytis cinerea* cannot grow in the modified atmosphere, which preserves the bioactive compounds of the fruit (Li *et al.*, 2012). During an investigation, thymol was more effective in preventing strawberry fruit rot compared to eugenol and menthol (Wang *et al.*, 2007).

Since there is no scientific report on the combined effect of MAP and volatile organic compounds on pomegranate arils, this study was conducted to determine the best atmospheric composition, either alone or in combination with thymol, to maintain bioactive characteristics, and antioxidant activity and extend the shelf life of pomegranate arils performed.

2. Materials and Methods

Fruit selection, storage and treatments applied

The pomegranates (cv. Rabbab) were harvested from Neyriz orchards (1605 m above sea level, 29°11’55.68” N 54°19’40.08” E), after reaching the mature stage (TSS/TA ≥16). The fruits were transferred to a postharvest lab at Shiraz University. A selection of fruits was made uniform in shape, color, and size. This was followed by disinfecting them in sodium hypochlorite (1%) for five minutes before washing them in distilled water. Pomegranate arils were manually plucked out of peels and mixed before packaging. Fifty g of arils were included in each unit of replication. They were packed in polyethylene + polyester (PE+PES) transparent, having dimensions of 150 × 250 mm, thickness of 90 microns, CO_2 transmission rate of 45-50 g/m^2/day/bar, O_2 transmission rate of 60-70 g/m^2/ day/bar, and water vapor transmission rate of 45 g/m^2/day/bar with three replicates. Then, four atmospheric compositions including 21% O_2 + 0.03% CO_2 + 78% N_2 (Passive- MAP), 5% O_2 + 5% CO_2 + 90% N_2 [Low O_2 atmosphere (LO_2A)], 70% O_2 + 10% CO_2 + 20% N_2 [High O_2 atmosphere (HO_2A)], 5% O_2 + 20% CO_2 ,75% N_2 [High CO_2 atmosphere (HCO_2A)] were selected to store the pomegranate arils. The packaging was performed using a vacuum packing machine (Dz-400 Wenzhou Zhonghuan Packaging Machine Co., Ltd, China) which was connected to a gas mixer. These packages were divided into two groups, with and without thymol (50 mg/L). The thymol applied (Purity ≥ 99%, CAS number 89-83-8) was purchased from Sigma-Aldrich Company.

After storing the samples (5±1°C, 92±3% RH), they were measured for variables every five days.

Total phenols content (TPC)

The Folin-Ciocalteu reagent was applied for measuring the TPC (Meyers *et al.*, 2003). Briefly, 100 µL of fruit juice was diluted with distilled water (1:25 ratio). Then, 100 µL sodium carbonate (2%) was added. After 3 minutes, Folin-Ciocalteu reagent (20 µL, 50%) was included and the sample remained for 30 minutes. Sample absorption was measured (750 nm) by a spectrophotometer (Epoch Biotech, Germany). The TPC concentration was reported as gallic acid (g/L fruit juice) (SM Fig. 1S).

Total anthocyanins content (TAC)

The anthocyanin concentration was determined by the pH differential method. Briefly, the aril sample extract was mixed with KCl buffer (0.025 M, pH 1.0) and NaOAc buffer (0.4 M, pH 4.5), separately. The absorbance was measured at 510 and 700 nm and the data were reported as mg cyanidin-3-glucoside per liter of fruit juice. For the calculation of TAC, the absorbance value (A) entered Equation. 1:

$$A= (A_{510} - A_{700}) \, pH_{1.0} - (A_{510} - A_{700}) \, pH_{4.5} \qquad \text{Eq. 1}$$

TAC based on the concentration of cyanidin-3-glucoside was calculated using Equation 2 (Lako *et al.*, 2007):

$$TAC\ (mg/L) = (A \times MW \times DF \times 1000 / \varepsilon \qquad \text{Eq. 1}$$

Where A represents absorbance value, MW represents cyanidin-3- glucoside molecular weight (449.2), dilution-factor (DF) (5), and ε (26,900) stands for the molar absorptive coefficient of cyanidin-3-glucoside.

Extraction of enzymatic extract

The amount of 500 mg of homogenized pomegranate arils in 50 mM potassium phosphate buffer (pH 7.2) containing 1% polyvinylpyrrolidone (PVP) and 1 mM ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 32869 × *g* for 15 min at 4°C. Every step of the enzyme extraction process was carried out on ice. Enzymatic tests for catalase, peroxidase, polyphenol oxidase, and total soluble protein were conducted using the supernatant.

Catalase (CAT) activity. For the assay, a mixture consisting of 50 mM potassium phosphate buffer (pH 7.2), 30 mM hydrogen peroxide and crude extract was prepared and its absorbance measured at 240 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamic Halo VIS-20 single beam, UK). Enzyme activity was described as the decrease in absorbance over time per U/mg protein by measuring the rate of conversion of hydrogen peroxide into water and oxygen molecules (Sun *et al.*, 2013).

Peroxidase (POD) activity. For the assay, a mixture of 50 mM potassium phosphate buffer (pH 7.2), hydrogen peroxide (% 1), guaiacol (4%) and crude extract was prepared and its absorbance measured at 470 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzyme activity was expressed as delta absorbance after 1 min reaction at 470 nm per U/mg protein (Sun *et al.*, 2013).

Polyphenol oxidase (PPO) activity. For the assay, a mixture consisting of 50 mM potassium phosphate buffer (pH 7), 0.02 M pyrocatechol solution, and the crude extract was prepared and its absorbance was measured at 420 nm using a spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzymatic activity was expressed as U/mg of protein (Silva and Koblitz, 2010).

