# Coating Effects of Orange and Pomegranate Peel Extracts Combined with Chitosan Nanoparticles on the Quality of Refrigerated Silver Carp Fillets

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# COATING EFFECTS OF ORANGE AND POMEGRANATE PEEL EXTRACTS COMBINED WITH CHITOSAN NANOPARTICLES ON THE QUALITY OF REFRIGERATED SILVER CARP FILLETS

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## ABSTRACT

The coating effects of orange and pomegranate peel extract combined with chitosan nanoparticles on the quality of silver carp (*Hypophthalmicthys molitrix*) fillets during refrigerated storage at 4C were evaluated. Solutions of orange and pomegranate peel extracts (1%, w/v) was used for dip pretreatment, and nanochitosan solution (2%, w/v) was used for the coating. The control and the coated fish samples were analyzed periodically for microbiologic (total mesophilic and psychrotrophic count), physicochemical (pH, total volatile basic nitrogen, thiobarbituric acid reactive substances) and sensory attributes. The results indicated that nanochitosan coating was effective for the preservation of silver carp fillets during refrigerated storage. However, a dip pretreatment in orange or pomegranate peel extract combined with nanochitosan coating were significantly inhibited the development of lipid oxidation in fish samples. In this context, pomegranate peel extract was significantly stronger than orange peel extract.

## PRACTICAL APPLICATIONS

The combination of orange or pomegranate peel extract and chitosan nanoparticles can be utilized as a safe preservative for fish under refrigerated storage.

## INTRODUCTION

Fish is perceived as an important part of a healthy diet among nutrition and food scientists as well as consumers (Brunsø *et al.* 2008). In the last decades, the consumption of this food group increased and became available to consumers far away from the coastal areas. However, fresh fish is a highly perishable product because of its biologic composition. Thus, the development of preservation methods in order to extend the shelf life of fresh fish products and carry them safely to the consumers would be desirable.

Edible films and coatings are possible opportunities to prolong the shelf life of perishable food products such as fish fillets. Chitosan (poly-b-(1–4)-D-glucosamine) and its derivatives, alone or in combination with biologic or not biologic materials are good candidates for this purpose. They have been found to be nontoxic, biodegradable, biofunctional and biocompatible biopolymer. Chitosan-based edible coating provides excellent oxygen barrier properties along with its antimicrobial activity (Lopez-Caballero *et al.* 2005; Kim and Thomas 2007; Kong *et al.* 2010; Ojagh *et al.* 2010; Domard 2011). Recently, we synthesized and used chitosan nanoparticles as edible coating for the preservation of silver carp fillets during refrigerated storage (Ramezani *et al.* 2015). We showed that both chitosan and nanochitosan coatings were effective for the preservation of silver carp fillets during refrigerated storage. However, nanochitosan exhibited higher antimicrobial activity than chitosan during the storage period. Furthermore, nanochitosan showed a stronger ability to inhibit the total volatile basic nitrogen (TVB-N) content than chitosan (Ramezani *et al.* 2015).

Moreover, chitosan and nanochitosan films and coatings can be used as a vehicle for incorporating natural or chemical antimicrobial agents, antioxidants, enzymes or functional substances such as plant extracts, probiotics, minerals or vitamins (Ye *et al.* 2008; Ojagh *et al.* 2010). As consumers are increasingly aware of the risk for health because of the

presence of chemical substances added to food for their preservation and antimicrobial properties, herbal extracts and essential oils are gaining interest for their application in food preservation. Combinations of chitosan and herbal extracts and essential oils such as cinnamon oil (Ojagh *et al.* 2010), tea polyphenols (Li *et al.* 2012) and rosemary extract (Li *et al.* 2012) have been used previously for extending the shelf life of fresh fish samples.

Chemical constituents and their bioactivities in peel, seed, leaf, pulp and juice of different fruits such as pomegranate (*Punica granatum* L.) and orange (*Citrus sinensis*) have been investigated. A range of polyphenols and antioxidants has been found in orange and pomegranate peels, juices, pulps and seeds (Bocco *et al.* 1998; Gil *et al.* 2000; Noda *et al.* 2002; Singh *et al.* 2002; Guo *et al.* 2003; Anagnostopoulou *et al.* 2005). Hence, in the present study, the coating effects of orange and pomegranate peel extracts combined with chitosan nanoparticles on the shelf life of refrigerated silver carp fillets were evaluated by determining physicochemical, microbiologic and sensory parameters.

