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Research Article
Effect of Chitosan Coating with Olive Leaf Extract on Shelf Life of Bighead Fish *(Hypophthalmichthys nobilis)* Chilled Fillet

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Abstract

Background and Objectives: Fish is an important source of protein and a rich source of essential components such as lipids, vitamins, minerals and antioxidants. The shelf life of fish products is limited by different factors such as enzymatic and microbiological spoilage. This study was aimed to evaluate chitosan film and olive leaf extract as possible candidates to improve shelf life of fish fillet. **Materials and Methods:** The effect of chitosan film containing the olive leaf extract as an herbal medicine on the shelf life of fish fillets during 16 days of maintenance in the refrigerator was investigated. The chemical and microbial characteristics including fat, protein, thiobarbituric acid, volatile nitrogen and total microbial count on fish fillets containing chitosan and olive leaf extract with control (T1) 1.5% chitosan (T2), 1.5% chitosan and 1% extract (T3) and 1.5% chitosan and 2% extract (T4) evaluated. **Results:** The shelf life of fish fillets coated with chitosan and olive leaf extract did not have a significant effect on the percentage of protein and fat. The amount of volatile nitrogen in the control sample increased at the end of the shelf life. This increase was observed in T4. The thiobarbituric acid level of the control sample was beyond the acceptable levels (1-2 mg kg^{-1} of malondialdehyde), but this increase was lower in T3. Based on the microbial analysis, T3 was not completely spoiled. While the control sample found to be corrupted on 12th day. **Conclusion:** Chitosan coating containing 1% extract had better performance than that of the 2%. This phenomenon can be caused by peroxidation property at high concentrations of the extract. Overall, chitosan film containing olive leaf extract increases the shelf life of fish fillets.

Key words: Bighead fish, olive leaf extract, shelf life, chitosan, malondialdehyde, fish fillet

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Seafood has a significant role in providing food to the world's people and by recognizing its nutritional suitability and superiority to other protein food their consumption is increasing day by day[1]. According to the latest reports from the Food and Agriculture Organization, the per capita consumption of fish in the world and Iran is 19 and 9.2 kg, respectively. Fish is one of the fast rotting food due to its high fatty acids with a few double bonds and high protein content. Keeping it in poor conditions, enzymatic and microbial activities cause spoilage and reduce the quality of fish meat. Therefore, its quality control is of particular importance and requires specific rules and standards[2]. One of the most important fish is the Bighead, which can be grown in offshore towns and in different climates. The high importance of these species is their role in the hydrothermal dual fish farming system with natural nutrition and good nutritional value as well as low production cost[3]. Compared to the meat and other meat products, fish and their products are more susceptible to bacterial and oxidative damage during storage due to high aqueous activity, near-neutral pH, relatively high amounts of free amino acids and the presence of autolytic enzymes[4]. Due to the unfavorable view of consumers about chemical additives and their harms in food, the tendency towards the use of natural additives has increased[5]. Therefore, edible coatings containing antimicrobial and antioxidant compounds reduce microbial activity and increase the shelf life of meat products and the use of proper antimicrobial compounds dose increases the quality of meat products[6].

