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Research Article

Antimicrobial effects of silver/copper/titanium dioxide-nanocomposites synthesised by chemical reduction method to increase the shelf life of caviar (*Huso huso*)

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Abstract

This paper focuses on the use of incorporation of silver (Ag) and copper (Cu) nanoparticles in titanium dioxide (TiO_2) nanocomposites, as Ag/Cu/TiO_2 nanocomposite was synthetized through the chemical reduction method against food borne microorganisms. The antimicrobial effect of Ag/Cu/Tio_2 nanocomposite demonstrated a considerable antimicrobial activity as order as *Escherichia coli* > *Staphylococcus aureus* > *Aspergillus niger*. The results of the agar diffusion test exhibited no halo growth around the discs of four nanocomposite samples, which indicated the perfect inhibitory effect of synthesized Ag/Cu/TiO2 nanocomposites against the afore-mentioned bacteria. Scanning electron microscopy (SEM) analysis exhibited that Ag and Cu nanoparticles were homogeneously distributed into the TiO_2 nanocomposite. Furthermore, the results of inductive coupled plasma mass spectrometry (ICP-MS) method showed that 0/168 ppm of Ag was released to caviar samples on 32nd day and 62nd days of the experiment. All findings indicated that the combined usage of Ag/Cu/Tio_2 nanoparticles could meet all desirable objectives including increase of antimicrobial activity and reduction release of Ag nanoparticles into the caviar, which extends caviar shelf life.

Keywords: Chemical reduction method, Nanocomposites, Shelf life, Caviar, Antimicrobial tests.

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Introduction

Packaging is an important part of food processing to protect food materials from physical, chemical, and biological damages. Conventional packing methods are passive obstacles designed to protect food products against the natural environment. However, some weaknesses in common food-packaging ingredients have caused broad attention to be directed toward nano-packaging. The use of nanoparticles use in this novel food packaging is associated with beneficial properties due to their microscopic size, which increases drug bioavailability (Singh *et al.*, 2017; Anvar *et al.*, 2019). For this purpose, two groups of substances (organic and inorganic) are applied, and between them inorganic materials have become more popular due to their stability in processing conditions in comparison to organic ones (Carbone *et al.*, 2016). Among inorganic nanoparticles that have antimicrobial attributes, Ag, Cu and TiO_2 are the most valuable materials (Toker *et al.*, 2013; Khoshnood *et al.*, 2017).

A variety of methods have been used for the synthesis of nano-sized particles, as techniques mentioned for Ag-NPs including microwave irradiation, thermal decomposition, and sono-chemical synthesis. A few approaches have previously performed for Cu-NPs production, such as manipulation of the reaction temperature in organic solvents, and another procedure (Chen and Sommers, 2001) showed that application of alkanethiolate monolayers to protect copper nanoparticles from oxidation. Although there are several methods for production of Ag-NPs and Cu-NPs, the most commonly used approach is chemical reduction which is performed in the current study.

Ag-NPs are known as potent inhibitors of a variety of microorganisms (Fortunati *et al.*, 2014). Evidence showed that the antimicrobial activity of Ag-NPs is associated with several mechanisms, including the production of reactive oxygen species, which resulted in the degradation of the cell as well as the release of silver ions from nanoparticles, which then leads to bacterial death (Guzmán *et al.*, 2009; Deus *et al.*, 2017). Cu-NPs exhibit great features, such as chemical stability and thermal resistance. In addition, the antimicrobial and anti-toxicity properties of Cu-NPs have been proven through a variety of mechanisms. Among them, there are the agglomeration of these nanoparticles in the cell wall and increase the oxidative stress. Due to their low price and broad range of application, Cu-NPs have been employed in food nano-packaging (Khan *et al.*, 2016). Titanium dioxide (TiO_2), a desirable inorganic material, is broadly employed in food nano-packaging due to its chemical stability, biocompatibility, and high photocatalytic performance (Peter *et al.*, 2016).

Some studies showed the effect of nanoparticles on immunity of shrimp (Kaviyani et al., 2020; Sadralsadati *et*

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al., 2020) against pathogen bacteria (Fakharzadeh *et al*., 2020; Salari Joo *et al.*, 2020), shelf life of aquatics (Kamani *et al*., 2020; Ghorabi and Khodanazary, 2020; Azari *et al*., 2020). The effects of nanoparticles on increasing the shelf life of aquatic products have been investigated using a variety of antimicrobial agents from various sources. A study confirmed the effect of nanocomposite consisting of fish protein isolate and fish skin gelatine films combined with 3% ZnO-NPs and 100% basil leaf essential oil on the shelf life extension of sea bass slices (Arfat *et al.*, 2015). The results of an investigation revealed that PLA/ZnO nanocomposite film used for packaging minced fish cakes could increase their shelf life by improving the antibacterial effect and acting as a as well as UV light barrier (Shankar *et al.*, 2018).

