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Caffeic acid as a preservative that extends shelf-life and maintains fruit quality of mulberries during cold storage

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Fruits can be easily infected and damaged by microbes. Cold storage is a popular approach used to extend the shelf-life of fruits. In this paper, the effect of caffeic acid on physiological parameters and shelf-life of mulberries (*Morus alba* L.) stored for 21 days at 4°C was evaluated. The results showed that the shelf-life was significantly improved in the mulberries treated with the different concentrations of caffeic acid solution for 5 min ($P < 0.05$). Certain physiological parameters, like phenolics, anthocyanins, flavonoids and Vitamin C were also significantly increased ($P < 0.05$) in the treated mulberries. The results showed that the rotting rate and the weight loss ratio were 47.0 and 6.6% in the 0.20 g/L caffeic acid-treated fruits after storing for 21 days at 4°C, respectively. While these two parameters were 79.0 and 9.7% in the control. The malondialdehyde (MDA) content was significantly lower ($P < 0.05$) in the 0.20 g/L caffeic acid-treated mulberries than that in the samples treated with 0.00, 0.10, 0.25 and 0.30 g/L caffeic acid. Moreover, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities in the caffeic acid treated mulberries were significantly higher than those in the control ($P < 0.05$). Therefore, caffeic acid, as a preservative, is favorable for elongation of the shelf-life, maintenance of the quality and inhibition of fruit decay in mulberries. This study is greatly informative to mulberry growers and commercial sellers.

Key words: Caffeic acid, mulberry fruits, cold storage, postharvest quality.

INTRODUCTION

Mulberries (*Morus alba* L.) are sweet and juicy fruits. The berries contain rich nutrients of sugars, proteins, vitamins and minerals, and abundant antioxidants of anthocyanins, flavonoids, and phenolic acids (Heinonen et al., 1998).

Cold storage can slow down the respiration of fruit and inhibit the reproduction of microorganism on fruits and vegetables (Saltveit and Morris, 1990). A successful cooling storage needs to ensure that the quality of the commodities is maintained desirable until they reach consumers (Formerhead, 2005). Low temperature effectively reduces enzyme activity and inhibits growth of microorganisms (Leccese et al., 2010). During the postharvest periods of mulberries, prompt cooling and favorable temperature (e.g., -1 to 4°C) are very important

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factors to restrain the undesirable quality changes (Saltveit and Morris, 1990). However, the flavor of the fruits stored by cold has greatly been changed thus quality in the stored fruits is not comparable with that in the fresh fruits (Galli et al., 2008). Nonetheless, cold is widely recognized as a healthy, safe and effective technology for fruit storage (Yang et al., 2016).

Various methods have been developed for preservation of mulberry fruits. Chen et al. (2015) demonstrated that the shelf-life in the 60 mg/L chlorine dioxide treated samples was extended to 14 days while it was 8 days in the control. However chlorine dioxide produces irritating odors and has unfavorable impact on the flavor of fruits so that it is not suitable for practical application (Huber et al., 2005). Oz and Ulukanli (2013) found that 1-MCP alone or plus $CaCl_2$ treatment reduced the browning rate and maintained the fruit color. Hu et al. (2014) observed that H_2S fumigation was able to slightly decrease soluble protein, acidity and ascorbate content. They also demonstrated that the activities of representative antioxidant enzymes in H_2S-treated samples were higher than those in the control samples during storage. However, hydrogen sulphide produces a strong odor of rotten egg that may negatively affect the flavor of mulberry fruits, limiting use of this reagent for storage in practice. Because these limitations existed in the previous studies, we screened some natural compounds that can be desirably used for storage of mulberries.

Caffeic acid (CA) is widely distributed in nature and possesses strong antioxidant activity (Wang et al., 2014). This natural compound shows a variety of potential pharmacological effects *in vitro* and in the model animals *in vivo* (Wang et al., 2009). Caffeic acid also shows immunomodulatory and anti-inflammatory activity. Ojeda-Contreras et al. (2008) found that caffeic acid phenethyl ester (CAPE) can prevent fungi from tomato fruits. Treated with CAPE, tomato fruits can be stored for 20 days at 25°C. Carreno et al. (2017) evaluated the cellular antioxidant activity (CAA) of CAPE, and found that CAPE has a cellular protective effect against reactive oxygen species (ROS). This study is aimed at evaluating the effects of different concentrations of caffeic acid on maintenance of postharvest fruit quality and to extend the shelf life of mulberry fruit through comprehensive analysis of several physiological parameters in the treated fruits with those in the control. This study is the first time to comprehensively analyze caffeic acid potentially used for preservation of mulberries during storage.