Nowadays, the use of different natural edible coatings alone or with an active ingredient (such as; microbial agents, antioxidants, etc.) in different nutrients is considered as a material with antimicrobial properties. In this regard, considerable research has been done on the development or application of biopolymers extracted from various natural products. Biopolymers such as; starch, cellulose derivatives, pectin, chitosan, gums and proteins of animal or vegetable origin are used to make thin films and coatings to cover fresh or processed foods to enhance their shelf life. Using edible coatings as a modern technology protects food from physical, chemical and biological damage, in addition to its benefits such as; the edibility, beautiful appearance, environmental friendliness, non-toxicity and cheapness. They act as a barrier to the exchange of gases, moisture and microorganisms and maintain the shelf life of the product from production to consumer reach. Chitosan is a new edible coating that has a multi-sugar structure. It is composed of glucosamine and N-acetyl glucosamine units (with beta-1 and 2 linkages) derived from crustaceans such as crabs and shrimp[7,8]. Chitosan is a non-toxic, biodegradable and biocompatible substance. It also has antimicrobial activity which encompasses a wide range of microorganisms including fungi, bacteria and viruses. Chitosan is insoluble in water but soluble in organic acids. This compound has very good coating properties, high antimicrobial activity and is very compatible with substances such as vitamins, minerals and antimicrobial agents[9]. However, the antibacterial properties of chitosan are attributed to the positive load of its molecules and the result of reacting with the negatively loaded molecules of the bacterial cell membrane[10]. However, there has been a decrease in the antibacterial activity of chitosan when used as a coating or film[11]. This eco-friendly technology is created on the product as a thin-film of the semi-permeable coating by spraying, brushing and immersing[12] and as a protective mechanism. It changes the internal atmosphere, regulates the transfer of oxygen, carbon dioxide and water vapor and minimizes breathing, evaporation and deterioration. Olive leaf extract is obtained from the olive tree, a useful plant that adorns the tables. In recent years, research on olive leaves has shown that the extract of olive tree leaves is an herbal medicine with numerous therapeutic effects[13]. The antimicrobial effects of olive leaf extract are due to the constituents of oleuropein, the most important phenolic compound of olive leaf[13]. These compounds and other phenolic compounds found in olive leaf extracts such as; parahydroxybenzoic, ferulic acid, caffeic acid, vanillic acid, procatecoic, synergic acid, paracomaric acid, quercetin, tyrosol, hydroxytyrosol and oleanolic acid have antimicrobial activity[14]. Therefore, olive leaf extract can be used as a natural preservative in many foods[15]. The antimicrobial properties of olive leaf extract are due to its phenolic compounds[16]. Studies on the effect of chitosan include the use of chitosan as a natural preservative in mayonnaise[17,18], the effect of chitosan on the chemical and microbial properties of chicken fillet[19], the effect of chitosan on rainbow trout[20] and the effect of chitosan coating with lemon essence on microbial quality of salmon[21]. Studies on olive leaf extract include the antioxidant effect of olive leaf extract on butter stability[22] and sunflower oil[23]. However, since there is no study on the use of olive leaf extract with chitosan on fish meat. Therefore, the aim of this study was to investigate the effect of chitosan coating with olive leaf extract at different concentrations on chemical and microbial properties of Bighead meat during storage at 1±4°C.

MATERIALS AND METHODS

Fish preparation: The study was carried out at Department of Agriculture and Food Industry, MehrAeen Higher Education Institute from September, 2016-October, 2017. Bighead fish was obtained from the live fish market and was immediately transported to the food lab and the fillet was prepared. After the head and tail cutting and separation of the abdominal discharge, skin and bone, fish fillets were prepared.

Preparation of chitosan coating solution: To prepare the solution, 1.5% chitosan coating (w/v) was prepared in 100 mL of 1% acetic acid solution (v/v). The mixture was placed on a magnetic stirrer for 3 h at room temperature for complete dissolution. Then glycerol was added in 0.75 mL as a plasticizer and mixed with a magnetic stirrer for 10 min. Finally, a filter paper (Whatman no. 3, UK) was used to remove impurities from the above solution. For uniform distribution, the olive leaf extract was mixed with tween 80 in chitosan solution.

Preparation of olive leaf extract: Olive leaves are harvested from the olive gardens in Roudbar and dried in the shade. Then it was powdered using a mill and passed through a 40-mesh sieve. Then the olive leaf powder is mixed with a water solvent at ambient temperature with a ratio of 1:10 for 3 h. The extract is smoothed with a filter paper (Whatman No. 1, USA). The extract was concentrated by rotary evaporator at 40°C. The amount of phenolic compounds in the extract was measured immediately and expressed as miligrams of tannic acid in grams of extract. Then the fillets are divided into 4 portions as shown in Table 1. Then each group was immersed in the solution for 30 sec and then it was removed from the solution. After about 2 min, they were again put in the film solution for another 30 sec. The fillets were then left to stand for 5 h at room temperature to form a coating. The fillets are then packed in polyethylene, transferred to the refrigerator and stored at 1±4°C for 16 days and were evaluated once every 4th day. All sample processes were performed in 3 replicates.

Table: 1: Primary components (%) of Bighead fish coating

Treatments	Chitosan coating (%)	Olive leaf extract (%)
T1 (control)	0.0	0
T2	1.5	0
T3	1.5	1
T4	1.5	2