## MATERIAL AND METHODS

### Preparation of Orange and Pomegranate Peel Extracts

Orange (*C. sinensis*) and pomegranates (*P. granatum*) were obtained from local markets. The peels were manually removed and sun-dried. Dried orange and pomegranates peels were ground into fine powder using a blender. To prepare water extract, the powder (20 g/L) was mixed with distilled water (25C) for 15 min with continuous stirring. The extract was filtered through Whatman filter paper No. 42 and dried in an oven at 50C for 12 h, until the solvent was completely evaporated. The extract powder obtained was subjected to analyses.

### Determination of Total Phenolics

The content of the total phenolics in orange and pomegranate peel extracts was estimated using a Folin–Ciocalteu method as described by Negi *et al.* (2003). Briefly, 0.2 mL of diluted sample was mixed with 1.0 mL of 10-fold diluted Folin–Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate solution. After standing for 30 min at room temperature, the absorbance was measured at 765 nm using an ultraviolet (UV)-visible spectrophotometer. The estimation of phenolic compounds in the extracts was carried out in triplicate and the final results were expressed as tannic acid equivalents (TAE) per gram of the extracts.

### Determination of Total Antioxidant Capacity

The total antioxidant capacity of orange and pomegranate peel extracts was evaluated by ferric reducing ability of plasma (FRAP) assay as described by Benzie and Strain (1996). Briefly, the FRAP reagent contained 2.5 mL of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6, and was freshly prepared and warmed at 37C prior to use. Aliquots of 40 μL diluted sample solution were mixed with 0.2 mL distilled water and 1.8 mL FRAP reagent. The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37C for 10 min. The 1 mM FeSO4 was used as the standard solution. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mM $FeSO_4$.

### Preparation and Characterization of Chitosan Nanoparticles

Chitosan nanoparticles were prepared based on the ionotropic gelation between chitosan and sodium tripolyphosphate. Briefly, chitosan with the deacetylation degree of 83% and molecular weight of 850 kDa was dissolved in 1% (v/v) acetic acid to obtain a 2% (w/v) chitosan solution. Sodium tripolyphosphate was dissolved in water to a concentration of 2%. Under magnetic stirring at room temperature, 4 mL of tripolyphosphate solution was added into 100 mL of chitosan solution. The mixture was stirred for 60 min, then, treated with sonication at 1.5 kW for 10 min before being used for further analysis (Du *et al.* 2009). Particle size and zeta potential were measured using a Zetasizer Nano-ZS-90 (Malvern Instruments, Malvern, UK). The analysis was performed at a scattering angle of 90° at 25C. For zeta potential measurements, samples were dispersed in water and measured under the automatic mode.

### Preparation and Treatment of Fish Samples

Fresh silver carp, varying from 450 to 500 g in weight, were purchased from a public market and transferred to the laboratory. After being gutted and washed, two fillets were obtained from each fish after removing the head and bone. Fillet samples were randomly assigned into five treatment groups and were given a dip treatment in distilled water (uncoated control: lot C), 1% glacial acetic acid (acid control: lot AC) and 2% nanochitosan solution (lot Nch) for 20 min. For the fourth and fifth lots, fillet samples were given a dip treatment in 1% (w/v) orange (lot O + Nch) or pomegranate (lot P + Nch) peel extracts for 60 min and then drained well. After that, they were individually coated by immersing in nanochitosan solution for 20 min. The coating solutions also consisted of 0.5% (v/v) glycerol as a plasticizer. All the fillets were then allowed to drain on a pre-sterilized metal net under a biologic containment hood

in order to form the edible coatings and stored at 4C for subsequent quality assessment. Physicochemical, microbiologic and sensorial analyses were performed at 3-day intervals to determine the overall quality of fish. Each lot repeated three times with three fish and the averages were used to determine the overall quality of fish.

### Microbial Analysis

A 10-g portion of the samples were aseptically transferred into sterile stomacher bag and homogenized in 90 mL of sterile saline (0.85% NaCl) for 60 s in a stomacher (Bagmixer 400W, Interscience, St. Nom, France). Tenfold serial dilution of fish homogenates were used for enumeration of bacteria. Total mesophilic (TMC) and total psychrotrophic (TPC) bacteria were enumerated using nutrient agar, incubated at 35C for 24 h, and at 7C for 10 days, respectively. The results were expressed as $\log_{10}$ cfu/g of the samples.

