ISSN: 0974-5823 Vol. 7 No. 4 April, 2022

International Journal of Mechanical Engineering

Effect of chitosan, salicylic acid, malva sylvestris and aloe vera extract on quality indices of *Citrus unshiu* Marc. during storage

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ABSTRACT

To investigate the effect of chitosan, salicylic acid, aloe vera gel, and mallow mucilage on the qualitative factors of *Citrus unshiu* Marc. and to determine their best concentration, a factorial experiment in a randomized complete design with three replications was conducted at the Islamic Azad University, Science and Research Branch. Treatments included chitosan (0.5, 1, and 1.5%), salicylic acid (1, 1.5, and 2 mM), aloe vera gel (15, 30, and 45%) and mallow mucilage (15, 30, and 45%). Fruits were immersed and then stored for 45 days (4 storage periods: 0, 15, 30, and 45 days). The measured characteristics included pH, titratable acid (TA), total soluble solids content (TSS), fruit weight loss, fruit carotenoids content, ascorbic acid content, antioxidant capacity and some enzymes activity. The results showed that pH, TSS, weight loss and carotenoids content increased and the amount of TA, ascorbic acid and antioxidant capacity decreased at storage time. The activity of superoxide dismutase (SOD) increased at day 15 and then decreased. There was an increasing trend in catalase enzyme (CAT) activity from day 0 to day 30 and reached the highest level during this period. There was also an increasing in peroxidase (POD) activity trend with increasing storage time. The lowest activity of this enzymes was achieved in 15% aloe vera treatment on day 45. The most effective treatment in maintaining pH, TA, TSS, carotenoids content, ascorbic acid, antioxidant capacity and POD enzyme was 2 mM salicylic acid (SA), which is recommended for use for fruits postharvest.

Key words: Mandarin, Postharvest, Citrus, Biodegradable, Antioxidant activity, Antioxidant enzyme.

1. INTRODUCTION

Citrus fruits are one of the world's subtropical fruits that have high economic value in Brazil, United States, and China, in a way that it is known as the citrus industry in the world. *Citrus unshiu* marc. has a good taste and marketability in Iran. This cultivar is suitable for cool subtropical regions and spreads in temperate regions of Japan, China, Spain and countries with suitable climatic conditions (Iglesias et al., 2007).

Postharvest technology of horticultural crops includes techniques, processes and treatments related to handling, processing, storage and transportation of fruits, which aim to prepare fruits to meet market needs, increase their commercial life, and reduce waste. Therefore, fruits must be properly prepared and treated with appropriate postharvest treatments to have relative resistance to long-term transportation and storage (Zacarias et al., 2020). Most fresh fruits and vegetables are known for their therapeutic value and health-promoting activities (Sinuraya et. al., 2021; Hanawi et. al., 2020; Almaiman et al., 2019). Therefore, organic products, produced without the use of synthetic chemicals, are receiving worldwide attention due to the presence of active compounds such as phenolic substances and their antioxidant properties (Suleria et al., 2015). In this regard, the use of healthy, natural and biodegradable compounds while improving the storage life of horticultural products, increases community health status indices.

One of the healthy methods for better control of postharvest diseases is the use of environmentally- and human-friendly compounds (Asghari, 2006). Aloe vera, scientifically named Aloe vera L., is a perennial and drought-tolerant plant whose gel is clear, odorless and sticky, and contains compounds such as vitamins, amino acids, enzymes, SA, anthraquinones, and saponins (Choi et al., 2001). *Malva sylvestris* is a biennial plant belonging the Malvaceae family that has long been used for food and medicinal purposes, and the most important active ingredients in its flowers inculde mucilage, flavonoids, tannins, phenolic compounds and anthocyanins (malvin, dolphinidin and malvidin) (Dehkordi, 2003). Salicylic acid (SA) is an endogenous plant growth regulator that causes a wide range of physiological and metabolic reactions in plants (Raskin, 1992). Exogenous application of SA protects plants against direct oxidative damage. SA induces a moderate stress at relatively low concentrations and creates an effect similar to oxidative stress in the plant but to a lesser extent. The rapid and transient increase in ROS is due to the increased antioxidant capacity that protect the plant from severe stress-induced damage (Horvath et al., 2007). SA treatment has also been shown to effectively reduce the respiration of harvested fruits and is also highly concentration dependent (Srivastava and Dwivedi, 2000; Mo et al., 2008). In addition, high SA concentrations affect the pore width and closes the fruit pores. Fresh weight and respiration rate have been found to be directly correlated with pore width (Manthe et al., 1992). Increasing the SA concentration reduces the activity of CAT and POD enzymes in bananas (Srivastava and Dwivedi, 2000). SA treatment also reduces the degradation of enzymatic and non-enzymatic antioxidants such as dehydroascorbate reductase and ascorbate peroxidase in the refrigerator (Huang et al., 2008).