MATERIALS AND METHODS

Mulberry fruits of Dashi (*M. alba* Lin.) were collected at 90% commercial maturity in Zhenjiang, Jiangsu Province (Yang et al., 2016). The fruits with uniform size, color and absence of visual damages were chosen for the experiments. Mulberries were randomized into six groups with 150 fruits each. Six treatments with graded concentrations of caffeic acid were arranged in this study, including 0.00 (as the control), 0.10, 0.15, 0.20, 0.25, and 0.30 g/L of caffeic acid, respectively. The fruits were dipped into the solutions for 5 min. The treated samples were drained by a plastic sieve for 1 h and a low rotation speed fan was used for a fast drying. Then the treated samples were wrapped with a 105×105×40 mm plastic box and stored at 4°C. The fruits were stored for eight time points at 0, 3, 6, 9, 12, 15, 18 and 21 days, respectively, and were collected at each time point for an immediate analysis of rotting rate and weight loss. Then the samples were frozen in liquid nitrogen and stored at -80 °C for further analysis of other physiological parameters.

Weight loss and rotting rate

The weight loss rate was measured by weighting the pre- and post-storage fruits, and expressed as a percentage of the initial weight.

The rotting rate was categorized into five groupings, where 1 = unaffected, 2 = trace (up to 5% surface affected), 3 = slight (5-20% surface affected), 4 = moderate (20-50% surface affected), and 5 = severe (> 50% surface affected) (Ayala-Zavala et al., 2004). Rotting rate (%) was calculated with an equation:

$$\text{Rotting rate (\%)}=\sum\frac{\text{rot scale}\times\text{number of fruit at the rot scale}}{\text{the highest rot scale}\times\text{total number of fruit in the treatment}}\times 100;$$

These measurements were performed in triplicates.

Evaluation of LD_{90}

Previous studies used the rotting rate as the only index for evaluation of storage ability. However, only this index alone cannot well reflect the actual value of the storage ability in fruits. In the study of insect toxicology and pathology, the LC_{50} or LD_{50} values for pesticides or pathogenic microorganisms are often needed to be calculated for evaluation of pesticide efficacy or microbe susceptibility (Walker et al., 2000). Similarly, we borrowed this strategy in this study for analysis of evaluation of caffeic acid to improve the mulberry storage ability. LD_{90} represents the storage days when percentage of the intact fruits reaches 90%. Probit software rooted in the SPSS19.0 for Windows module (probability unit regression) was used for estimation of LD_{90}.

Physiological parameters

The total anthocyanin content of the fruit extracts was determined by using a previously described method (Proctor, 1974), with a slight modification. To isolate the anthocyanin, 1.5 g of mulberry fruit was added to 36 ml of 77% (v/v) ethanol containing HCl (1%, v/v), and then ultrasonically homogenized for 2 h at 50°C and 400 W. The extracting solution was diluted with KCl-HCl buffer (pH 1.0) and HAC-NaAC buffer (pH 4.5) and mixed well. Then, the extracts were maintained at room temperature for 30 min. Absorption was measured at 512 and 700 nm in buffers at pH 1.0 and 4.5. Anthocyanin content of the mulberry fruits was calculated with an equation:

$$\text{Anthocyanin content (mg/g FW)} = \frac{[(A_{512} - A_{700})_{pH1.0} - (A_{512} - A_{700})_{pH4.5}] \times V \times n \times M}{\varepsilon \times m}$$

where V (mL) is the total volume of extracting solution, n is the dilution ratio of the extracting solution, M (g/mol) is the molecular mass of cyanidin-3-*O*-glucoside, ε is the extinction coefficient of cyanidin-3-*O*-glucoside, and m (g) is the mass of the sample. These measurements were performed in triplicates.

Vitamin C content was determined using the protocol of Malik and Zora (2005), with slight modifications. The content of Vitamin C content was calculated on a 100% (W) ascorbic acid standard curve and expressed as mg of ascorbic acid (AA) per 100 g of the fresh weight (FW).