### Physicochemical Analyses

**Determination of TVB-N.** TVB-N was determined through direct distillation of the fish homogenates after the addition of MgO. The distillate was collected in a flask containing a 2% aqueous solution of boric acid and a mixed indicator consisted of 0.1% methyl red and 0.1% bromocresol green in ethanol. Afterward, the boric acid solution was titrated with 0.1 N $H_2SO_4$ solution (Goulas and Kontominas 2005). The TVB-N value (mg N/100 g of fish) was determined according to the consumption of sulfuric acid.

**Determination of Thiobarbituric Acid Reactive Substances.** The thiobarbituric acid reactive substances (TBARS) value in the samples was determined using a spectrophotometric method (Wrolstad *et al.* 2005). Fish sample (5 g) was mixed with 100 mL of 10% trichloroacetic acid (w/v) and homogenized in a blender for 30 s. After filtration, 2 mL of the filtrate were added to 2 mL of 0.02 M aqueous TBA in a test tube. The test tubes were incubated at 100C for 45 min; then the absorbance was measured at 532 nm using UV-Vis spectrophotometer. TBARS value was calculated from the standard curve of malondialdehyde and expressed as mg malondialdehyde/ kg of fish.

**Determination of pH.** A 5-g sample of the fish fillet was homogenized thoroughly with 45 mL of distilled water and the homogenate was used for pH determination.

### Sensory Evaluation

The overall acceptability of silver carp fillets was determined using a 5-point scale considering texture, color and odor of the samples. Panelists scored for sensory characteristics, such as color discoloration (5, no discoloration; 1, extreme discoloration); odor (5, extremely desirable; 1, extremely unacceptable/off-odors) and texture (5, firm; 1, very soft). The averages of these scores were defined as overall acceptability (5, extremely desirable; 1, extremely unacceptable). Shelf life criteria assumed that rejection would occur when the sensory attributes declined below 4.0 (Ojagh *et al.* 2010).

### Statistic Analysis

All measurements were replicated three times for each lot and mean values ± standard deviations were reported for each case. Analysis of variance (ANOVA), Duncan's test and Kruskal–Wallis test were performed using the Statistical Package for the Social Sciences (SPSS) 16 (SPSS, Inc., Chicago, IL), to evaluate the significance of differences among mean values.

## RESULTS AND DISCUSSION

### Extraction Yield, Total Phenolics and Total Antioxidant Capacity of Orange and Pomegranate Peel Extracts

Orange and pomegranate peel is normally used as cattle feeds with low value or directly disposed in the field, which could cause environmental problem. It has been shown that orange and pomegranate peel is a good raw material for producing natural antioxidants because of its high content of antioxidants (Bocco *et al.* 1998; Anagnostopoulou *et al.* 2005; Qu *et al.* 2010). In general, extraction solvent, temperature and solid–liquid ratio are influential parameters for extraction process (Lapornik *et al.* 2005; Bucic-Kojic *et al.* 2007). Water as an environmental friendly solvent has been reported to be very effective for antioxidant extraction from pomegranate peel, seed and marc (Singh *et al.* 2002; Qu *et al.* 2010). In addition, by considering the antioxidant activity of water extract of pomegranate peel, Qu *et al.* (2010) reported that, the water/peel ratio of 50/1 (w/w), temperature of 25C, and extraction time of at least 2 min, resulted in the highest phenolics content and antioxidant activity. Therefore, water was used as the extraction solvent in this research. Extraction yield, total phenolic and total antioxidant capacity of the orange and pomegranate peel extracts were shown in Table 1.

### Particle Size and Zeta Potential of Chitosan Nanoparticles

Size (including size distribution) and zeta potential are essential characteristic parameters for nanosuspensions

| | Extraction yield (%) | Total phenolic (mg TAE/g) | Total antioxidant capacity (mmol $Fe^{2+}$/g) |
|---|---|---|---|
| 1% Orange peel extract | 32 ± 2.5 | 364 ± 10.3 | 4.9 ± 0.1 |
| 1% Pomegranate peel extract | 40 ± 1.5 | 350 ± 6.4 | 6.7 ± 0.09 |