Chitosan is a non-toxic, biodegradable, functional and biostructural compound that has strong antimicrobial and antifungal activity and is able to effectively control fruit rot. This compound has also been able to easily form coatings in fruits and vegetables and reduce the respiration rate in them by regulating the permeability of carbon dioxide and oxygen (Kurita, 2006). It has been reported that chitosan coating reduced fruit juice loss by half compared to the control group (Gao et al., 2018). Chitosan has been reported to effectively delay postharvest ripening characteristics of Chinese cherries such as weight loss, rot rate, SSC, firmness, and TA (Xin et al., 2017). Chitosan treatment (1%) is effective in preventing weight loss, reduction of respiration rate and also reduction of fruit rot rate in two plum cultivars (Stanley and Giant). It also prevents fruit weight loss and retains TA, fruit pH and firmness in two cultivars by reducing respiration rate (Bal, 2018). Examination of effect of chitosan and SA on the postharvest quality of grapefruit showed that the chitosan and SA increased the activity of chitinase, phenylalanine ammonia lyase (PAL), peroxidase, 1-β, and 3-glucanase, SOD, and polyphenol oxidase, and also increased the synthesis of the content of total phenolic compounds (Shi et al., 2018).

A study was performed on the effect of application of chitosan coatings and peppermint essential oil on Tommy Atkins mango. Results showed a lower decrease in TA and increase in pH values in coated mangoes than control fruits during storage and mangos coated with chitosan essential oils had a lower sugars and higher organic acids, which reduced fruit weight and firmness and led to a delay in changes in catechin and procyanidin B_1 and B_2 content during the storage period (de Oliveira et al., 2020). In one study, aloe vera gel was used alone or in combination with sage essential oil in tomatoes. The results showed a decrease in ethylene production and release in tomatoes coated with 10% aloe vera, while the use of essential oil increased ethylene release and respiration rate. Aloe vera coating reduced acidity, β-carotene, lycopene content and increased fruit firmness. High concentrations of sage essential oil increased fruit weight loss, but reduced fruit redness, chroma, SSC, acidity, β-carotene and lycopene. Aloe vera gel and sage essential oil treatment led to no change or even an increase in total phenolic content and antioxidants during the storage period (Tzortzakis et al., 2019). In another study on the effect of SA and aloe vera gel on Thomson Novell oranges, it was reported that the treated fruits had less weight loss and higher firmness, SSC, TA, vitamin C, and total phenol content (Rasouli et al., 2019). Aloe vera gel led to a further increase in CAT, SOD, and ascorbate peroxidase activity (Ali et al., 2019).

The results of research on the effect of malva sylvestris mucilage and thyme essential oil on the storage life of pear cultivar of Shahmiveh pear cultivar showed that *Malva sylvestris* mucilage controlled fruit weight loss percentage by increasing fruit juice and increased firmness and decreased SSCs compared to the control group at any stage of the storage period (Alikhani et al., 2010). The mallow (*Malva sylvestris* L.) belongs to the Malvaceae family and the most important active ingredients of its flowers include mucilage, flavonoids, tannins, phenolic compounds and anthocyanins (malvin, delphinidin, and malvidin). Phenolic compounds and anthocyanins participate in a variety of plant defense mechanisms (Dehkordi, 2003).

Considering the high importance of citrus fruit storage life and also improving the export status of these fruits from the north of the country, the aim of the present study was to improve the storage life of *Citrus unshiu* Marc. based on the use of biodegradable human-friendly compounds.

2. MATERIALS AND METHODS

2.1. Plant material and experimental design

After complete color development and physiologically mature, fruits were harvested from a commercial orchard in Miandorod, Sari city, at the end of November 2017, and after proper arrangement, were immediately transferred to the laboratory. The fruits were washed with tap water and infected fruits were removed and healthy and uniform fruits in terms of size and color with no physical injuries and disease symptoms selected. The aim of this experiment was to investigate the effect of chitosan, SA, aloe vera gel and mallow mucilage treatments on the quality factors of *Citrus unshiu* marc. and to determine their best concentration, factorially in a completely randomized design. Experimental treatments, including chitosan (0.5, 1, and 1.5%), SA (1, 1.5 and 2 mM), aloe vera gel (15%, 30%, and 45%) and mallow mucilage (15%, 30%, and 45%) were immersed for 3 minutes. The fruits were then spread on a wire rack to dry. Afterwards, the fruits were transferred to cold storage at 7°C and 90% relative humidity and stored for 45 days (four storage periods: 0, 15, 30 and 45 days).

2.2. Quality parameters determination

To measure the fruit pH, the pH meter (3520 Bench pH Meters, UK) was adjusted with buffer solutions with pH 4 and 7. Filtered fruit extract was poured into a small beaker where the device electrodes were placed and the extract pH was read and recorded. The total soluble solids content (TSS) was measured with a refractometer (Brix TE-RM50B, Victoria, Australia). Titratable acidity (TA) was assessed by titration of 5 mL juice from a sample made up of 3 fruits with 0.1 N NaOH until the organic acids were neutralized at pH 8.1-8.3 with an pH meter (3520 Bench pH Meters, UK). Results were expressed as a percentage of citric acid (Silva et al., 2017).

$$\text{Total acids (\%)} = \frac{1 \times \text{acid equivalent weight} \times \text{NaoH normality} \times \text{NaOH volume}}{10 \times \text{Sample weight}} \times 100$$

2.3. Weight loss determination

In order to measure the weight loss percentage of *Citrus unshiu* marc. separate samples of fruit in 3 replications of each treatment were kept for the evaluation of fruit weight loss (%) at the end of the experiment. Physiological loss in weight (PLW) was recorded with using a digital scale with an accuracy of 0.001 g by subtracting final weight from the initial weight and then expressed as percent weight loss with reference to the initial weight. The weight loss percentage of each repetition was calculated using the following equation:

$$\text{Fruit weight loss (\%)} = \frac{A - B}{A} \times 100$$

A: fruit weight before storage, B: fruit weight after the end of the storage (Arnal and Del Rio, 2004).