Phenylalanine ammonia-lyase (PAL) activity. Extracts prepared from 500 mg homogenized pomegranate arils in 50 mM of sodium borate buffer (pH 8.8), 5 mM β-mercapto-ethanol, and 1% PVP buffer, followed by centrifugation at 28341×*g* at 4 °C for 20 min and the supernatant was used for enzyme assays. For assay, a mixture consisting of sodium borate buffer (pH 8.8), and 20 mM L-phenylalanine, and crude extract was incubated at 37°C for 60 min. The reaction was stopped by addition of 6 mol/L HCl. The absorbance of the samples before and after incubation at 290 nm was measured by spectrophotometer (UV-visible spectrophotometer, Dynamica Halo VIS-20 single beam, UK). The enzymatic activity was expressed as per U/mg of protein (Liu *et al.*, 2016). Total soluble protein was measured using the Bradford (1976) method. One mL of Bradford reagent with 100 µL enzymatic extract was mixed completely and its absorption measured at 595 nm. Protein content was estimated using calibration curve of bovine serum albumin (BSA) (SM Fig. 2S) (Bradford, 1976).

Hydrogen peroxide (H_2O_2) content. To measure the H_2O_2 content, 500 mg of pomegranate arils were homogenized with 5 mL of trichloroacetic acid (TCA) (1% w/v) and was centrifuged at 24149×*g* for 15 min. Then, the supernatant was mixed with 10 mM potassium phosphate buffer (pH 7) and 1 mM potassium iodide, and its absorbance at 390 nm was detected using a microplate spectrophotometer (Microplate spectrophotometer, Epoch Biotech, Germany). The standard curve of different concentrations of H_2O_2 was used to calculate the H_2O_2 content and was expressed as mmol/L fruit juice (Nukuntornprakit *et al.*, 2015).

Determination of microbial contamination

A stomacher was used for one minute to homogenize 10 g of pomegranate arils with 90 mL of physiological solution (0.9%). Dilutions (0.01, 0.001, and 0.1) were made using physiological solutions. For both aerobic mesophilic and psychrophilic bacteria, microbial culture was carried out on plate count agar medium (PCA). For mold and yeast, it was carried out on yeast extract glucose chloramphenicol agar (YGC Agar). Every step was performed in a sterile environment using two duplicates of every dilution. Molds and yeasts were incubated at 25±1°C for five days (ISO, 2008), aerobic mesophilic bacteria at 37±1°C for 48 hours, and psychrophilic bacteria at 6.5±1°C for five days (NP-4405, 2002). Log CFU per gram of pomegranate arils was used to calculate the number of microbial colonies.

Sensory quality

Overall acceptance test (flavor, color, and texture) carried out by 10 trained panelists provided hedonic evaluations (Test aimed at measuring the overall hedonic perception of a product by consumers). Quality scores defined based on 5= highest quality score, 3= limit of acceptance and 1= poorest quality value (Watts *et al.*, 1989).

Statistical analysis

The experiment was conducted as a three-factor factorial design, including different atmosphere compositions (21% O_2 + 0.03% CO_2 + 78% N_2, 5% O_2 + 5% CO_2 + 90% N_2, 70% O_2 + 10% CO_2 + 20% N_2 and 5% O_2 + 20% CO_2 +75% N_2), concentrations of thymol (0 and 50 mg/L), and storage period (0, 5, 10, 15, 20 and 25) arranged according to a completely randomized design (CRD) based on a completely randomized design (CRD) , having three replicates. SAS software enabled the analysis of variance (Two-Way ANOVA). Mean values were evaluated for significant differences by Duncan's multiple range test (P≤0.05). Principal component analysis (PCA) was performed using the factoMineR ver. 2.4 package to explain the relationship between the different measured parameters. Cluster analysis was performed using the factoextra package for data-mining and grouping treatments which were more similar to each other.

3. Results

TPC and TAC

The results showed statistical significance in the main effects and reciprocal effects of two and threefold treatments on TPC (P<0.01) (Table 1). On the 15th day of storage, when all treated arils were consumable, the highest TPC (796.13 mg GAE/L) occurred in arils packaged under the HO_2A containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. TPC in arils packaged with HO_2A containing thymol was 25.04%, 21.51%, and 6.55% more than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. TPC in arils packaged with HO_2A containing thymol was 28.55% more than HO_2A without thymol (Fig. 1A).

The results showed statistical significance main effects and reciprocal effects of two and three-fold treatments on TAC (P<0.01) (Table 1). On the 15th day of storage, when all treated arils were consumable, the highest TAC (154.53 mg/L) occurred in arils packaged with HO_2A containing thymol, although it had no statistical significance (P<0.01) compared to arils packaged with HCO_2A containing thymol. TAC in arils packaged with HO_2A containing thymol was 17.69%, 9.75%, and 7.15% higher than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. TAC in arils packaged with HO_2A containing thymol was 15.93% more than HO_2A without thymol (Fig. 1B).