**TABLE 1.** EXTRACTION YIELD, TOTAL PHENOLIC AND TOTAL ANTIOXIDANT CAPACITY OF ORANGE AND POMEGRANATE PEEL EXTRACTS

(Muller *et al.* 2001). Size distribution profile of the chitosan nanoparticles is shown in Fig. 1A. Chitosan nanoparticles had a mean diameter of 108.6 nm with a narrow size distribution (width: 11.3 nm; polydispersity index: 1.000). As shown in Fig. 1B, the chitosan nanoparticles had a zeta potential of +49.50 mV (Fig. 1B). For a physically stable nanosuspension solely stabilized by electrostatic repulsion, a zeta potential of ±30 mV is required as a minimum (Muller *et al.* 2001). All these data suggested that chitosan nanoparticles prepared here were stable.

## Microbial Analysis

In a living fish, bacteria are generally present in the skin, gills and gut, but they are prevented from entering the muscle. Once a fish dies, autolysis begins and bacteria can enter and decompose the muscle (Li *et al.* 2012). Changes in TMC and TPC bacteria during refrigerated storage are shown in Fig. 2. The initial TMC and TPC in the fish flesh ranged from 3.36 ± 0.13 to 3.61 ± 0.31 and 3.27 ± 0.14 to 3.56 ± 0.21 $\log_{10}$ cfu/g, respectively. Values of TMC and TPC increased gradually within each treatment lot during the storage period. In the first 6 days of storage, the groups treated with Nch, O + Nch and P + Nch had significantly lower TMC and TPC than the uncoated control, lot C ($P < 0.05$). On days 9 and 12, TMC and TPC of fish treated with Nch, O + Nch and P + Nch were significantly lower than the uncoated (lot C) and acid (lot AC) controls ($P < 0.05$), indicating the antimicrobial activity of the chitosan nanoparticles. TMC and TPC of fish treated with Nch, O + Nch and P + Nch did not exceed the maximal permissible limit of 7.0 $\log_{10}$ cfu/g until the end of storage period, while these values were higher than 7.0 $\log_{10}$ cfu/g in uncoated (lot C) and acid (lot AC) controls on days 9 (except for TMC of lot AC) and 12. Although, during the last 6 days of storage, TMC and TPC in O + Nch lot were slightly lower than Nch and P + Nch lots, no significant difference in TMC and TPC values was observed between Nch, O + Nch and P + Nch lots during the refrigerated storage ($P > 0.05$).