Although there are various investigations on the antimicrobials effects of nanoparticles used in food packaging (Fortunati *et al.*, 2014; Deus *et al.*, 2017; Zhang *et al.*, 2017), based on our knowledge, there is no report regarding the effect of TiO_2 nanocomposite loaded on nano-silver and nano-copper as caviar packaging to prolong its shelf life. Caviar is one of the most expensive natural food product around the world and is considered as a valuable source of nutrients, including amino acids and omega-3 fatty acids, which are important for body metabolism and nervous system function (Adeli and Namdar, 2015). The Caspian Sea has a global reputation as high-quality caviar producer (Harris and Shiraishi, 2018). However, current caviar packaging methods resulted in some undesirable consequences. Therefore, employing proper packaging would increase the shelf life of caviar while maintaining its quality.

In this study, the effect of titanium nanocomposites loaded on Ag-NPs and Cu-NPs as food packaging on extending the shelf life of caviar was examined. We also used the method of inductive coupled plasma mass spectrometry (ICP MS) method to obtain information about the release of nanoparticles from the polymer to the caviar.

Materials and methods

Materials

In this study, all media (Nutrient broth, MacConkey broth, Mueller-Hinton Agar, Plate Count Agar, TCB and YGC agar) were purchased from Merck, Germany. Low-density polyethylene film was obtained from the Iran Petrochemical Company. The microbial strains applied were *Escherichia coli* 1399 (ATCC 25922), *Staphylococcus aureus* 1431 (ATCC 25923) and *Aspergillus niger*, which were prepared from the microbial network of Tehran University of Medical Science, Iran.

Production of nanoparticles by chemical reduction

Synthesis of silver nanoparticle

For the production of AgNPs, according to the procedure described by Guzman *et al*. (2009) the following materials was used: silver nitrate solution (purity

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99.98%, 0.005 M) as a starting material, citrate of ammonium solution (purity 99.5% , 0.34 M) as a stabilizing agent and hydrazine hydrate solution (0.001 M) as a reducing agent. The citrate of ammonium mixture was added to the silver nitrate solution, which had already boiled while magnetically stirring (1500 rpm) for 1 hour at 50-60 ^{o}C until Ag-NPs` colloid formed. The colour change from yellow pale to red revealed the generation of Ag-NPs (Guzmán *et al.*, 2009). Later, the size dispersal of the colloidal Ag-NPs was depicted using dynamic light scattering (DLS).

Synthesize of copper nanoparticle

The Cu-NPs were produced using pentahydrate of copper sulphate (0.1 M) as precursor material, starch as polymer to coat the copper nanoparticles and ascorbic acid (0.2 M) as a reducing agent according to the method explained by Khan *et al*. (2016). Briefly, copper sulphate pentahydrate was added to the starch solution and robustly stirred for 30 min. Ascorbic acid and sodium hydroxide solution were added to the prepared mixture at 80° C for two hours, while stirring had been continued. Solution colour turned from yellow to yellowish-brown, indicating the reaction was completed and the settlement of the solution considered at room temperature. The supernatant was then removed.

Preparation of TiO_2 nanocomposites loaded on g/Cu NPs

The Ag/Cu/TiO_2 nanocomposites were produced (Lotfi *et al.*, 2019) using twin screw extruders (Brabender, DSE 20) to mix all nanoparticles and then apply the coating via a melt mixing method. This method is a desirable technique for preparing the nanoparticles/ nanocomposites of a thermoplastic and elastomeric polymeric matrix in which the polymer is molten and mixed with nanoparticles utilizing an extruder in the presence of inert gas. The temperature and rotating speed of the extruder were set at 190ºC and 44 rpm, respectively. The parameters in the melt mixing method were adjusted as follows: the speed of machine and the pressure of the blowing film were set at 60 rpm and 1.5×10^{5} pascals, respectively. The films were kept in a sanitary place to improve their qualities. Figure 1 shows the extruder machine and its heating program, respectively.

Structure and morphology of the nanoparticles

Scanning electron microscopy (SEM. Hitachi, Japan) was used to examine the dispersal and morphology of the synthesized nanoparticles on the polymer at a voltage of 20 kV and a pressure of 1 Torr. For this purpose, a sample suspension was prepared by dissolving 2×2 cm^2 pieces of polymer in acetonitrile solvent. This was followed by evaporating the solvent, the residual objects were placed on sputter coater holding argon gas loaded on gold