Table 1 - Results of variance analysis for the effect of MAP, Thymol and storage time on the TPC and TAC of pomegranate aril

Source of variations	Degrees of Freedom (df)	Mean of squares	
		TPC	TAC
Storage time (S)	5	2715771.76 **	46557.58 **
MAP	3	13991.19 **	1542.59 **
Thymol (T)	1	757373.01 **	63881.39 **
S × MAP	15	1661.14 **	166.41 **
S × T	5	95210.57 **	12444.42 **
MAP × T	3	2183.04 **	125.56 **
MAP × T × S	15	2365.83 **	122.26 **
Error	72	274.21	27.98
C.V. (%)	—	2.01	4.14

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

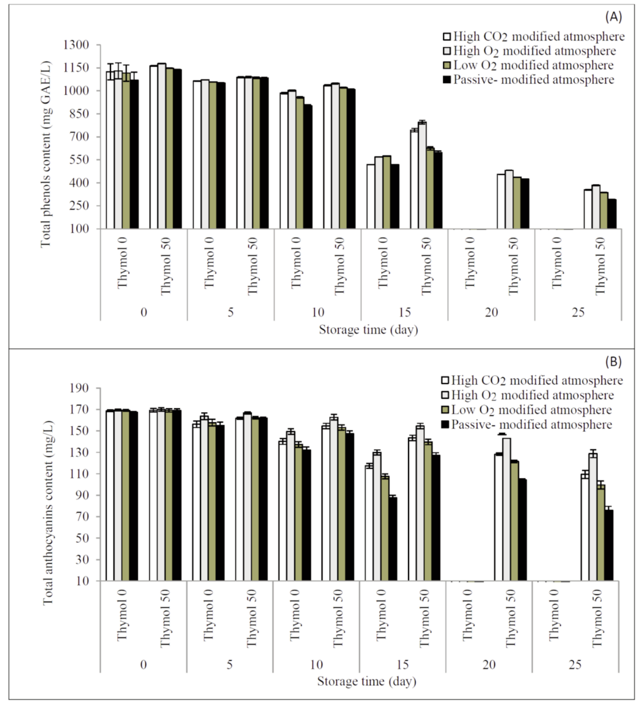

Fig. 1 - Interaction effects of modified atmosphere, thymol and storage time on TPC (A) and TAC (B) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means. Duncan's multiple

CAT, POD, PPO and PAL activity

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on CAT activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were con-

Table 2 - Results of variance analysis for the effect of MAP, Thymol and storage time on the antioxidant enzymes activity and H_2O_2 content of pomegranate aril

Source of variations	Degrees of freedom (df)	Mean of squares				
		CAT	POD	PPO	PAL	H_2O_2content
Storage time	5	33495.00 **	1205.78 **	326.27 **	1129.05 **	195.40 **
MAP	3	2166.53 **	238.75 **	112.78 **	124.03 **	8.56 **
Thymol	1	16966.43 **	2809.24 **	737.47 **	471.44 **	308.61 **
Storage time× MAP	15	149.33 **	59.42 **	11.14 **	12.95 **	1.16 **
Storage time× Thymol	5	1171.73 **	1209.52 **	574.24 **	52.62 **	361.15 **
MAP× Thymol	3	217.47 **	105.69 **	13.10 **	10.22 *	0.34 ns
MAP× Thymol × Storage time	15	58.90 **	48.00 **	8.75 **	6.56 *	1.68 **
Error	72	24.00	1.74	2.30	3.60	0.24
C.V. (%)	—	6.11	6.09	10.37	10.10	3.64

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

sumable, the highest activity (89.16 U/mg protein) occurred in arils packaged under the HO_2A containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. CAT activity in arils packaged with HO_2A containing thymol was 40.28%, 23.71%, and 16.53% higher than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. CAT activity in arils packaged with HO_2A containing thymol was 31.85% more than HO_2A without thymol (Fig. 2A).

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on POD activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were consumable, the lowest activity (25.00 U/mg protein) occurred in arils packaged under the HO_2A containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. POD activity in arils packaged with HO_2A containing thymol was 33%, 11%, and 11% lower than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. POD activity in arils packaged with HO_2A containing thymol was 20.92% lower than HO_2A without thymol (Fig. 2B).

Statistical significance was observed in the main effects and reciprocal effects of two and three-fold treatments on PPO activity (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were consumable, the lowest activity (13.90 U/mg protein) occurred in arils packaged under the HO_2A containing thymol, although it had no statistical significance (P< 0.01) compared to arils packaged with LO_2A and HCO_2A containing thymol. PPO activity in arils packaged with HO_2A containing thymol was 51.65%, 18.92%, and 15.82% lower than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. PPO activity in arils packaged with HO_2A containing thymol was 32.73% lower than HO_2A without thymol (Fig. 2C).

Statistical significance was observed in the main effects and reciprocal effects of two-fold (except for the modified atmosphere × thymol interaction effect) on PAL activity (P<0.01), whereas the reciprocal effects of three-fold treatments were significant at P< 0.05 (Table 2). On the 15th day of storage, when all treated arils were consumable, the highest activity (23.41 U/mg protein) occurred in arils packaged under the HO_2A containing thymol, although it had no statistical significance (P< 0.05) compared to those packaged with HCO_2A containing thymol. PAL activity in arils packaged with HO_2A containing thymol was 35.36%, 27.29%, and 13.24% higher than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. The PAL activity in arils packaged with HO_2A containing thymol was 18.45% higher than HO_2A without thymol (Fig. 2D).