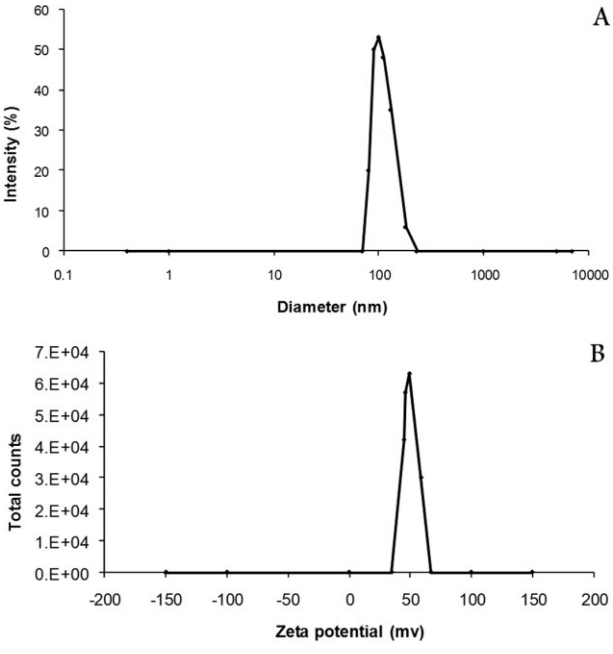


**FIG. 1.** PARTICLE SIZE (A) AND ZETA POTENTIAL DISTRIBUTION (B) OF CHITOSAN NANOPARTICLES

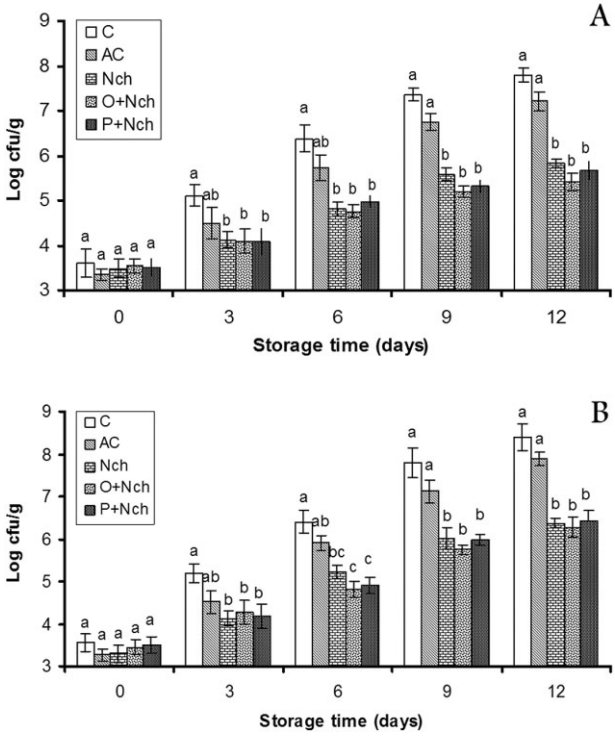


**FIG. 2.** CHANGES IN TOTAL MESOPHILIC (A) AND TOTAL PSYCHROTROPHIC (B) COUNTS OF FISH SAMPLES DURING REFRIGERATED STORAGE. MEANS WITH DIFFERENT SMALL LETTERS IN THE SAME DAY REPRESENT SIGNIFICANT DIFFERENCE AT $P < 0.05$

Chitosan is well known for its broad antimicrobial properties against bacteria and fungi. The antimicrobial properties of chitosan coating have been reported in the literature (Tsai *et al*. 2002; Lopez-Caballero *et al*. 2005; Ojagh *et al*. 2010). Tsai *et al*. (2002) found that pretreatment of fish fillets (*Oncorhynchus nerka*) for 3 h with 1% chitosan solution retarded the increase in the counts for mesophiles, psychrotrophs, coliforms, *Aeromonas* spp. and *vibrio* spp. Lopez-Caballero *et al*. (2005) reported that a coating consisting of a blend of chitosan dissolved in acetic acid and gelatin exerted an inhibitory effect on the gram-negative flora of fish patties. According to Ojagh *et al*. (2010), a chitosan coating enriched with cinnamon oil extended the shelf life of fresh rainbow trout during the refrigerated storage by retarding the enhancement of the total viable and psychrotrophic counts.

Unlike chitosan and its derivatives, studies on the antimicrobial properties of chitosan nanoparticles are still limited. Qi *et al*. (2004) reported that chitosan nanoparticles exhibit higher antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* than chitosan on account of the special character of the nanoparticles, likely the nanoparticle's larger surface area and higher affinity with bacteria cells, which yields a quantum-size effect. Conversely, Sadeghi *et al*. (2008) reported that chitosan nanoparticles have less inhibition effect on *S. aureus* than the polymers in free soluble form as nanoparticles have less positive charge available to bind to the negative bacterial cell wall. We recently indicated a higher antimicrobial activity of nanochitosan than chitosan, where during the refrigerated storage of silver carp fillets, lower values of TMC and TPC were observed in nanochitosan-coated than chitosan-coated samples (Ramezani *et al*. 2015).

## Physicochemical Analysis

**TVB-N.** The TVB-N value, which is mainly composed of ammonia and primary, secondary and tertiary amines, is widely used as an indicator of meat deterioration. Its increase is related to the activity of spoilage bacteria and endogenous enzymes (Ojagh *et al*. 2010; Li *et al*. 2012). Changes of TVB-N values in fish samples during storage were presented in Fig. 3. The initial TVB-N values ranged from $11.4 \pm 2.9$ to $13.3 \pm 4.6$ mg N/100 g of fish. TVB-N values of silver carp fillets increased progressively with storage time in all the treatments. However, it was obviously observed that the rate of TVB-N enhancement was significantly slower in samples coated with Nch, O + Nch and P + NCh than the controls. On days 9 and 12, coated groups (Nch, O + Nch and P + Nch) had significantly ($P < 0.05$) lower TVB-N values than that of the controls (lots C and AC); however, there were no significant difference ($P > 0.05$) in TVB-N values among Nch, O + Nch and P + Nch treatment groups during the refrigerated storage. Li *et al*. (2012) reported that in the preservation of large yellow croaker, using rosemary extracts or tea polyphenol dip could be attributed to either a more rapidly reduced bacterial population or decreased capacity of bacteria for oxidative deamination of nonprotein nitrogen compounds or both. Ojagh *et al*. (2010) found that pretreatment of rainbow trout with 2% chitosan as well as 2% chitosan incorporating 1.5% cinnamon oil could retard the increase in the TVB-N. Fan *et al*. (2009) found the chitosan coating lowered TVB-N values distinctly and hence slowed spoilage of silver carp. A stronger ability of nanochitosan coating than chitosan coating to inhibit the enhancement of TVB-N content in silver carp fillets during the refrigerated storage was observed previously (Ramezani *et al*. 2015).