The total flavonoid content of mulberry fruits was determined by the spectrophotometric method described by Lu et al. (2012), with a slight modification. Total flavone was extracted from 1.5 g mulberry fruits using 20 mL of 95% (v/v) ethanol, and the mixture was sonicated for 2.5 h. The liquid extract (1 mL) or a standard solution of rutin was transferred to a 10 mL volumetric flask with 0.3 mL of 5% (w/v) $NaNO_2$. After standing at room temperature for 6 min, 0.3 mL of 10% (w/v) $Al(NO_3)_3$ was added to the solution, and the mixture was homogeneously mixed and allowed to stand for 6 min. Finally, 4 mL of 4% (w/v) NaOH was added. The 60% (v/v) ethanol was added into the solution till a final volume of 10 mL then stood for 12 min. The absorbance of the samples was measured at 510 nm. The total flavonoid content was calculated as mg/g FW. All samples were analyzed in triplicates.

The polyphenol content of the mulberry fruits was measured using Folin-Ciocalteu reagent (Slinkard and Singleton, 1977). Polyphenols were extracted from 1 g mulberry fruit using 70 mL of 60% (v/v) ethanol then an additional ultrasonic extraction for 3 min. The filtered residue was extracted again, and the two extracts were mixed to detect the polyphenol content of mulberry fruits. The polyphenol content was expressed as milligrams of gallic acid equivalent (GAE) per kilogram. Two millilitres of Folin-Ciocalteu's phenol reagent was added into 5 mL of the extracting solution, mixed well and then 15% (w/v) Na_2CO_3 was added into the mixture till the final volume of 25 mL. The mixture solution was allowed to react at 45°C for 40 min and then cooled to room temperature. Subsequently, the absorbance of the samples was detected at 765 nm. Three replicates were used to determine the results of each assay.

The standard curves of vitamin C, polyphenols and total flavone were Y = 59044300 X - 19356.5, R^2 = 0.9987; A = 71.92 C - 0.053, R^2 = 0.9987; A = 5.449 C + 0.00562, R^2 = 0.9918, respectively, where Y is the peak area, X and C are concentrations (mg/mL), and A is the absorbance (Malik and Zora, 2005).

MDA content

Malondialdehyde (MDA) content in the fruits was determined using the method of Dhindsa et al. (1981) with slight modification. Fresh tissue (0.2 g) from mulberry was homogenised with 1 mL of 10% (m/V) trichloroacetic (TCA). The reaction mixture was spun at 12,000 ×g for 10 min. The supernatant (0.5 mL) was mixed with 0.5 mL of 0.6% (W/V) thiobarbituric acid (TBA), incubated at 100°C for 20 min and quickly cooled down. After centrifugation at 3000 ×g for 10 min, the absorbance of the supernatant was detected at 532, 450 and 600 nm using I3 Spectramax (USA). The MDA content of the mulberry fruits was calculated with equation:

$$\text{MDA content } (\mu \text{ mol/g FW}) = \frac{[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] \times V}{m}$$

where V (L) is the total volume of the extracts, the total volume of the reaction mixture solution and the volume of the extracted solution in the reaction mixture solution, and m (g) is the mass of the sample.

DPPH activities

The antioxidant activity of the mulberry fruits was evaluated using the method of Cheung et al. (2003), with a slight modification. To determine free radical scavenging activity, samples were extracted with methanol. One hundred and ninety microlitres of 0.6 mM DPPH radical were dissolved in methanol. Ten microlitres of mulberry fruit extract or (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid standard solution were added to 0.6 mM DPPH radical in methanol, mixed well and kept at room temperature for 1 h in the dark. The absorbance was measured spectrophotometrically at 517 nm. The percent in reduction in DPPH was expressed as mg Ve per g fresh weight of mulberry fruits.

Statistical analysis

The experimental values of three replicates were expressed as the means ± standard deviation. To estimate statistically any significant differences among the mean values, the data were analysed with a one-way analysis of variance (ANOVA test). Statistical comparisons of the data were based on the Pearson correlation coefficient, and levels lower than 0.05 were considered significant. *R* programming language was used to determine the significance of the differences between the samples.