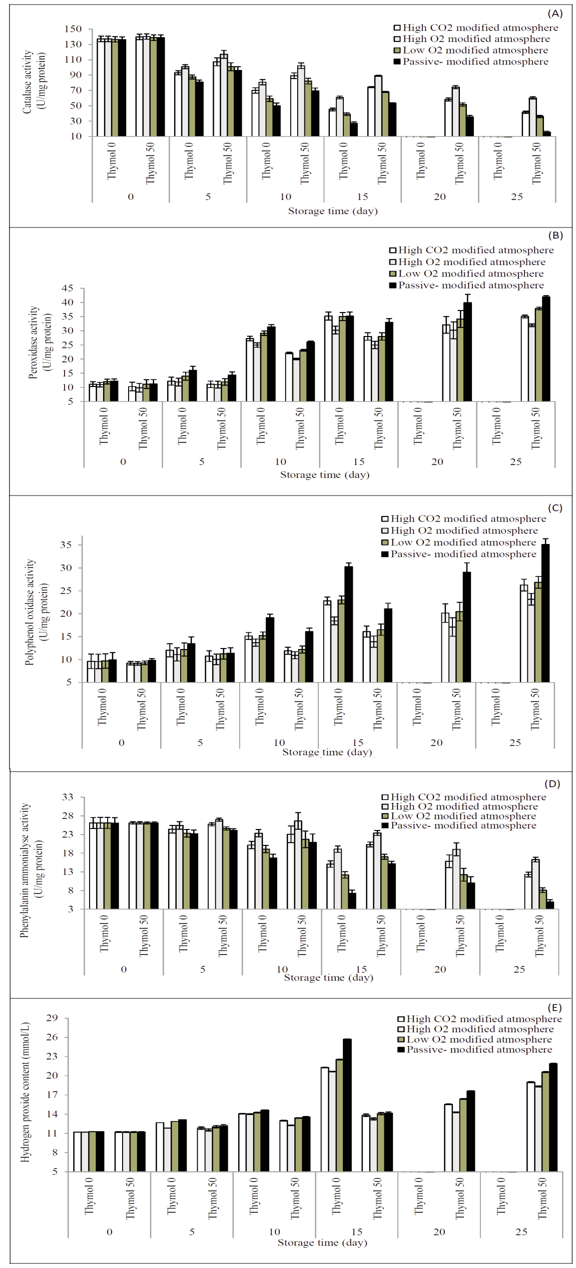

Fig. 2 - Interaction effects of modified atmosphere, thymol and storage time on CAT activity (A), POD activity (B), PPO activity (C), PAL activity (D) and H_2O_2 content (E) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means. Duncan's multiple range test (P<0.01). Interaction effects of modified atmosphere, thymol and storage time on PAL activity (D) of pomegranate arils. Data are the mean ± SE (n=3). Duncan's multiple range test (P<0.05).

H_2O_2 content

Significant effects were observed in both the main effects and reciprocal effects of two-fold treatments (except for modified atmosphere × thymol which was not significant), and reciprocal effects of three-fold on H_2O_2 content (P<0.01) (Table 2). On the 15th day of storage, when all treated arils were consumable, the least amount of H_2O_2 (13.25 mmol/L) occurred in arils packaged under the HO_2A containing thymol, although it had no statistical significance (P<0.01) compared to those packaged with passive MAP, LO_2A, and HCO_2A containing thymol. H_2O_2 level in arils packaged with HO_2A containing thymol was 6.86%, 6.26%, and 4.37% lower than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. H_2O_2 level in arils packaged with HO_2A containing thymol was 55.92% lower than HO_2A without thymol (Fig. 2E).

Microbial contamination

Significant effects were observed in both the main effects and reciprocal effects of two-fold treatment (except for modified atmosphere × storage time and modified atmosphere × thymol, which were not significant) on aerobic mesophilic bacteria at P<0.05 and P<0.01, respectively (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number of aerobic mesophilic bacteria (1.20 Log CFU/g) occurred in arils packaged under the HO_2A containing thymol, there was no significant difference between arils packaged in HO_2A, LO_2A, and HCO_2A containing thymol simultaneously (P<0.05). The number of aerobic mesophilic bacteria in arils packaged with HO_2A containing thymol was 21.36%, 17.5%, and 12.5% lower than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. The number of aerobic mesophilic bacteria in arils packaged with HO_2A containing thymol was 12.5% lower than HO_2A without thymol (Fig. 3A).

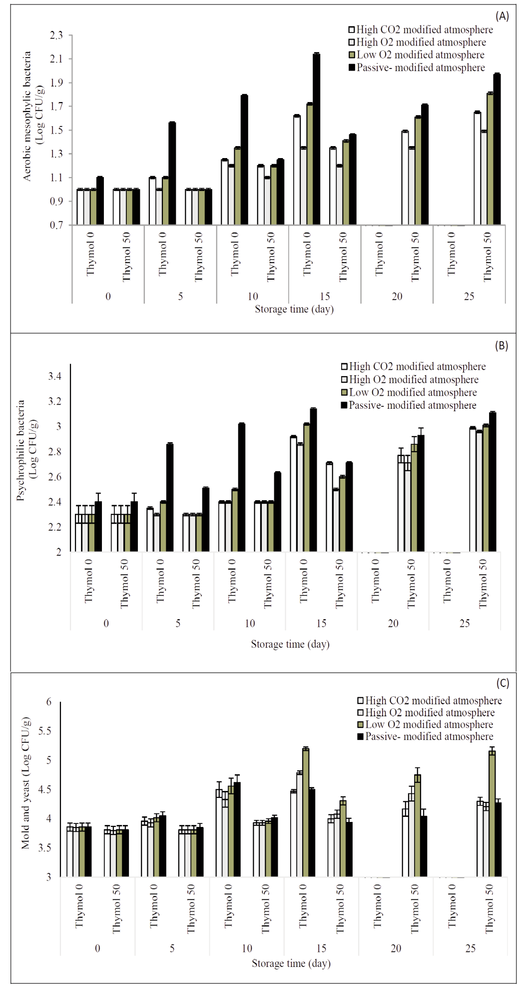

Fig. 3 - Interaction effects of modified atmosphere, thymol and storage time on aerobic mesophilic bacteria (A) of pomegranate arils. Data are the mean ± SE (n=3). Duncan's multiple range test (P<0.05). Interaction effects of modified atmosphere, thymol and storage time on psychrophilic bacteria (B) and mold and yeast (C) of pomegranate arils. Data are the mean ± SE (n=3). Vertical bars represent the standard errors of the means.