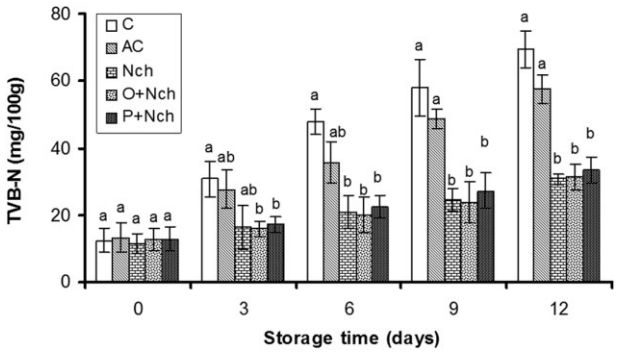


**FIG. 3.** CHANGES IN TOTAL VOLATILE BASIC NITROGEN (TVB-N) VALUES OF FISH SAMPLES DURING REFRIGERATED STORAGE. MEANS WITH DIFFERENT SMALL LETTERS IN THE SAME DAY REPRESENT SIGNIFICANT DIFFERENCE AT $P < 0.05$

**TBARS.** TBA value has been widely used as an indicator for the assessment of degree of lipid oxidation. In Fig. 4, the TBA values for the different treatment groups during

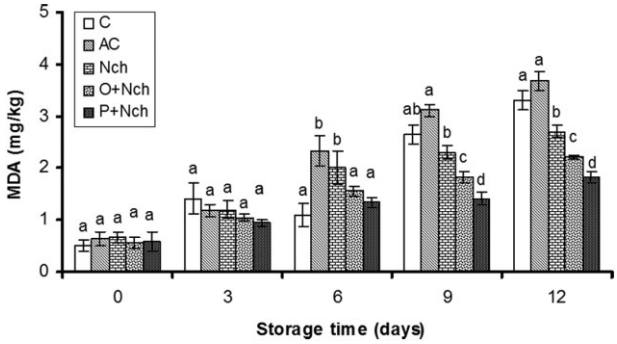


**FIG. 4.** CHANGES IN THIOBARBITURIC ACID (TBA) VALUES OF FISH SAMPLES DURING REFRIGERATED STORAGE. MEANS WITH DIFFERENT SMALL LETTERS IN THE SAME DAY REPRESENT SIGNIFICANT DIFFERENCE AT $P < 0.05$

refrigerated storage are presented. The initial TBA values ranged from 0.51 ± 0.11 to 0.66 ± 0.12 mg MDA/kg of fish. TBA values of the controls (C and AC) and coated samples (Nch, O + Nch and P + Nch) increased with storage time. By the end of the storage period (day 12), coated samples had significantly lower TBA value than the controls ($P < 0.05$), while Nch, O + Nch and P + Nch-coated samples reached TBA value of 2.71 ± 0.13, 2.21 ± 0.05 and 1.82 ± 0.11 mg MDA/kg of fish, in comparison with 3.31 ± 0.19 and 3.68 + 0.18 mg MDA/kg of fish in C and AC groups, respectively. Furthermore, on the last 6 days of storage, P + Nch-coated samples showed significantly lower TBA values than the O + Nch-coated samples ($P < 0.05$) and O + Nch-coated samples showed significantly lower TBA values than the Nch-coated samples ($P < 0.05$).

Both antioxidant and oxygen barrier properties of chitosan have been reported previously (Sathivel *et al.* 2007; Fan *et al.* 2009; Ojagh *et al.* 2010). In addition, using a combination of chitosan and herbal extracts such as rosemary extract (Li *et al.* 2012), tea polyphenols (Li *et al.* 2012) and cinnamon oil (Ojagh *et al.* 2010) significantly reduced the degree of lipid oxidation in fish tissue. Similarly, in the present study, the lower TBA value in O + Nch and P + Nch lots than Nch lot could be due to the high antioxidant activity of orange and pomegranate peel extract. Moreover, the higher antioxidant activity of pomegranate peel extract compared with orange peel extract (Table 1), resulted in significantly lower TBA value in P + Nch lot ($P < 0.05$).