2.4. Determination of antioxidant capacity and carotenoids content

To measure the antioxidant capacity, 4 ml of methanol 80% was added to 0.5 g of fruits powdered and crushed with liquid nitrogen. The fruit texture was centrifuged with methanol at 9500 rpm for 20 minutes. Then 100 µl of the extract was added to 3400 µl of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution prepared from Sigma Aldrich Co. The resulting mixture was kept at room temperature in the dark place for one hour and then read with spectrophotometer at 517 nm according to the following formula:

$$\text{Antioxidant capacity (\%)} = \frac{\text{Sample}}{\text{Control}} \times 100$$

Ascorbic acid concentration of fruit extract was also measured based on reduction in 6,2-dichlorophenol indophenol (DCPIP) induced by ascorbic acid using a spectrophotometer at a wavelength of 520 nm (Bor et al., 2006).

Total carotenoids content was determined by using a spectrophotometer (Lambda EZ 201, USA). The sample mixture was centrifuged at 4000 rpm for 15 minutes. The supernatant was removed and its absorption was measured by spectrophotometer at 450 nm wavelength (Abeysinghe et al., 2007).

2.5. Determination of antioxidant enzymes activity

To evaluate the activity of SOD, CAT, and POD enzymes, 5 g was removed from a healthy part of the mesocarp of mandarin and was immediately frozen in liquid nitrogen until enzyme assays were performed, and then stored at -80 °C (Jin et al., 2009).

To extract the enzymatic extract, 1 gram of the sample texture was grinded with 3 ml of sodium phosphate buffer (pH 6.7) in a cold porcelain mortar and made into a uniform mixture. The resulting mixture was immediately centrifuged at 18,000 rpm at 4 °C for 15 minutes. After complete settling, the supernatant was used to measure the activity of the above enzymes (Mac-Adam et al., 1992).

After preparation of enzymatic extracts, SOD activity was determined by measuring the ability of each extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). To measure SOD activity, the reaction solution included 50 mM phosphate buffer (pH 7.5), 13 mM methionine, 75 mM nitroblue tetrazolium, 0.1 mM EDTA, and 2 mM riboflavin. The enzymatic reaction solution was kept in complete darkness. A total of 2.5 ml of SOD reaction medium with 200µl of enzyme extract was placed under fluorescent lamp for 10 minutes. Two series of tubes containing 2.5 ml of reaction medium plus 200µl of extraction buffer were prepared. One series in the dark and one series with the samples were placed under a fluorescent lamp for 10 minutes. After 10 minutes, all samples were transferred to darkness and the absorption rate was measured at 560 nm using a spectrophotometer (Shimidazucarry 50) (Giannopolitis and Ries, 1977).

Pereira et al. (2012) method was used to measure CAT activity. To measure CAT activity, the reduction of H_2O_2 in 240 nm/min was studied. The reaction mixture consisted of 0.1 M phosphate buffer (pH=7) and 240 mM H_2O_2. The process begins by adding 0.1 ml of enzymatic extract. Enzyme activity was expressed as the enzyme unit in protein gram (Ug^{-1} Protein).

For this purpose, 3 ml of 0.1 M sodium phosphate solution and 50 µl of pure guaiacol liquid ($C_7H_8O_2$) and then 50 µl of 3% hydrogen peroxide (H_2O_2) were added to the enzyme extract and changes in light absorption at 436 nm were immediately recorded using a spectrophotometer at 15-second intervals for 3 minutes. After adding oxygenated water and guaiacol mixture, the solution turned brownish red. To calculate the POD activity, the last absorption number is subtracted from the first read absorption number and divided by 3 (Mac-Adam et al., 1992).

2.6. Statistical Analysis

Statistical analysis of data obtained from this study and comparison of means was performed using SAS 9.1 and MSTAT-C software and graphs were made using Excel software. The results were tested by analyzing the variance of GLM and comparing the means using Duncan's multiple range test.

3. RESULTS

The results of ANOVA show that the storage periods as well as the treatments had a significant effect on pH, TA acid, SSC, fruit weight loss, carotenoids, ascorbic acid, fruit antioxidant capacity (P<0.01). The interaction between storage period and treatments had a significant effect on pH, TA, and ascorbic acid (P<0.01) but had no effect on other measured traits (Table 1).

Table 1. Analysis of variance of the effect of salicylic acid (SA), chitosan (Chi), mallow mucilage (PM), and *Aloe vera* gel (AG) treatments on the measured characteristics of *Citrus unshiu* marc. during cold storage at 7°C for 45 days.

S.O.V	DF	pH	TA	TSS	Fruit WL	Carotenoids	Ascorbic acid	Antioxidant A.
Period (P)	3	4.29^{**}	643.6^{**}	0.025^{**}	121.2^{**}	106.1^{**}	3279.5^{**}	7748.8^{**}
Treatment (T)	12	0.265^{**}	16.59^{**}	0.001^{**}	0.18^{**}	4.25^{**}	73.13^{**}	26.32^{**}
T × P	36	0.058^{**}	3.47^{**}	0.0003^{ns}	0.06^{ns}	1.04^{ns}	26.28^{**}	11.72^{ns}
Error	104	0.1	2.31	0.031	0.042	1.12	10.04	7.81
CV.	-	6.88	10.94	5.56	8.04	9.43	4.98	3.42

* and **: significant at <0.05 and <0.01. ns: non-significance.