Table 3 - Results of variance analysis for the effect of MAP, Thymol and storage time on the microbial load of pomegranate aril

Source of variations	Degrees of Freedom (df)	Mean of squares		
		Aerobic mesophilic bacteria	Psychrophilic bacteria	Mold and yeast
Storage time	5	20.40 **	0.78 **	7.71 **
MAP	3	0.33 **	0.56 **	0.32 **
Thymol	1	50.39 **	0.05 *	21.67 **
Storage time× MAP	15	0.02 *	0.49 **	0.04 **
Storage time× Thymol	5	11.79 **	0.38 **	13.24 **
MAP× Thymol	3	0.03 NS	0.92 **	0.03 **
MAP× Thymol × Storage time	15	0.03 *	0.45 **	0.04 **
Error	72	0.01	0.01	0.006
C.V. (%)	—	8.05	4.11	2.09

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

We observed statistical significance in the main effects and reciprocal effects of two and three-fold treatments on psychrophilic bacteria (P<0.01) (except for the effects of thymol, which were significant at P<0.05) (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number (2.50 Log CFU/g) occurred in arils packaged under the HO_2A containing thymol, although it had no statistical significance (P<0.01) compared to those packaged with LO_2A containing thymol. The number of psychrophilic bacteria in arils packaged with HO_2A containing thymol was 8.14%, 4%, and 8.4% lower than passive MAP, LO_2A, and HCO_2A containing thymol, respectively. Thymol reduced the number of psychrophilic bacteria in arils packaged in HO_2A by 14.4% compared to HO_2A without thymol (Fig. 3B).

We observed statistical significance in the main effects and reciprocal effects of two and three-fold treatments on mold and yeast (P<0.01) (Table 3). On the 15th day of storage, when all treated arils were consumable, the lowest number (3.94 Log CFU/g) occurred in arils packaged under the passive MAP containing thymol, which differed significantly (P<0.01) from the other treatment groups simultaneously. The number of mold and yeast in arils packaged with passive MAP containing thymol was 3.43%, 8.58%, and 1.5% lower than HO_2A, LO_2A, and HCO_2A containing thymol, respectively. Thymol reduced the number of mold and yeast in arils packaged in passive MAP containing thymol by 12.4% compared to passive MAP without thymol (Fig. 3C).

Overall acceptance

Acceptability had a similar pattern and decreased during cold storage. The variance results indicated that with the exception of the interaction effect of the modified atmosphere × thymol and the three-fold interaction effects, which was not significant, both the main effect and the two-fold interaction effects on acceptability were significant (P<0.01) (Table 4). The highest quality score (4.50) was recorded in arils packaged in HO_2A containing thymol and the lowest quality score (2) was recorded in arils packaged in passive MAP without thymol on the fifteenth day of storage, when all treatments were edible, with significant (P<0.01) differences from the other treatments at the same time. Acceptability in arils packaged in HO_2A containing thymol was 26%, 17%, and 6% higher than passive MAP, LO_2A, HCO_2A containing thymol, respectively. Acceptability in arils packaged in HO_2A containing thymol was 10% more than in HO_2A without thymol (Fig. 4).

Table 4 - Results of variance analysis for the effect of MAP, Thymol and storage time on the acceptability of pomegranate aril

Source of variations	Degrees of Freedom (df)	Mean of squares
		Acceptability
Storage time (S)	5	32.59 **
MAP	3	16.91 **
Thymol (T)	1	5.77 **
S × MAP	15	2.11 **
S × T	3	2.22 **
MAP × T	3	0.21 NS
MAP × T × S	9	0.15 NS
Error	360	0.45
CV (%)	—	16.54

*, **, NS = Significantly difference at 5% and 1% of probability level, and non-significantly difference, respectively.

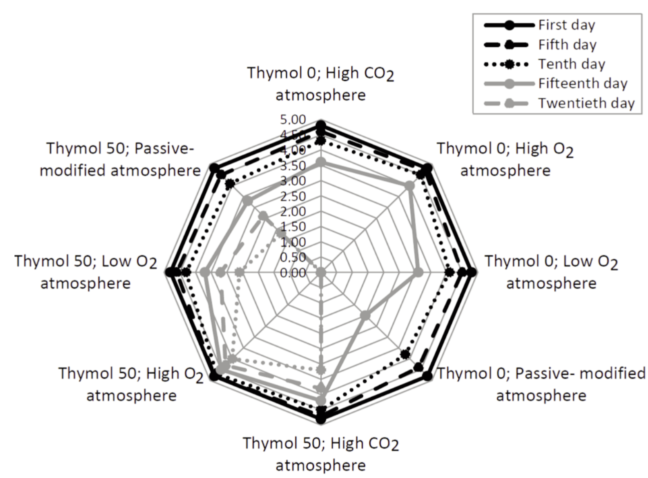

Fig. 4 - Mean comparison effects of treatment at 5°C on ready-to-eat pomegranate arils acceptability during storage.