**pH.** Changes in pH values during the refrigerated storage were shown in Fig. 5. During the storage time, the pH values decreased and then increased gradually. Similar observations were made by Fan *et al.* (2009) and Li *et al.* (2012). The decrease of pH value might be attributed to the dissolution of $CO_2$ in the fish sample, while the increase was postulated to be caused by an increase in volatile bases (e.g., ammonia and trimethylamine) produced by either endogenous or microbial enzymes (Manat *et al.* 2005). In this study, until day 6 of storage, no significant difference was found among the coated and the control fillet samples; however, on days 9 and 12, the pH value of the uncoated samples (lot C) was significantly higher than AC, Nch, O + Nch and P + Nch lots, which might result from a high level of volatile basic amines in the muscle tissue of the uncoated controls.

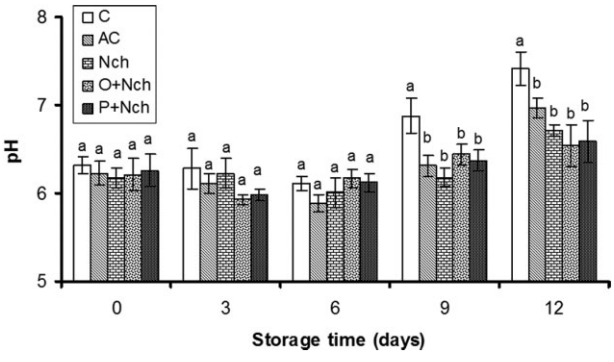


**FIG. 5.** CHANGES IN PH VALUES OF FISH SAMPLES DURING REFRIGERATED STORAGE. MEANS WITH DIFFERENT SMALL LETTERS IN THE SAME DAY REPRESENT SIGNIFICANT DIFFERENCE AT $P < 0.05$

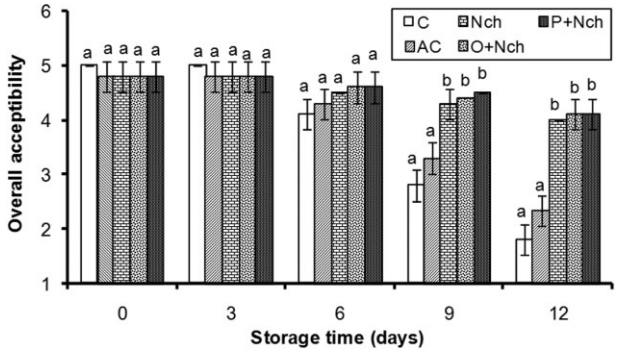


**FIG. 6.** CHANGES IN OVERALL ACCEPTABILITY VALUES OF FISH SAMPLES DURING REFRIGERATED STORAGE. MEANS WITH DIFFERENT SMALL LETTERS IN THE SAME DAY REPRESENT SIGNIFICANT DIFFERENCE AT $P < 0.05$

## Sensory Evaluation

The results of the sensory assessment of samples are given in Fig. 6. Samples were considered to be acceptable for human consumption until the sensory score reached 4 (Ojagh *et al.* 2010). As shown, until day 6, there was no significant difference in the overall acceptability among all treatment groups ($P < 0.05$); however, compared with the fish fillets coated with Nch, O + Nch and P + Nch, the overall acceptability of the control samples (lots C and AC) decreased sharply from days 6 to 12, and they had significantly lower scores on days 9 and 12 ($P < 0.05$). Li *et al.* (2012) observed a significant decrease in the overall acceptability after 8 days of storage of untreated large yellow croaker, which agreed well with a concomitant shift in bacterial counts. Similarly, in this study, sensory evaluation results appeared to be correlated to microbial and chemical value analyses.

# CONCLUSION

Results of the present study showed that dipping in orange or pomegranate peel extract combined with nanochitosan coating were significantly inhibited the development of lipid oxidation in fish samples. In this context, pomegranate peel extract was significantly stronger than orange peel extract.

Moreover, a combination of orange or pomegranate peel extract and chitosan nanoparticles extended the shelf life of fish fillets under refrigerated storage.

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