RESULTS

Effects of caffeic acid (CA) treatments on physiological parameters of mulberry fruits during storage at 4 °C for 21 days

As shown in Table 1, the levels of anthocyanin in mulberries showed an increased trend during storage within 21 days. On the 21st days of the storage, the mulberries treated with 0.20 g/L caffeic acid had the highest level of anthocyanin compared with the remaining treatments. In most cases, the treatments with 0.20 and 0.30 g/L caffeic acid showed the best abilities to maintain anthocyanin in the stored mulberries compared with the remaining treatments (Table 1).

The polyphenol and flavonoid contents of mulberry fruits varied with storage days and different concentrations of caffeic acid (Table 1). On the 21st day of the storage, the fruits treated with 0.15 and 0.20 g/L caffeic acid had the highest level of polyphenol and flavonoid contents as compared with the remaining

Table 1. Effects of caffeic acid (CA) treatments on physiological parameters of mulberry fruits during storage at 4°C for 21 days.

Storage days (day)	CA concentration (%)	Anthocyanin (mg/g FW**)	Vitamin C content (mg/100 g FW)	Polyphenols (mg/g FW)	Total flavone (mg/g FW)
0	-	1.08±0.03	168.19±0.83	10.09±0.42	2.127±0.678
21	Control	1.29±0.03^{b}	166.44±1.27^{c}	11.85±0.64^{d}	2.13±0.15^{d}
21	0.10 g/L	1.21±0.03^{b}	179.95±1.05^{c}	10.28±1.27^{e}	1.92±0.26^{e}
21	0.15 g/L	1.13±0.03^{c}	248.17±2.98^{a}	14.09±2.16^{a}	4.17±0.49^{a}
21	0.20 g/L	1.82±0.03^{a}	258.61±1.78^{a}	13.48±0.49^{b}	3.02±0.16^{b}
21	0.25 g/L	0.99±0.03^{c}	175.80±1.65^{c}	11.55±0.41^{d}	2.14±0.24^{d}
21	0.30 g/L	1.34±0.03^{b}	197.29±0.60^{b}	12.96±0.37^{c}	2.58±0.25^{c}

[1)]The data are represented as the mean ± SD of three replicate samples. Means in same column with different letters are significantly different ($P < 0.05$) determined with Duncan's multiple range test. [2)] FW indicates fresh weight.

Table 2. Effects of caffeic acid on LD_{90} of the mulberry fruits stored at 4 °C for 21 days.

Treatment	LD_{90} (days)*
Control	6.4
0.10 g/L CA	9.4
0.15 g/L CA	10.9
0.20 g/L CA	11.8
0.25 g/L CA	8.9
0.30 g/L CA	11.8

[1)]LD_{90} represents the storage time until the rate of the good fruits reaching at 90%.

treatments. Table 1 also showed changes of the total vitamin C content in the mulberries within 21 days of the storage of all treatments. The initial vitamin C content of mulberries was 168.19 mg/100 g. On the 21st day of the storage, vitamin C content in the mulberries treated with 0.20 g/L caffeic acid was the highest (258.61 mg/100 g) among the treatments, and extensively higher than that in the control (166.44 mg/100 g). Therefore, the 0.20 g/L caffeic acid showed the best ability to inhibit vitamin C from degradation in the stored mulberries (Table 1).

Effects of caffeic acid on LD_{90} of the mulberry fruits stored at 4°C for 21 days

Mulberry is a soft fruit and easily to decay by mechanics and microbe factors (Yang et al., 2016). The mulberry fruits treated with 0.20 g/L caffeic acid had a significantly ($P < 0.05$) lower weight loss compared with the control (Figure 1). After storage for 15 days, the mulberry fruits treated with 0.20 g/L caffeic acid had a significantly ($P < 0.05$) lower rotting rate compared with the control (Figure 2). On the 21st day, 0.30 g/L caffeic acid showed the lowest rotting rate, followed by 0.20, 0.25, 0.15, 0.10 and 0.00 g/L treatments. The LD_{90} values of the 0.20 g/L and 0.30 g/L caffeic acid treatments were 11.8 and 11.7 days, respectively. However, LD_{90} in the control was 6.8 days (Table 2). Therefore, the 0.20 and 0.30 g/L caffeic acid showed the best potential to impede the weight loss and fruit decay compared with the control after the storage of 15 days.