3.1. pH and titratable acid

With increasing storage time, the pH of fruit juice was gradually increased and reached the highest level on day 45. Results showed a further increase in fruit juice pH in the control treatment increased as compared to other treatments and the treatments were able to maintain the fruit acidity during the storage period. SA (1 and 2 mM) and then chitosan 1% had the greatest inhibiting effect on the increase of fruit pH. At the end of storage period, SA treatment (1 and 2 mM) maintained the fruit pH at 4.75 and 4.64, respectively. SA treatment (2mM) maintained the acidity of *Citrus unshiu* marc. in cold storage and pH values of control increased by 19% as compared to fruits treated with 2mM SA (Table 2).

The trend of changes in TA content of fruits is completely opposite to the pH changes; in other words, with increasing storage time, the fruit TA content gradually decreased and reached the minimum level on day 45. TA level was lower in the control treatment than other treatments, which indicated more decomposition of organic acids during storage. With regard to this index, SA (2 mM) also had the greatest effect on the organic acid preservation, which was not significantly different from other treatments except control at the end of storage (Table 2).

3.2. Total Soluble Solids (TSS)

The total soluble solids (TSS) level, regardless of the type of treatments, was at its lowest level, which gradually increased over time (Table 2). At the baseline, the TSS level was in a limited range and there was no statistically significant difference between the treatments in this regard, but the effect of treatments on TSS gradually increased from day 15 and reached it maximum level at the end of storage period. The TSS content was higher than in the control treatment other treatments at the end of storage period (day 45), which is due to the decrease in fruit juice and thus an increase in sugar concentration. The highest TSS stability was observed in the 2mM SA treatment with an average of 16.69 °Brix, which was not statistically different from 1.5% chitosan, 30% mallow mucilage, 1 mM SA and 30% and 45% aloe vera gel treatments with the average TSS content of 17.3, 17.08, 17.63, 17.71, and 17.59 °Brix, respectively.

3.3. Fruit weight loss

The fruit weight loss percentage on the day 15 increased significantly compared to the baseline and reached an average of about 3%, and such increase continued with a slight slope until the day 45. The fruit weight loss percentage in the control treatment increased significantly over time, but this increase occurred slowly in other treatments and continued until the end of the storage period. There was no significant difference between the treatments on day 30, but there was a sudden and faster weight loss in the treated fruit on the day 45, which was significantly different from the 1% chitosan and 45% mallow mucilage treatments.

There was a slower upward trend in weight loss in fruits treated with 1% chitosan, 45% aloe vera gel, and 45% mallow mucilage on the day 30. These treatments prevented evaporation by creating physical coating and closing the pores of the skin surface on the day 45 and thus prevented further weight loss (Fig. 1).

Table 2. Effect of salicylic acid (SA), chitosan (Chi), mallow mucilage (PM) and Aloe vera (AG) treatments on some characteristics of *Citrus unshiu* marc during cold storage at 7 °C for 45 days. Similar letters show no statistically significant differences.