Using a correlation matrix designed from measured characteristics across various treatments PCA was performed. With 94.71% of the variance covered, the two principal components (PC1 and PC2) are shown in figure 5. Only 3.91% of the total variance was explained by PC2, whereas PC1 was estimated to account for the maximum amount at 90.80%. As shown in figure 5, there was a close correlation between the contents of TPC and TAC as well as the activities of CAT and PAL. These attributes were apparently associated with passive MAP, LO_2A, HO_2A, HCO_2A containing thymol and HO_2A without thymol. Increases in the bioactive compounds and antioxidant activity were related to the enhancement of shelf life of ready-to-eat pomegranate arils. A close relationship existed among POD activity, PPO activity, H_2O_2 content, number of aerobic mesophilic

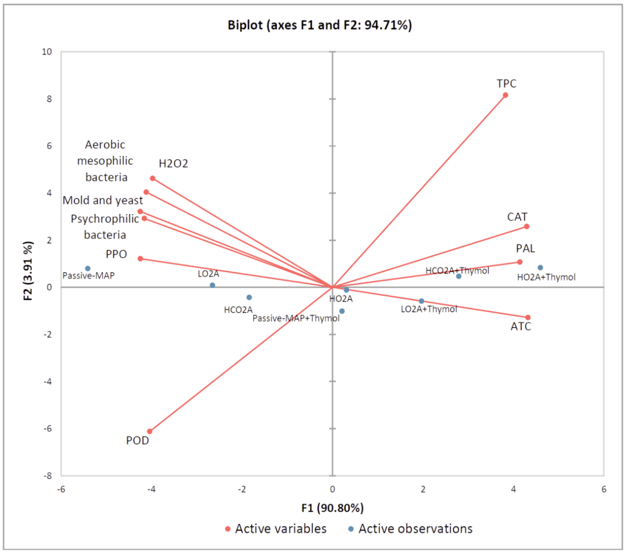

Fig. 5 - PCA of measured attributes of ready-to-eat pomegranate arils with different treatments.

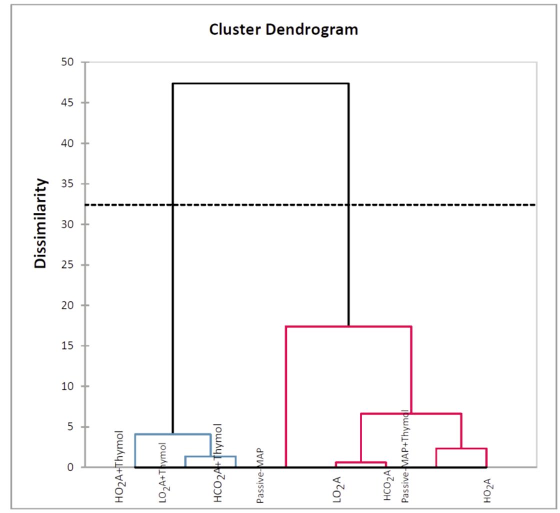

Fig. 6 - Dendrogram of 8 modified atmospheres based on evaluated traits by ward method.

bacteria, psychrophilic bacteria and yeast and mold. These attributes were apparently associated with passive MAP, LO_2A, HCO_2A without thymol. Increases in POD and PPO activity levels, H_2O_2 content and microorganism's contamination was negatively related to the shelf life of pomegranate arils (Fig. 5).

Overall, PCA analysis showed that the relative variables were affected by MAP, although, the effects of passive MAP containing thymol, modified atmospheres containing thymol and HO_2A without thymol were more, because these treatments showed a close relationship with bioactive compounds and antioxidant systems. The results of PCA and biplot diagram were consistent with the grouping obtained from cluster analysis. Cluster analysis divided the treatments into two main groups in terms of similarity of the evaluated traits. Treatments of LO_2A containing thymol, HO_2A containing thymol, and HCO_2A containing thymol were in one group, and the rest of the treatments were in another group. According to the results of groupings and the importance of traits in postharvest storage, the LO_2A, HO_2A, and HCO_2A containing thymol could be considered the best atmospheric composition (Fig. 6).

4. Discussion and Conclusions

Changes in postharvest storage conditions can lead to abiotic stress and the synthesis or accumulation of polyphenols (Senica *et al.*, 2018). HO2A influences the metabolism of secondary compounds and results in the synthesis or accumulation of phenolic compounds (Zheng *et al.*, 2007). The findings of our research were consistent with those reported by Zheng and colleagues, who observed a higher TPC in high O_2-treated Chinese bayberries from day 6 until the end of storage (Zheng *et al.*, 2008). Myrtle fruits exposed to 60-80% O_2 showed higher TPC and quality characteristics compared to fruit stored in passive MAP, indicating the positive impact of HO_2A on fresh produce (Fadda *et al.*, 2017). Storing strawberries at low temperatures and in HO_2A (90 kPa) maintained the phenolic content, improved the antioxidant capacity, and enhanced the quality of the fruit for up to 20 days (Van de Velde *et al.*, 2019 a, b). Our results suggest that HO_2A preserved the polyphenol content and cellular integrity by reducing the levels of superoxide and H_2O_2 (Yang *et al.*, 2020). The phenolic compounds of pomegranate cv. wonderful packed in HO_2A were found to be higher than those in passive MAP, according to our results (Belay *et al.*, 2017). Additionally, cinnamaldehyde was shown to maintain higher levels of phenolic compounds in ready-to-eat pomegranate arils (Ranjbar and Ramezanian, 2022). Thymol seems to act as a signaling molecule that increases the TPC by generating a signal similar to mild stress.