Effects of caffeic acid on MDA content of the mulberry fruits stored at 4°C for 21 days

MDA is the product of membrane lipid peroxidation, which can reflect the degree of cell ageing (Liu et al., 2011). All treatments showed an increasing trend of MDA content in the mulberries with extension of the storage days (Figure 3). On the 21st day, the MDA content of control rapidly increased to 13.88×10^{-5} μ mol/g, and MDA in the 0.20 g/L caffeic acid-treated mulberry fruits was the lowest (10.53×10^{-5} μ mol/g, $P < 0.05$) compared with other treatments (Figure 3).

Effects of caffeic acid on DPPH content of the mulberry fruits stored at 4°C for 21 days

As shown in Figure 4, the DPPH radical scavenging activities of mulberry fruits had an increase-decline-increase-decline trend during storage within 21 days. On the 21st days of the storage, the mulberries treated with 0.20 g/L caffeic acid had the highest level of DPPH

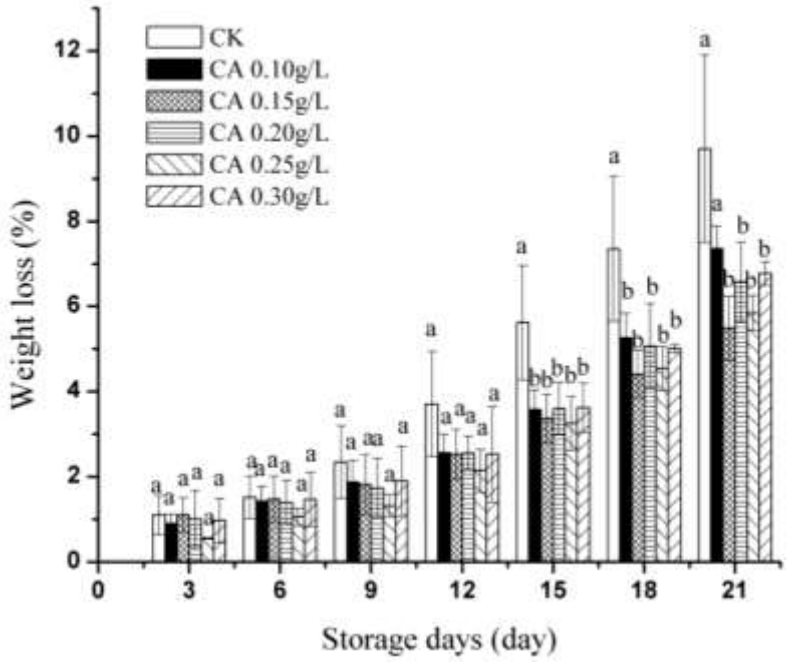

Figure 1. Changes in weight loss of mulberry fruits treated with 0.00, 0.10, 0.15, 0.20, 0.25 and 0.30 g/L caffeic acid when stored at 4°C for 21 days. Data is presented as 'mean ± SD' of three replicates. Difference small letters (a or b) above the bars show significant differences at $P < 0.05$.