Storage Time	Treatments	pH		TA		TSS		Car.		As. Acid		Anti. C.		POD	
Start Day	control	4.48	h-o	0.31	d-n	10.16	l-s	9.00	q	72.75	a-e	93.22	ab	14.70	g
	AG 15%	4.31	j-q	0.34	a-g	8.87	o-s	9.31	q	73.92	a-d	93.87	ab	26.53	c-g
	AG 30%	4.05	o-r	0.34	a-e	9.19	n-s	10.06	j-q	70.40	a-h	94.87	ab	21.88	d-g
	AG 45%	4.09	n-r	0.35	a-d	9.18	n-s	9.50	n-q	76.27	a	93.93	ab	22.54	d-g
	PM 15%	4.31	j-q	0.34	a-f	9.47	m-s	8.75	q	70.99	a-g	94.09	ab	21.52	d-g
	PM 30%	4.15	m-q	0.35	a-d	8.73	p-s	9.78	k-q	75.09	ab	93.87	ab	28.79	b-g
	PM 45%	4.27	k-q	0.35	abc	9.09	o-s	9.70	l-q	69.81	b-i	94.51	ab	29.17	b-g
	SA 1mmol/l	4.04	o-r	0.36	abc	9.10	o-s	9.23	q	70.99	a-g	93.35	ab	28.02	c-g
	SA 1.5mmol/l	3.97	pqr	0.34	a-e	8.10	rs	8.77	q	75.09	ab	94.13	ab	21.77	d-g
	SA 2mmol/l	3.93	qr	0.36	a	8.60	p-s	9.74	k-q	69.81	b-i	93.51	ab	27.43	c-g
	Chi 0.5%	4.05	o-r	0.35	a-d	8.00	s	9.60	m-q	71.57	a-g	95.29	a	26.15	c-g
	Chi 1%	3.68	r	0.33	a-i	8.33	qrs	9.54	n-q	69.23	b-j	93.83	ab	22.32	d-g
	Chi 1.5%	4.18	m-q	0.34	a-f	9.01	o-s	9.45	opq	69.23	b-j	94.22	ab	24.30	d-g
15th Day	control	4.92	c-h	0.30	i-r	14.40	f-i	10.36	g-q	65.71	g-m	89.42	b	23.97	d-g
	AG 15%	4.35	j-q	0.32	c-m	13.83	g-k	10.28	h-q	68.64	c-k	91.40	ab	20.27	efg
	AG 30%	4.39	i-o	0.32	c-m	12.10	i-n	11.66	e-m	72.16	a-f	89.94	ab	32.42	a-g
	AG 45%	4.16	m-q	0.34	a-h	11.16	k-q	12.58	a-f	71.57	a-g	89.94	ab	37.85	a-e
	PM 15%	4.37	i-q	0.33	b-k	13.64	g-k	10.15	i-q	72.16	a-f	90.81	ab	34.55	a-g
	PM 30%	4.54	f-n	0.33	a-i	10.87	k-s	9.40	pq	73.33	a-e	91.59	ab	16.98	fg
	PM 45%	4.31	j-q	0.34	a-g	14.14	g-j	10.85	f-q	66.29	f-m	90.94	ab	35.91	a-f
	SA 1mmol/l	4.44	i-o	0.34	a-h	11.74	i-o	10.39	g-q	74.51	abc	91.01	ab	35.43	a-f
	SA 1.5mmol/l	4.37	i-q	0.33	b-j	11.38	j-p	10.30	h-q	69.81	b-i	91.17	ab	27.77	c-g
	SA 2mmol/l	4.17	m-q	0.35	abc	10.99	k-r	12.35	a-h	70.40	a-h	91.40	ab	17.90	efg
	Chi 0.5%	4.59	e-m	0.30	h-q	13.83	g-k	9.80	k-q	68.05	d-l	91.59	ab	33.36	a-g
	Chi 1%	4.30	i-q	0.33	a-i	12.74	h-l	9.46	opq	67.47	e-l	90.68	ab	34.23	a-g
	Chi 1.5%	4.42	i-p	0.31	f-p	11.43	j-p	8.80	q	71.57	a-g	90.26	ab	21.64	d-g
30th Day	control	4.95	b-g	0.27	pqr	19.63	ab	11.83	d-k	51.04	u-x	72.14	d	41.80	a-d
	AG 15%	4.49	h-o	0.30	h-q	17.98	abcd	12.13	b-j	63.95	i-n	73.18	d	25.34	c-g
	AG 30%	4.43	i-o	0.31	e-o	17.25	b-f	12.68	a-f	59.25	n-s	77.54	cd	33.77	a-g
	AG 45%	4.53	f-n	0.29	l-r	15.47	d-h	12.22	b-i	62.77	k-p	78.48	cd	26.21	c-g
	PM 15%	4.74	c-j	0.30	g-q	18.37	a-d	11.67	e-m	63.36	j-o	78.48	cd	34.87	a-g
	PM 30%	4.71	c-k	0.31	d-n	15.76	c-g	11.58	e-n	58.67	n-s	80.95	c	17.32	fg
	PM 45%	4.64	d-l	0.33	b-j	18.34	a-d	11.77	e-l	61.01	m-r	79.91	cd	22.98	d-g
	SA 1mmol/l	4.44	i-o	0.30	h-q	12.79	h-l	12.00	c-j	62.19	l-q	78.68	cd	34.16	a-g
	SA 1.5mmol/l	4.32	j-q	0.32	c-l	14.65	e-i	12.33	a-h	57.49	o-t	79.23	cd	33.93	a-g
	SA 2mmol/l	4.25	l-q	0.34	a-f	14.17	g-j	13.05	a-e	63.95	i-n	85.30	bc	28.91	b-g
	Chi 0.5%	4.52	g-n	0.31	f-p	18.56	abc	11.80	d-l	56.91	p-u	83.36	bc	49.61	a
	Chi 1%	4.49	h-o	0.29	k-r	15.65	c-h	11.44	e-p	57.49	o-t	78.81	cd	34.46	a-g

	Chi 1.5%	4.46	i-o	0.29	l-r	12.36	i-m	10.41	g-q	64.53	h-n	76.99	cd	34.90	a-g
45th Day	control	5.76	a	0.27	r	20.77	a	13.23	a-e	40.48	y	53.29	g	44.66	abc
	AG 15%	5.35	b	0.29	j-r	18.19	a-d	12.97	a-e	48.69	wx	62.87	ef	18.52	efg
	AG 30%	5.13	bc	0.27	qr	17.72	bcd	12.45	a-g	55.73	r-v	66.99	e	41.47	a-d
	AG 45%	4.97	b-f	0.27	qr	17.59	bcd	14.04	abc	55.73	r-v	60.76	f	21.75	d-g
	PM 15%	5.08	bcd	0.30	g-q	18.38	a-d	13.15	a-e	50.45	vwx	59.85	f	33.67	a-g
	PM 30%	4.81	c-i	0.28	o-r	17.09	b-f	13.91	a-d	55.73	r-v	64.10	ef	22.42	d-g
	PM 45%	5.03	b-e	0.29	m-r	17.93	a-d	14.16	ab	52.21	t-x	66.02	e	29.48	b-g
	SA 1mmol/l	4.98	b-e	0.30	i-r	17.64	bcd	12.98	a-e	54.56	s-w	64.56	ef	22.59	d-g
	SA 1.5mmol/l	4.75	c-j	0.29	l-r	17.83	a-d	13.42	a-e	51.63	t-x	66.18	e	30.50	a-g
	SA 2mmol/l	4.64	d-l	0.28	n-r	15.69	c-h	14.40	a	56.91	p-u	67.93	e	19.05	efg
	Chi 0.5%	5.01	b-e	0.28	n-r	19.51	ab	12.88	a-f	46.93	x	59.62	f	48.51	ab
	Chi 1%	4.98	b-e	0.28	n-r	18.93	ab	12.47	a-g	53.97	s-w	65.99	e	29.90	a-g
	Chi 1.5%	4.94	b-g	0.28	o-r	17.31	b-e	11.52	e-o	56.32	q-v	59.79	f	27.99	c-g