Physicochemical tests: Soxhlet apparatus was used to measure protein and fat changes in the sample and treatments over the shelf life[24]. Thiobarbituric Acid (TBA) was measured (mg kg^{-1} malondialdehyde) as an indicator of lipid corruption, 10 g of the sample was weighed and mixed with 50 mL of distilled water. The resulting mixtures were transferred to distillation Erlens with 47.5 mL of distilled water. About 2.5 mL normal hydrochloric acids with anti-foaming and anti-boiling agents were added to the mixture and Erlens was connected to the distiller. The mixture was heated and 50 mL of the distilled material was removed from the mixture after boiling. About 5 mL of the distilled material and 5 mL of TBA reagent were transferred to test tubes and placed in boiling water for 35 min after complete shaking. At the same time, all these steps were repeated for the control sample. The specimens were then cooled for 10 min after being boiled for 35 min and their optical density[25] was read at 1 cm cells in contrast to the control at a wavelength of 538 nm. Total Volatile Basic Nitrogen (TVB-N) in mg/100 g, 10 g sample with 2 g magnesium oxide as a catalyst and 300 mL distilled water and several glass pearls were poured inside Kjeldahl digestion balloon and attached to the other parts of the Kjeldahl machine (KjelFlex K-360, Swiss). At the bottom of the Erlenmeyer flask receptor beneath the refrigerant (such that the end of the refrigerant was in solution), 25 mL of 2% boric acid and a few drops of 0.1% methyl orange were poured. In this case, the color will be red. Then the digestive balloon heated so that its contents boil within 10 min. Distillation continued 25 min after boiling in which case all the volatile alkalis in the meat are distilled and absorbed into the contents of the Erlenmeyer flask (Duran, Germany) receptor. It will turn the solution color blue (because of the alkaline environment). Then, the heat cuts off and the distilled solution was titrated with 0.1 N sulfuric acid until red. Now given that, each mililiter of sulfuric acid 0.1 N is equivalent to 0.0014 g or 1.4 mg N, the amount of volatile alkali was calculated in milligrams percent from the relationship: (100*1.4* Acid 0.1 dosage for sample = Volatile alkali of Total Volatile Nitrogen (TVN))[25].

Total Viable Counts (TVC) were determined on Nutrient Agar (Merck, Darmstadt, Germany) using a conventional pour technique. For this purpose microbial test including the total viable count of microorganisms in Log CFU g^{-1} and the culture of 0.1 mL sample was done for 48 h incubation with a incubator (Memmert INB200, Germany) at 35°C.

All reagents were purchased from Merck and Sigma Company.

Statistical analysis: Descriptive statistics using mean and standard deviation were used to describe the quantitative characteristics. Analysis of Variance ANOVA was used to compare the mean responses of Bighead or olive leaf extract and chitosan coating solution and if necessary the Duncan *post hoc* GLM test ($\alpha = 0.05$) was used. Data analysis was performed using SPSS v.21 software.

RESULTS AND DISCUSSION

Protein and fat: The results of the chemical composition of Bighead fish containing chitosan coating incorporated with olive leaf extract are shown in Table 2. Analysis of the Bighead fish samples showed that the increase of concentration olive leaf extract in the coated Bighead fish samples did not affect significantly ($p>0.05$) on protein, fat and moisture content. The moisture content of all samples was approximately 67%.

Total Volatile Basic Nitrogen (TVB-N): The number of phenolic compounds in the powder of olive leaf extract was measured immediately and it was obtained as 0.259 mg equivalent of tannic acid in a gram of extract. The results of volatile nitrogen measurements are shown in Fig. 1. According to the results, there was a significant difference between treatments and the interaction of time on treatments ($p<0.05$). Fish samples coated with 1.5% chitosan and 1% olive extract had a significant difference with respect to other samples over time ($p<0.05$). The maximum acceptable total volatile basic nitrogen is stated to be 25 g of nitrogen per 100 g of fish meat[4]. The results showed that in all treatments, the volatile nitrogen content increased during storage. Such an increase in the volatile nitrogen content indicated the degree of meat spoilage and then the accumulation of non-protein compounds including ammonia, primary and secondary amines. There was no significant difference ($p<0.05$) between treatments at days 0 and 4 (except for the control sample). However, on days 8, 12 and 16 of storage, there was a significant difference ($p<0.05$) between T1 and the other treatments. As can be seen in Fig. 1, on day 8 the maintenance of volatile nitrogen for all treatments exceeded the limit. The reason for this may be the relationship between the amount of total volatile basic nitrogen and the bacterial load as shown in the chart. In addition, in terms of comparing the significant differences between the whole samples in a 16 days control sample storage process, there was an increase in the age from 0-16 days. Sample coated without olive leaf extract had no significant difference until day 4. However, from day 8 onwards, the fish meat age increased significantly compared to the control group. Sample containing 1% olive leaf extract had no significant difference except for day 16. It had a lower age than the first two samples. In the sample containing 2% olive extract from day 8 onwards, the difference was significant with control and chitosan containing only samples. Among the various parts of the olive tree, the olive leaf is the richest source of phenolic compounds and