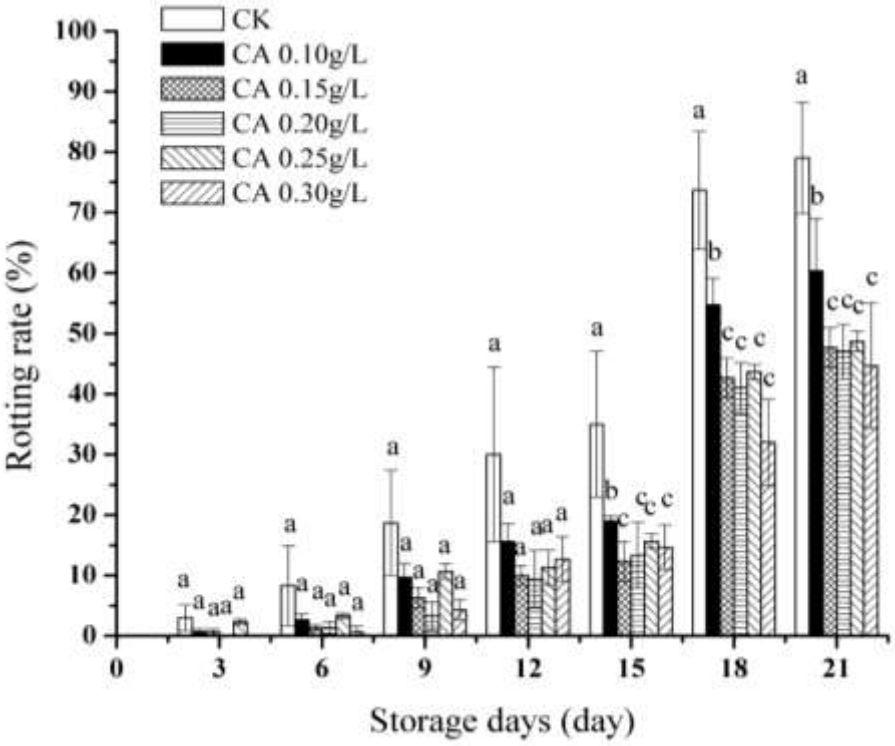

Figure 2. Rotting rate of mulberry fruits treated with 0.00, 0.10, 0.15, 0.20, 0.25 and 0.30 g/L caffeic acid when stored at 4°C for 21 d. Data is presented as 'mean ± SD' of three replicates. Difference small letters (a or b) above the bars show significant differences at $P < 0.05$.

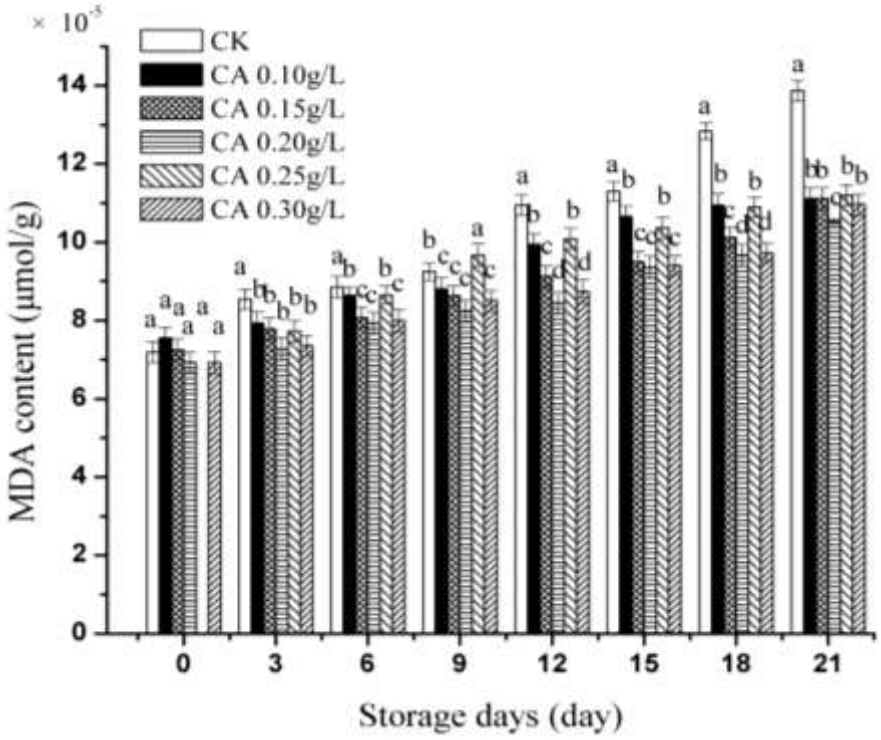

Figure 3. Changes of MDA content in the mulberry fruits treated with 0.00, 0.10, 0.25 and 0.30 g/L caffeic acid when stored at 4°C for 21 days. Data is presented as 'mean ± SD' of three replicates. Difference small letters (a or b) above the bars show significant differences at $P < 0.05$.