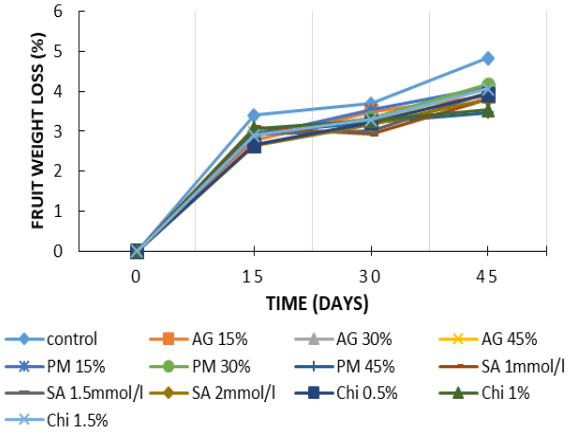

Fig. 1. Effect of salicylic acid (SA), chitosan (Chi), mallow mucilage (PM) and Aloe vera (AG) treatments on weight loss percentage of *Citrus unshiu* marc during cold storage at 7 °C for 45 days.

3.4. Fruit carotenoids

The carotenoids content, regardless of the effect of treatments, increased in fruit texture over time, which was often due to juice content. Overall, there was no specific pattern for changes in carotenoid content of treated fruits and this variable trend continued from day 0 to the end of storage period. There was low carotenoids content in the 1.5% chitosan treated group compared to other treatments that was a significant and common problem between the four measurement periods. Finally, the highest carotenoids content was observed in 2mM SA treatment at the end of storage period, which was not significantly different from all treatments except 1.5% chitosan treatment (Table 2).

3.5. Ascorbic acid

The ascorbic acid content gradually decreased over time and finally reached its minimum level at the end of the storage period. Overall, there was also no specific pattern in changes in the ascorbic acid content, similar to carotenoids, in the treated groups and this variable trend continued from day 0 to the end of storage period. There was no significant difference between control fruits and treated fruits in terms of ascorbic acid content in the first and second measurements, but, the ascorbic acid content in control fruits decreased from the day 30 to day 45 and reached its lowest level on the day 45 (Table 2).

The highest ascorbic acid content was observed in 2 mM SA-treated fruits with an average of 56.9 $mg.g^{-1}$ fresh weight at the end of storage period, which was significantly different from control treatment, 15% aloe vera gel, 15% mallow mucilage, and 0.5% chitosan with ascorbic acid content of 40.48, 48.69, 50.45, and 46.93 mg/g fresh weight, respectively (Table 2).

3.6. Antioxidant capacity

There are no significant differences between treatments in terms of their antioxidant capacity at the beginning of storage and on the day 15, but treatments were more effective over time, and finally the differences were more significant at the end of storage period. There was a decreasing trend in changes of the antioxidant capacity of *Citrus unshiu* marc. during the storage period. The antioxidant capacity was decreased by nearly half in some treatments at the end of storage period. Overall, there was a significant difference between all treatments with the control treatment on day 45 and reached its maximum level in 2mM SA treatment (67.93%) which was not significantly different from 30 and 45% aloe vera, 30% and 45% mallow mucilage, 1 and 1.5 mM SA, and 1.5% chitosan treatments (Table 2).

3.7. Activity of antioxidant enzymes

Storage period had a significant effect on total protein content and SOD ($P<0.05$) and on CAT and POD ($P<0.01$). The studied treatments had a significant effect on total protein and POD ($P<0.01$) but had no significant effect on SOD and CAT enzymes. The interaction of treatment and storage period was significant only on POD ($P<0.05$) (Table 3).

Table 3. Analysis of variance of the effect of salicylic acid (SA), chitosan (Chi), mallow mucilage (PM) and Aloe vera (AG) treatments on enzymatic factors of Citrus unshiu marc. during cold storage at 7°C for 45 days.

S.O.V	Mean squares			
	DF	SOD	CAT	POD
Period (p)	3	0.241^{*}	0.217^{**}	438.4^{**}
Treatment (T)	12	0.063^{ns}	0.044^{ns}	269.2^{**}
P × T	36	0.05^{ns}	0.027^{ns}	146.75^{*}
Error	104	0.068	0.035	98.21
CV.	-	13.19	15.15	14.44

* and **: significant at < 0.05 and <0.01. ns: non significance

3.8. Superoxide dismutase (SOD) and catalase (CAT) activity

The activity of SOD increased on the day 15 compared to the beginning of storage period, but it decreased from the day 15 onwards, and finally reached the lowest level on the last day of the storage period (Ug^{-1} Protein 1.88) (Fig. 2).

According to Figure 8, there was an increasing trend in changes of CAT activity from day 0 (start of storage) to the day 30 and reached the highest level (U g^{-1} Protein 0.455) during this period, but showed a slight decrease at the end of storage period. The lowest CAT level activity was observed at the beginning of storage period (0.276 U g^{-1} Protein).