Table 2: Protein and fat analysis of the Bighead fish samples (on fresh weight basis)

Treatments	Protein (%)	Fat (%)
T1 (control)	87.05±0.01[a]	11.56±0.01[a]
T2	87.03±0.01[a]	11.59±0.01[a]
T3	87.15±0.01[a]	11.58±0.01[a]
T4	87.00±0.01[a]	11.58±0.01[a]

Matched letter in each row indicate non-significant difference at 5% error level. Mean, after an average of triplicate trial±standard error at $p<0.05$

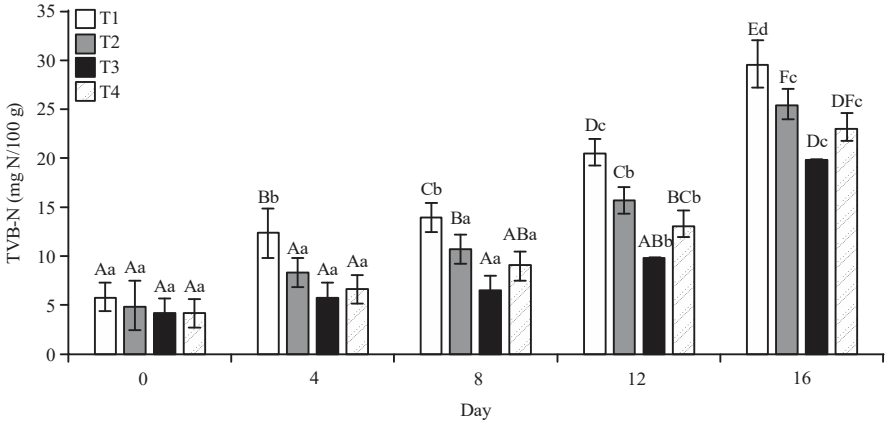

Fig.1: Amount of TVB-N changes in the samples over time

T1: Control, T2: 1.5% chitosan, T3: 1.5% chitosan enriched with 1% olive extract, T4: 1.5% chitosan enriched with 2% olive extracts, the uppercase letters show the comparison over 16 days and the lowercase letters show the comparison between each period, mismatched letters in each row indicate a significant difference at 5% error level

oleuropein is the most abundant phenolic compound in the leaf. Over a period of up to 8 days, there was a direct and linear relationship between the concentration of olive leaf extract and antioxidant power because initially by increasing the concentration up to 8 days, the antioxidant and anti-radical capacity of the extract increased. However, the effect of a 2% concentration of olive leaf extract on the 12th and 16th day compared to 1% olive leaf extract reduced the antioxidant and phenolic potency of the extract. This phenomenon can be attributed to the peroxidative activity of this extract. Some substances containing phenolic compounds at high concentrations exhibit peroxidative properties[13,26] as increasing the extract concentration by up to 1% is likely to produce a synergistic effect with the antioxidant present in the chitosan coating. However, increasing the extract concentration to 2% reduced the synergistic effect with the synthetic antioxidants present in chitosan. In a study, Abdeldaiem[27] investigated the effect of irradiation and an edible coating containing ethanolic extract of papaya on enhancing the shelf life of a chicken. The volume of volatile nitrogen in the control group at day 6 and 9 was 26.15 and 35.73 mg/100 g, respectively. However, in the group containing the coatings, this amount was 22.57 and 27.29 mg/100 g, respectively on these days.