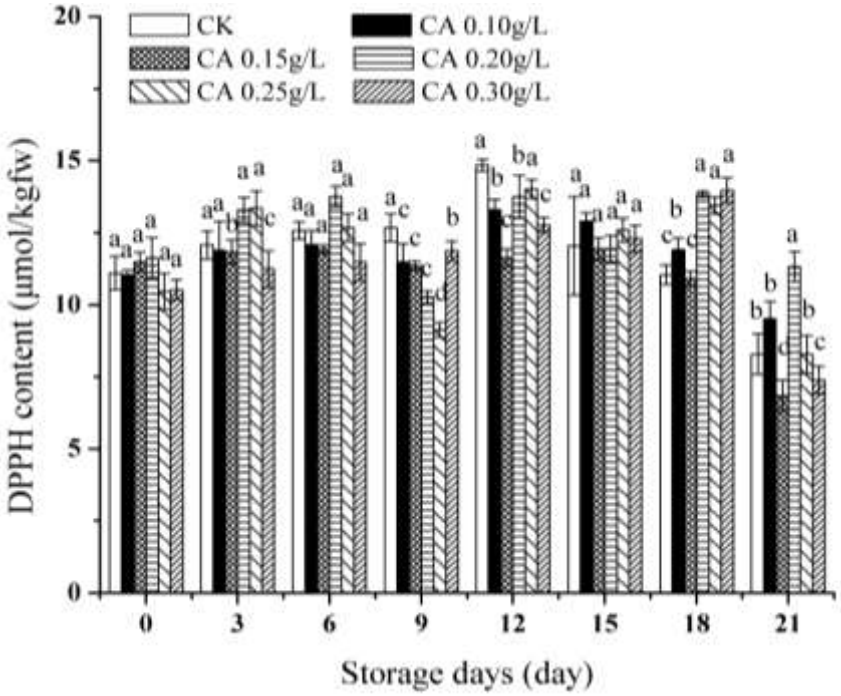

Figure 4. DPPH scavenging activities of mulberry fruits treated with 0.00, 0.10, 0.15, 0.20, 0.25 and 0.30 g/L caffeic acid when stored at 4°C for 21 days. Data is presented as 'mean ± SD' of three replicates. Difference small letters (a or b) above the bars show significant differences at P<0.05

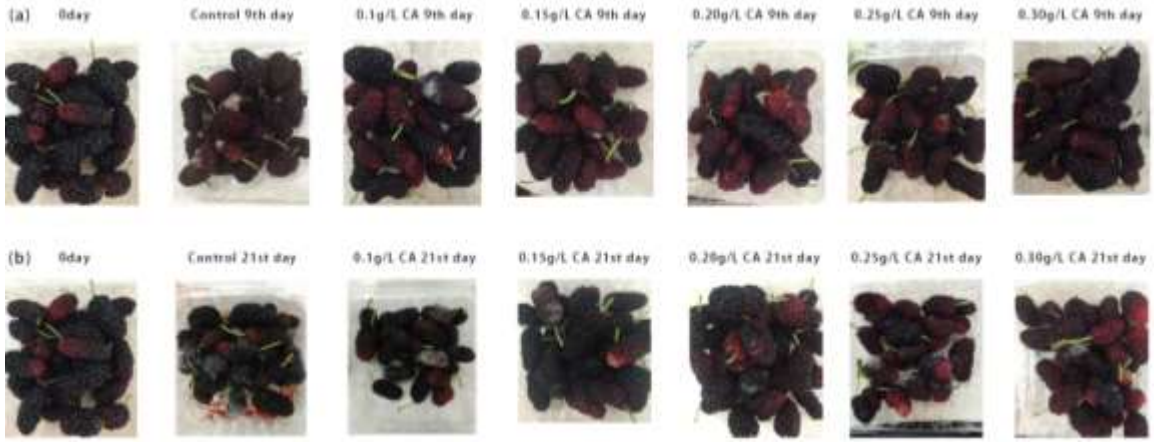

Figure 5. Appearance quality of the mulberry fruits treated with 0.00, 0.10, 0.15, 0.20, 0.25 and 0.30 g/L caffeic acid when stored at 4°C on day 9 (a) and day 21 (b).

content as compared with the remaining treatments. The control and caffeic acid-treated mulberry fruits exhibited similar changes in the DPPH radical scavenging activity during storage (Figure 4).

Effects of caffeic acid on overall appearance quality of the mulberry fruits stored at 4°C for 21 days

The overall appearance quality in Figure 5b shows that the control fruits were almost entirely decayed and infected by pathogens. But half of the mulberry fruits treated by 0.15g/L caffeic acid were decayed. However, the 0.20, 0.25 and 0.30 g/L caffeic acid treatments had the best preservation efficacy without obvious pathogens on the surfaces of the mulberry fruits. These observations were in accordance with the previously measured parameters in Figures 1 and 2.