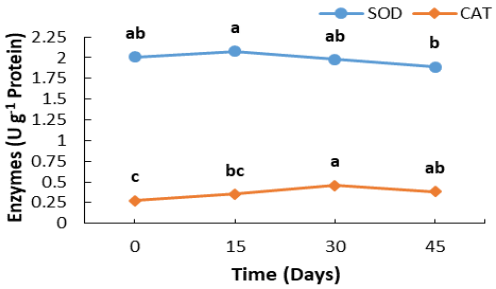

Fig. 2. Changes in activity of superoxide dismutase and catalase in *Citrus unshiu* marc. during cold storage at 7 °C for 45 days. Columns with similar letters show no statistically significant differences.

3.9. Peroxidase (POD) activity

There was an increasing trend in changes of POD activity during the measurement periods with prolonging storage period and reached its highest level on days 30 and 45. POD activity was measured from day 0 in the studied treatments and results showed no significant differences in the activity of this enzyme in any treatments, but the differences became apparent from the day 15 onwards, so that it was more noticeable on days 30 and 45. The highest POD activity was obtained on day 30 in the 0.5% chitosan treatment (49.6U g^{-1} Protein), which was statistically different all treatments, except with 15% and 45% aloe vera treatments, 30% and 45% mallow mucilage and 2mM SA treatments. The lowest POD activity was obtained in the 30% mallow treatment on the day 30, which indicates that the above treatment was effective in reducing oxidants (Table 2).

4. DISCUSSION

The trend of changes in TA content of fruits is completely opposite to the pH changes, that is, the TA content gradually decreased and reached its minimum level on day 45 with increasing the storage period. In this regard, it has been reported that changes in pH of fruit extract during fruit ripening are mostly due to leakage of organic acids from vacuoles into the cell cytoplasm. Also, the pH values of the fruit extract increases and changes from acidic to alkaline considering excessive fruit ripening (Pelayo et al., 2003). The increase in citric acid upon ripening or decrease in acidity may be due to their conversion to sugars and their greater use in fruit metabolism processes (Doreyappa and Huddar, 2001). Since organic acids are considered as the main substrate in the respiratory mechanism, the decrease in TA content during storage can be due to metabolic changes in the fruit or the consumption of organic acids during the respiration process (Echeverria and Valich, 1989). Typically, TA and total organic acid content decreases during fruit ripening. However, TA level remained unchanged in SA-treated kiwifruit compared to control fruits during storage period (Kazemi et al., 2011).

In the present study, the baseline TSS content, regardless of the treatment type, was at its lowest level, which gradually increased over time. The highest stability of TSS content was observed SA-treated group, which was not significantly different from the chitosan, mallow mucilage, and aloe vera gel treated groups. Over time, TSS levels have increased significantly. Cold storage has been reported to increase TSS levels (Tareen et al., 2012). The TSS level is an important factor in the quality of citrus fruits and its reduction reduces the product quality and marketability. Du et al., (1997) reported that the use of chitosan prevents fruit respiration. The reduction in TSS level is due to the reduction in the carbohydrates and pectin content, the partial hydrolysis of proteins, and the breakdown of glycosides into smaller units during respiration (Bal, 2018). Applying aloe vera coating and reducing the respiration intensity delays the increase of TSS content (Vargas et al., 2006). In this study, 30% and 45% aloe vera delayed the increase in TSS level of the tangerine. In this regard, studies reported delayed the increase in SST level in cherry and grape fruits coated with aloe vera gel (Marpudi et al., 2011).

In a study, Jannati et al. (2014) found higher TSS level in strawberries treated with thyme essential oil than control fruits. Abdolahi et al. (2010) studied the effect of different plant essential oils on the quality characteristics of grapes and stated that fruits treated with different plant essential oils had lower TSS levels than control fruits.

In this study, fruit weight loss gradually increased over time. The increasing trend in weight loss percentage in fruits treated with 1% chitosan, 45% aloe vera gel, and 45% mallow mucilage is slower from day 3 onwards. These treatments prevented evaporation physically by forming coating and closing the pores of the skin surface and also prevented further the weight loss. As fruit aging increases, respiration rate increases as well. The weight loss percentage is mainly related to transpiration and respiration, and water loss through transpiration depends on the difference in water vapor pressure between the fruit texture, around the fruit, and the storage temperature (Hernández-Muñoz et al., 2006). It is possible that SA immersion of mandarin washes the cuticle layer of the fruit thereby opening the pores and leads to water evaporation from the fruit surface. However, studies show that aloe vera can be effective in controlling weight loss of cherry fruit (Martinez-Romero et al., 2006) and pomegranate seeds (Nabigol and asghari, 2013). In combination with chitosan, this gel acts as a protective layer on the product and prevents the loss of fruit juices by increasing water holding capacity (Shah and Hashmi, 2020). Also, it has been reported that chitosan treatment forms a coating the blueberries surface and thus prevents water evaporation from the fruit surface and reduces the fruit weight loss percentage (Deng et al., 2016). Chitosan treatment reduces the weight loss percentage during storage period, which may be due to the physical inhibition of chitosan due to its constituent properties, this treatment thus protects the fruit from water loss during storage period by controlling biochemical changes in apple metabolism (Li et al., 2015). It has also been reported that the essential oils of medicinal plants act like a coating by forming a thin oily layer around the skin surface of the fruit, preventing water from evaporating from the skin, and maintaining the fruit skin moisture (Ghafouri et al., 2016).