Thiobarbituric acid (TBA): The results for the measurement of parameters related to TBA are presented in Fig. 2. There was a significant difference between treatments with each other and the interaction of time on treatments ($p<0.05$). The effect of 1.5% chitosan treatment containing 1% olive extract compared to the other treatments, the control sample against the time period and their interaction on the reduction of life expectancy and nutritional value of the sample were better ($p<0.05$). As can be seen, the amount of TBA in all treatments showed a significant increase ($p<0.05$). There was no significant difference ($p<0.05$) between treatments on day 0 of storage. However, on days 4, 8, 12 and 16, there was a significant difference between the control and the other samples. In addition, between periods, 2 and 1% of olive leaf extract were better than other samples (control sample and a sample containing only chitosan). Primary by-products of the oxidation of fats are hydroperoxides which are unstable compounds and do not play a role in the unfavorable taste of fish. After breaking, the hydroperoxides produce substances such as aldehydes, ketones, alcohols, hydrocarbons, esters, furan and lactones. With increasing shelf life, the samples containing olive extract had a better condition than the sample without olive leaf extract which is due to the antioxidant and phenolic potency of olive leaf extract and increased shelf life of the product[28]. However, increasing the extract to 2% had no significant effect on increasing shelf life compared to the 1% sample. This phenomenon can be attributed to the peroxidative properties of this extract because some substances containing phenolic compounds at high concentrations exhibit peroxidative activity. Extracts also lack purity which can decrease the effectiveness of the extract by increasing its percentage[13,29]. The results of this study are consistent with the findings of Kamil *et al.*[30] who investigated the antioxidant effect of chitosan on Herring fish meat. Peroxide and thiobarbituric acid index in Herring samples containing 200 ppm chitosan decreased by 61 and 52% after 8 days of storage, respectively[30]. In co-administration of chitosan and vegetable extracts in sardine, beef, lamb and pork under cold storage conditions, it was reported that chitosan samples containing herbal extracts showed lower TBA levels than the uncoated ones during storage[31].

Microorganisms count (Total viable count): Results of analysis of chitosan-containing and olive leaf extract coating showed significant effect over shelf life. Statistical analysis of data showed that the total count of microorganisms in the control sample had a significant difference with the total count of microorganisms (Fig. 3). In addition, by increasing the amount of olive extract in the treatments, the total count of microorganisms decreased by 1% ($p<0.05$). The lowest count of total microorganisms was in samples containing 1 and 2% of olive extract, respectively.

Over time in some treatments, the overall count of microorganisms increased. Of course, this increase was more noticeable in the control treatment. At the end of the period, the highest bacterial load was approximately 4.5 CFU g^{-1}. A comparison between treatments showed that chitosan treatment containing 1 and 2% olive leaf extract had significantly lower bacterial load compared to the other treatments ($p<0.05$). Although, the initial microbial load of freshwater fish varies and depends on factors such as water status and ambient temperature. High levels of microbial load can be found in the raw product depending on the storage and manipulation conditions. As can be seen in the chart, the total number of microorganisms at the beginning of the period was less than 4 CFU g^{-1} for all treatments, indicating that the quality of the fillets used was good. The permissible amount for a total of 7 microorganisms is recommended for seismic fish. At 16th day of storage, the fillets in the olive leaf extract treatments had less than 7 microorganisms, while the rate of microorganism loads[4,32] in the control sample and only chitosan samples reached above 7. There are various reports about the time allowed for the fish fillets to

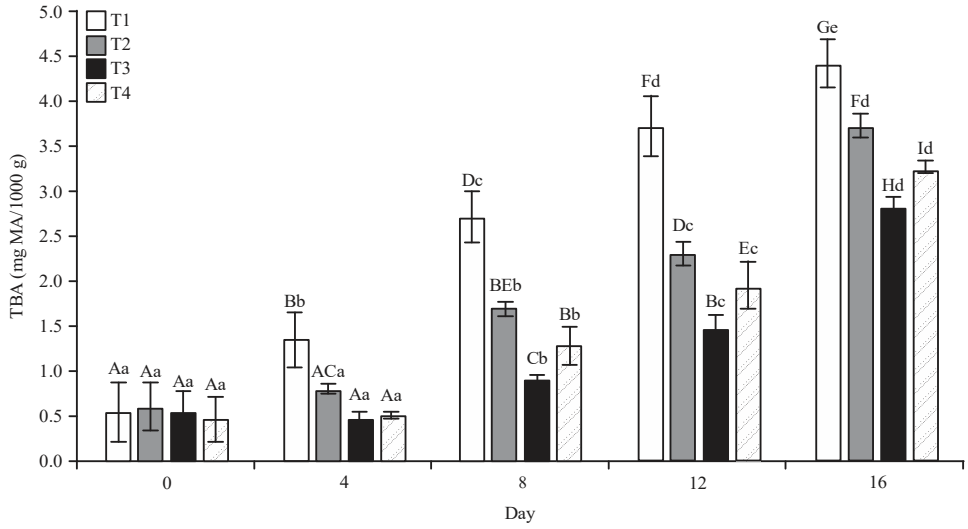

Fig. 2: Amount of TBA changes in the treatments over time

T1: Control, T2: 1.5% chitosan, T3: 1.5% chitosan enriched with 1% olive extract, T4: 1.5% chitosan enriched with 2% olive extracts, the uppercase letters show the comparison over 16 days and the lowercase letters show the comparison between each period, mismatched letters in each row indicate a significant difference at the 5% error level