DISCUSSION

Many fruits and vegetable containing natural anthocyanins demonstrate positive efficacy on human healthcare. Our study indicates that total anthocyanin in the stored mulberries was extensively affected by caffeic acid treatment and the cold storage period. The accumulation of pigment and anthocyanin content in mulberries were increased with extension of the storage time (Chen et al., 2015). An enhanced anthocyanin content during storage was previously reported for raspberries, strawberries, low bush blueberries and high bush blueberries (Kalt et al., 1999). This phenomenon may be due to the continued biosynthesis of phenolic compounds after harvest, and it is related to the ripening processes (Wang and Gao, 2013).

Polyphenol and flavonoid contents in fruits are affected by numerous factors and varied among species, cultivars, temperature, climactic and environmental conditions during the growth period (Kalt, 2005). The polyphenol and flavonoid contents of the stored mulberries had an increasing trend along with storage time (Table 1). This observation is in agreement with the report by Chen et al. (2015). This phenomenon was also found in the stored raspberries, strawberries, low bush blueberries and high bush blueberries without coatings (Kalt et al., 1999).

Vitamin C in the fruits is an unstable compound and easily oxidized during postharvest (Hassanpour, 2014). Therefore, it is reasonable that Vitamin C in both the treated and untreated samples was decreased during the storage (Table 1). However, our study also shows that caffeic acid effectively inhibited decomposition of Vitamin C in the stored mulberries (Table 1).

CAPE is a propolis constituent that has gained attention due to its broad pharmacological activities (Zhang et al., 2014), including antibacterial, antiproliferative, antiparasitic and antioxidant effect, among others (Wang et al., 2014; Alday-Provencio et al., 2015). CAPE is more biologically effective than other natural hydroxycinnamic acid derivatives because of its structural properties, possessing better bioavailability in lipophilic systems due to its partition coefficient (Zhang et al., 2014). In this paper, we fund that caffeic acid can decrease the rotting rate; it may be that caffeic acid is able to protect the mulberries from pathogen infection. Data of three parameters, weight loss, rotting rate and LD90 (Figures 1 and 2 and Table 2) suggest that caffeic acid may impede the aging process and maintain quality of the stored mulberries, which further indicates that caffeic acid may decrease transpiration and respiration processes in fruits (Zhu et al., 2008). The results indicated that caffeic acid had an obvious effect on decrease of the weight loss in mulberry fruits. This is probably due to the fact that caffeic acid has the capacity against water evaporation in the mulberry fruits (Wang et al., 2014). A lower rotting rate in the caffeic acid treated

mulberry fruits may be attributed to the antibacterial ability of caffeic acid (Wang et al., 2009). The rotting rate was often used for evaluation of the preservation consequence in horticultural crops (Yang et al., 2016). However, only having this index alone may not reflect the overall economic values of mulberries. For this reason, LD90 was used in this study for evaluation of caffeic acid capacity on maintenance of the storage fruit quality in mulberries. Although several parameters, e.g., anthocyanins, polyphenol content and flavonoid content were increased with the storage time within 20 days (Table 1), LD90 suggested the optimum time for storage of the mulberry fruits treated with caffeic acid was for 11 days or shorter (Table 2).

MDA is often used as an index representing cell oxidative damage suffering from lipid peroxidation (Xu et al., 2009). MDA was suppressed by salicylic acid in 'Qingnai' plum fruits (Luo et al., 2011) and cucumbers (Hu et al., 2009). However, few previous studies have been conducted concerning evaluation of caffeic acid on suppression of MDA during the cold storage. The DPPH radical scavenging activities are associated with the contents of phenols and anthocyanins (Chen et al., 2014). The DPPH radical scavenging activities fluctuated during storage. Our study showed that DPPH radical-scavenging was improved by caffeic acid treatments, in agreement with the previous report by Concellónab et al. (2012).

From all these evidences, caffeic acid is suggested to use as a preservative for the cold storage of the mulberry fruits.

Conclusions

The cold stored mulberry fruits pre-treated with caffeic acid had higher anthocyanin, phenolic compounds, flavonoid and Vitamin C contents and lower rotting rate, weight loss and MDA than the control fruits. The results reveal that the caffeic acid treatments have a positive effect on preservation of the mulberry fruits. This natural compound is suggested for use as a preservative to extend the shelf-life and maintain the fruit quality for the cold storage of the mulberry fruits. Further investigation is also needed to elucidate the underlying molecular basis of the caffeic acid with the capacity to improve the preservation quality of the stored mulberry fruits.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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