In this study, the carotenoids content in the fruit texture increased over time, regardless of the effect of treatments, which was often due to water loss. Also, the highest carotenoids content was observed in 2mM SA treatment at the end of storage. Accordingly, SA treatment has been reported to induce antioxidant activity and has increased resistance to living and non-living stresses in various plants (Huang et al., 2008). Application of SA on the shoots of pepper plant induces the carotenoid accumulation in fruits (Elwan and El-Hamahmy, 2009). Pre-harvest treatment of SA has been reported to slow down the degradation of lycopene and β-carotene in Cara Cara Thompson Novel orange during storage (Huang et al., 2008), which is consistent with the results of the present study. Degradation of carotenoids in control fruits compared to chitosan-treated fruits in the present study may be due to higher respiration at the end of storage. It has also been reported that chitosan increases the intrinsic concentration of CO2 in papaya fruits (Ali et al., 2013) and increasing CO2 levels reduces ethylene synthesis and delays fruit aging, thereby reducing the degradation of chlorophyll and carotenoids (Martínez et al., 2006). In addition, the use of oral coatings along with plant essential oils on the crop surface can delay chlorophyll degradation during storage and thus reduce fruit discoloration due to chlorophyll degradation and stimulation of the synthesis of other pigments such as carotenoids and lycopene (Vishwasrao and Ananthanarayan, 2016).

In the present study, the ascorbic acid content, regardless of the effect of the treatments, gradually decreased over time and finally reached its lowest level at the end of the storage period. Also, the highest ascorbic acid content was observed in 2mmol SA-treated fruits at the end of storage period, which was not significantly different from the control treatment, 15% aloe vera gel, 15% mallow mucilage, and 0.5% chitosan. SA-treated fruits have been reported to show high ascorbate and dehydroascorbate contents (the oxidized form of ascorbic acid) (Huang et al., 2008). Vitamin C level also decreased during the storage period of guavas, but this decrease was lower in chitosan treatments (Hong et al., 2012). Chitosan-treated pepper fruits and plant essential oils have been reported to have the highest vitamin C content among treatments and controls at the end of storage period. Since a decrease in

ascorbic acid content can be strongly related to O_2 content, the chitosan can reduce O_2 emissions, thereby slowing fruit ripening and thus better preserving vitamin C content and delaying the sweet pepper aging (Abbasi et al., 2009).

The activity of SOD enzyme increased on the day 15 compared to the beginning of storage, but it decreased from the day 15 onwards and finally reached the lowest level on the last day of the storage period (U g^{-1} Protein 1.88). There was an increasing trend in changes in catalase activity from day 0 (beginning of storage) to the day 30 and reached the highest level (U g^{-1} Protein 0.455) during this period; however, there was a slight decrease at the end of storage period. The highest POD activity was obtained on day 30 in the 0.5% chitosan-treated group with an average of 49.6 U g^{-1} Protein, which is not significantly different from all treatments, except with 15 and 45% aloe vera treatments, 30% and 45% mallow mucilage and 2 mM SA. Accordingly, SA treatment has been reported to affect antioxidant enzymes such as SOD, POD, and CAT (Srivastava and Dwivedi, 2000). According to researchs, SA protects the plant from oxidative damage by increasing its antioxidant capacity by acting on H_2O_2. It also reduced frost-related damage by inducing antioxidant systems and stimulating the production of heat shock proteins (Wang et al., 2006). Postharvest application of SA in citrus fruits increases the activity of antioxidant enzymes such as dehydroascorbate reductase, glutathione reductase, SOD as well as glutathione and ascorbate, thereby delaying lipid peroxidation. Studies have shown that aloe vera has antimicrobial, antioxidant, antiviral and anti-inflammatory properties (Pal et al., 2013; Asghari et al., 2013). According to the results of this study, aloe vera was able to maintain its antioxidant activity until the forty-fifth day of the storage period. They found that aloe vera gel treatment prevents the reduction of antioxidant activity in grapes during the storage period (Reynolds and Dweck, 1999).

5. CONCLUSION

The results of analysis of experimental data in the present study showed that the best treatment to increase the storage life of *Citrus unshiu* marc. during the 45-day storage was 2 mM SA treatment, which was more effective than other treatments. Application of 2 mM SA preserved the predominant organic acids of the fruit and prevented the increase of pH of *Citrus unshiu* marc. fruits. It also preserved TSS in fruits and prevented the breakdown of carotenoids and ascorbic acid and maintained antioxidant activity. In addition to 2mM SA, 30% and 45% aloe vera gel, 45% mallow mucilage, and 1.5% chitosan also preserves organic acids and TSS and prevents the fruit pH. The upward trend in weight loss in fruits treated with 1% chitosan, 45% aloe vera gel and mallow mucilage was slower than control fruits. 45% Mallow mucilage along with 2 mM SA preserved the fruit carotenoids and along with 1.5% chitosan, 30% and 45% aloe vera gel and 2 mM salicylic acid, in addition to preventing the breakdown of ascorbic acid and fruit carotenoids, maintain the total antioxidant capacity of fruits during storage for 45 day.

The activity of antioxidant enzymes was at optimal level until the day 30, which indicates that there was no stress in fruits during this period. However, prolonged storage (more than 30 days) has resulted in a further production of ROS and led to damage to fruit texture. Also, 15% aloe vera treatment had the highest effect on the incidence of postharvest stress in fruits.

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