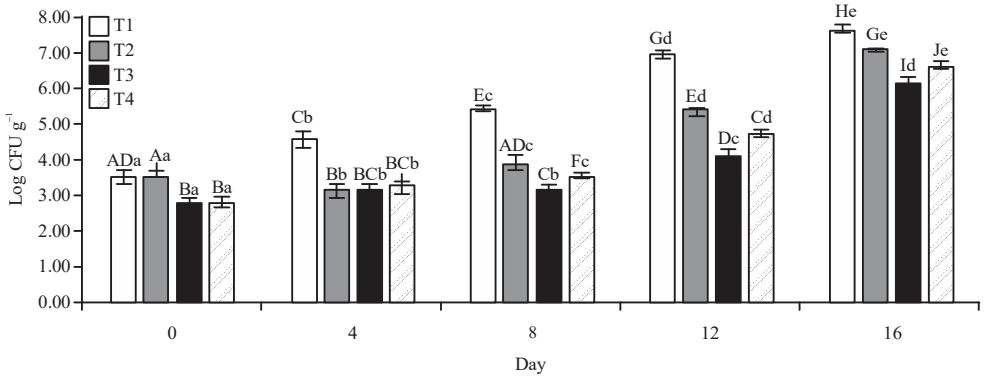

Fig. 3: Changes in the total count of microorganisms in the samples over time

T1: Control, T2: 1.5% chitosan, T3: 1.5% chitosan enriched with 1% olive extract, T4: 1.5% chitosan enriched with 2% olive extracts, the uppercase letters show the comparison over 16 days and the lowercase letters show the comparison between each period, mismatched letters in each row indicate a significant difference at the 5% error level

reach the total load. Kostaki *et al.*[33] reported that a total load of microorganisms in the seabass (*Dicentrarchus labrax*) fish fillets reached 7 during 7 days of storage at 4°C. Ojagh *et al.*[29] reported that a total load of microorganisms in the salmonid fish fillets was more than allowed during the 12 days of storage. Kykkidou *et al.*[34] reported that a total load of microorganisms in the Mediterranean swordfish fillets was more than allowed in 6 days of storage at 4°C. Generally, the total load of microorganisms for most treatments increased with time. This increase was higher in control treatment and was the highest at the end of the period. The least amount of total microorganisms was in chitosan coating treatments containing 1 and 2% olive leaf extract[29,33,35]. These 2 treatments had no significant difference over the whole storage period. This indicates that the two levels mentioned are almost identical in terms of their effect on the overall microorganisms load reduction process. The lower load of microorganisms in chitosan-coated treatments with olive leaf extract indicates that the coating with olive leaf extract has an effective role in reducing a total load of microorganisms. This study was in agreement with the results of a study on chitosan-coated carp fillets, which increased and decreased chitosan-coated fillet life and growth of micro-organisms, respectively[36]. The results of this study are in line with the result of Khabar and Golestan[28], Rafiee *et al.*[23] and Jafarian *et al.*[37] on canola oil sunflower oil preservation.

The results showed that the use of chitosan-coating with olive leaf extract for storage of Bighead fish fillet during storage was able to increase the antioxidant potential of Bighead fish fillet. Among the treatments, 1% extract had the best result compared to the sample containing 2% olive leaf extract. This led to an increase in shelf life in the present study.

CONCLUSION

Analyzing the data of this study showed that the addition of olive leaf extract to chitosan coating increased the antimicrobial and antioxidant properties of the coating so that the process of microbial spoilage and oxidation in the coatings has significantly delayed. This also reduced the amount of total volatile basic nitrogen, thiobarbituric acid index and reduced the overall bacterial count. It should be noted, however, that the tests alone do not confirm the positive performance of this study. It requires a lot of studies and research in this field.

SIGNIFICANCE STATEMENT

This study discovers the synergistic effect of olive leaf extract and chitosan combination that can be beneficial on the shelf life of Bighead fish (*Hypophthalmichthys nobilis*) chilled fillet. Nowadays due to the unfavorable view of consumers about the dangers of chemical additives in food, the tendency to use natural additives has increased. Oleuropein is the most important phenolic found in olive leaf extract and olive leaf extract is used as a natural preservative in many foods. This study will help the researcher to uncover the critical areas of coating of fish with chitosan that many researchers were not able to explore. Thus a new theory on the enhancement of shelf life of fish may be arrived at.

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