Under modified atmospheric conditions, the stability of anthocyanins is higher due to lower oxidation (Banda *et al.*, 2015). Moradinezhad *et al.* (2020) found that pomegranates packaged in HO_2A had a higher anthocyanin content by the end of the storage time. Storage of blackberries, sweet cherries, cherries and strawberries in the modified atmosphere compared to the normal atmosphere increased in anthocyanin content (Dziedzic *et al.*, 2020). The production of anthocyanin in blood orange fruits stored at HO_2A (70%) is a known physiological response to oxidative stress (Baenas *et al.*, 2014). Similar changes in anthocyanin accumulation and PAL activity indicate that the anthocyanin biosynthesis pathway is controlled by PAL through the supply of cinnamic acid (Dziedzic *et al.*, 2020). The decrease in total anthocyanin during storage is due to hydrolytic reactions that convert anthocyanin glycosides to chalcones, which are degraded to phenolic acid aldehydes (Aguilera *et al.*, 2016). Essential oils reduce the reactivity of anthocyanins with O_2 by saturating the inner space of the package.

SOD, CAT and POD are responsible for inhibition of free radicals related to low temperature stress (Ahmad, 2014). Ayhan and Esturk, 2009 found that an increase in antioxidant activity was reported in pomegranate arils cv. Hicaznar stored under HO_2A (70 kPa). At HO_2A, the activity of H_2O_2 inhibitors, including CAT and SOD was higher (Liu and Wang, 2012). Our results suggest that CAT activity is related to the content of phenolic acids (Liu *et al.*, 2021) and similar results have been obtained for kiwifruit (Liu *et al.*, 2019) and dragon fruit (Pasko *et al.*, 2021).

Low-temperature stress in pomegranate fruit is linked to the production of oxygen free radicals such as superoxide and H_2O_2. Therefore, to prevent oxidative stress damage at low temperatures, alternative respiratory systems or the inhibition and decomposition of toxic substances are necessary (Fung *et al.*, 2004). Essential oil, known for its high antioxidant properties, appears to be effective in delaying the lipid peroxidation process and inhibiting oxygen free radicals (Rodriguez-Garcia *et al.*, 2016). Current results, using the electron spin resonance and oxygen radical absorbance capacity (ORAC) assays, have shown that thymol has the ability to increase enzymatic and non-enzymatic antioxidants to inhibit oxygen free radical production in fruit tissue (Wang *et al.*, 2007). Consistent with our findings, the activity of antioxidant enzymes increased in mangos packaged with MAP containing thymol (Perumal *et al.*, 2017). Additionally, cinnamaldehyde increased antioxidant capacity and delayed the reduction in nutritional quality of citrus fruit (Gao *et al.*, 2018). Both carvacrol and anethole increased SOD and CAT activity in raspberries (Jin *et al.*, 2012), and grapefruit extract increased catalase activity in grapes (Xu *et al.*, 2019). It appears that essential oils stimulate the antioxidant mechanism or the production of secondary metabolites and increase antioxidant capacity.

POD is a crucial enzyme in fruit tissue browning that utilizes H_2O_2 as a catalyst for the oxidation of phenolic compounds (Singh *et al.*, 2018). It accelerates the breakdown of phenols when PPO is present (Richard-Forget and Gauillard, 1997). Our findings indicate that reducing H_2O_2 levels to increase access to high O_2 helps maintain polyphenolic content, cell integrity, and decreases POD activity (Yang *et al.*, 2020). HO_2A also delays the peak activity of POD by preventing oxidative stress (Wang *et al.*, 2020).

It has been reported that higher activity of POD and PPO in melon is related to metabolic activity and accelerated respiration rate (Menon and Ramana Rao, 2012). The production of oxygen free radicals and cell membrane damage lead to the reaction of phenolic compounds and PPO, which leads to tissue browning (Mishra *et al.*, 2012). High levels of O_2 could reduce browning and inhibit PPO and POD activity which was in accordance with previous research (Li *et al.*, 2014). One of the main purposes of using essential oils is to delay the activity of PPO enzyme and prevent browning (Marandi *et al.*, 2010).

Microorganisms cause damage to the structure of the cell membrane, leading to the proximity of phenolic compounds and the enzyme PPO, resulting in browning. Essential oils can delay the activity of PPO and prevent the browning of fruit tissue by reducing microorganisms and membrane damage (Marandi *et al.*, 2010). PPO activity is inhibited at low pH (Hithamani *et al.*, 2018), and essential oils can reduce the activity of PPO by lowering the pH. Additionally, the antioxidant activity of essential oils can decrease the decomposition rate of pigments and prevent the browning of fruits caused by PPO activity (Serrano *et al.*, 2005). Treatment of grapefruit with grapefruit extract has been shown to prevent the increase of PPO (Xu *et al.*, 2009).

PAL activity leads to an increase in the synthesis of polyphenolic phytoalexins, which results in a decrease in the oxidation of phenolic substrates by reducing the activity of PPO (Galani *et al.*, 2017). The high ratio of PAL to PPO leads to the accumulation of phenols and increased activity of the antioxidant system, resulting in less accumulation of ROS. This also helps maintain membrane integrity by preventing the peroxidation of unsaturated fatty acids, ultimately reducing pomegranate browning (Martinez-Espla *et* *al.*, 2018). Therefore, HO_2A is effective in reducing the enzymatic browning of arils during storage by boosting the activity of the antioxidant system and increasing the PAL to PPO activity ratio (Martinez-Espla *et al.*, 2018). Our findings suggest that thyme essential oil enhances the activity of the PAL enzyme in avocado fruit (Assis *et al.*, 2001).

The low amount of H_2O_2 in fruits packed in HO_2A is related to the mechanism of H_2O_2 inhibitory enzymes and non-enzymatic antioxidant. Research shows that the activity of H_2O_2 inhibitory enzymes is higher at high O_2 concentrations (Liu and Wang, 2012). Mitochondrial dysfunction due to the accumulation of ROS is the leading causes of senescence (Qin *et al.*, 2009). In the normal atmosphere, anthocyanins and phenol decreases due to increased activity of PPO, POD and accumulation of H_2O_2 (Luo *et al.*, 2017).

According to our results, the lowest number of aerobic mesophilic bacteria was observed in HO_2A and HCO_2A, whereas the highest number was in the normal atmosphere (Moradinezhad *et al.*, 2020). Since the growth of anaerobic microorganisms occurs at very low O_2 levels and the growth of aerobic microorganisms happens at atmospheric O_2 concentrations (around 21 kPa), HO_2A inhibits both aerobic and anaerobic microorganisms. The inhibitory effect of HO_2A on aerobic mesophilic bacteria is linked to the toxicity of high O_2 concentrations (Tomas-Callejas *et al.*, 2011), which can cause damage to DNA and nucleoproteins in microorganisms (Moradas-Ferreira *et al.*, 1996). Additionally, the reduction of microbial load in HO_2A is attributed to ROS produced at a partial pressure of O_2 (Zhang *et al.*, 2013), which damages the antioxidant system of microorganisms. Our results also show a decrease in the number of aerobic mesophilic bacteria in minimally processed pomegranates cv. Hicaznar under HO_2A (70 kPa) (Ayhan and Esturk, 2009).

According to our results, HO_2A reduced the psychrophilic bacteria in melon slices (Oms-Oliu *et al.*, 2008). In products prone to mold growth, high oxygen has a strong inhibitory effect on mold growth (Rojas-Grau *et al.*, 2009). Our findings indicate that modified atmospheric packaging of pomegranate arils has reduced the number of molds and yeasts at 5°C (Ayhan and Esturk, 2009). HCO_2A is effective in inhibiting aerobic microorganisms, especially gram-negative bacteria and molds, but is not very effective in inhibiting yeasts (Al-Ati and Hotchkiss, 2002). Inhibition of mold growth at a 10% CO_2 concentration has been reported, but no fungicidal effect was observed (Poubol and Izumi, 2005).

Thymol is a natural volatile monoterpenoid phenol and the main active ingredient in the oil extracted from the species *Thymus vulgaris* L. The antimicrobial activity of essential oils is attributed to their high monoterpenes content, which have antibacterial and antifungal properties (Bouaziz *et al.*, 2009). Our research revealed that cinnamaldehyde in arils stored in a modified atmosphere significantly reduced microbial agents (Ranjbar and Ramezanian, 2022). Generally, essential oils are more effective at low pH levels. At low pH, the hydrophobic nature of essential oils increases, allowing them to easily dissolve in cell membrane lipids and cause the leakage of cell contents (Burt, 2004).

Storage at HO_2A prevents enzymatic browning and flavor changes due to control of anaerobic conditions (López-Gálvez *et al.*, 2015). Our findings suggest that hot air treatment and a modified atmosphere containing pure O_2 and pure CO_2 on pomegranate arils show that the modified atmosphere containing 80% O_2 + 20% N_2 and the heat treatment at 45 °C, compared to a modified atmosphere containing 20% CO_2 + 80% N_2 and the heat treatment at 55 °C, had a better effect on physicochemical properties and pomegranate quality (Maghoumi *et al.*, 2013). Additionally, the quality of cherries (Wang *et al.*, 2014) and blood oranges (Molinu *et al.*, 2016) was affected by HO_2A. On the other hand, the antioxidant properties of strawberry fruit were improved by HO_2A (Odriozola-Serrano *et al.*, 2010), which aligns with our findings. Our results also indicate that thyme oil has a positive effect on the quality and overall acceptance of organic bananas (Vilaplana *et al.*, 2018). The main advantage of essential oils is their strong antioxidant properties that prevent changes in taste due to the release of free radicals (Dorman and Deans, 2000).

According to our results, the combined application of thymol and eugenol in the passive MAP of cherries had no organoleptic effect (Serrano *et al.*, 2005).

The results of this research demonstrated that MAP, especially MAP with a high O_2 concentration, is a valuable technique for maintaining the nutritional quality, and antioxidant activity, and controlling the microbial load of pomegranate arils within the acceptable range for commercial purposes. A synergistic effect was found when using HO_2A, which contains thymol, on the qualitative characteristics of ready-to-eat pomegranate arils. This includes preserving phenolic compounds, antioxidant enzymatic activity, delaying enzymatic browning, and maintaining visual appearance. Additionally, the application of HO_2A packaging is effective in preserving bioactive compounds that help maintain fruit quality, appearance, taste, and health-promoting properties.

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