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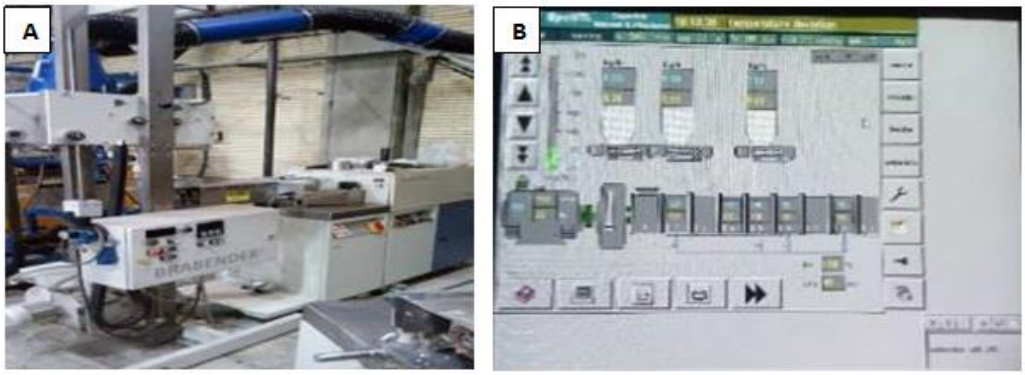

Figure 1: The extruders machine (A) and it`s heating program (B).

coating (Khan et al., 2016). Afterward, samples were shifted to the SEM chamber to determine the structure and morphology of the produced nanoparticles.

Antimicrobial assessments of the Ag/Cu/TiO_2 nanocomposites

The antimicrobial activity of nanocomposite based on Ag- Cu-NPs was assessed against *E. coli* 1399 (ATCC 25922), *S. aureus* 1431 (ATCC 25923) and *A. niger* by applying three methods consist of minimum inhibitory concentration (MIC), agar diffusion disc and colony count tests as described consequently.

Minimum inhibitory concentration test

Two pathogenic bacteria, *E. coli* and *S. aureus*, were employed for a minimum inhibitory concentration test according to the broth microdilution method (Kalemba and Kunicka, 2003). A total of 96 well-plates were used, 100 µL of culture medium was added to each well. The culture mediums for *E. coli* and *S. aureus* were MacConkey broth and TCB, respectively. A 0.5 McFarland standard suspension of each isolate was utilized as an inoculum. One hundred µL of chloramphenicol, an antimicrobial solution was added to each well while the antimicrobial suspension was serially diluted. This was followed by adding 10 µL of bacteria to each well. The first well was considered as a positive control consisted of a culture medium together with bacteria, meanwhile, the second well was considered as a negative control, as it contained a culture medium with an antimicrobial solution. Afterward, the 96-well plates were covered with aluminium foil and incubated at 37°C for 24 h. Later, the turbidity in each well was defined using a spectrophotometer at 530 nm and compared with the positive control well to determine the minimum inhibitory concentrations (MIC) which required preventing at least 50% of bacteria growth.

Disc diffusion method

To investigate the antimicrobial activity of coating by disc diffusion method, first nanocomposite samples were cut into 1 × 1 cm^2 pieces and dissolved in 10 mL of an ethanol solvent, which had

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already reached its boiling point while the mixture was magnetically stirred to produce a uniform suspension. Then, polyethylene film was added into the xylene and stirred until dissolved completely.

To have an appropriate inoculum of *E. coli* and *S. aureus*, fresh culture plates of bacteria were spread onto the surface of a petri dish covering Mueller-Hinton agar that had already solidified and dried in the incubator and standardized to 0.5 on the McFarland scale. Four paper discs (1 cm in diameter) were incorporated with 20 µL of nanoparticles and were placed in a petri dish. The plates were then incubated at 37 $^{\circ}$C for 20 h. Ultimately, the potential halo growth was measured with a digital Calliper (Bauer *et al.*, 1966).

Microorganism colony count

The prepared nanocomposite film was tested against three pathogenic agents (two bacteria and one fungus) *S. aureus* (gram-positive bacteria), *E. coli* (gram-negative bacteria) and *A. niger* via the colony counting approach. Firstly, the above bacteria were cultured in nutrient broth with a concentration equal to 0.5 on the McFarland degree and were then diluted to 1×10^6 µL. Then, 1 g of canned caviar (Beluga, *Huso huso*) purchased from the Sarcheshmeh region near Tehran, Grand Bazaar, was covered using nanocomposite, cut into 2×2 cm^2 pieces and ultimately refrigerated as control samples. Consequently, 1 gr caviar was mixed with 9 mL Ringers serum and then serially diluted to make seven desired dilutions for each sample. One plate for each concentration of each bacteria was used and all samples were incubated at 37 $^{\circ}$C for 48 h while incubation condition was set at 25 $^{\circ}$C for 4-5 days for *A. niger*. The medium culture for *S. aureus* and *E. coli* was Plate Count Agar whereas YGC agar applied for *A. niger*. For each sample, daily visual examinations and colony counting were performed on days 3, 5, 7, 8, 9 and 10 (Gallocchio *et al.*, 2016).

Characterization of Ag and Cu nanoparticles

A Fourier transform infrared (FTIR) spectrum (Thermo Scientific Nicolet FT-IR) within the range of 400–4000 cm^{-1} was employed to acquire information related to the chemical groups found on the nanocomposite to gain a better understanding of their intermolecular interactions (Fig. 2 A-B). In addition, the dynamic light scattering (DLS) approach was used to determine the distribution of the Ag-NPs (Fig. 3) following the procedure of Chen and Sommers (2001).

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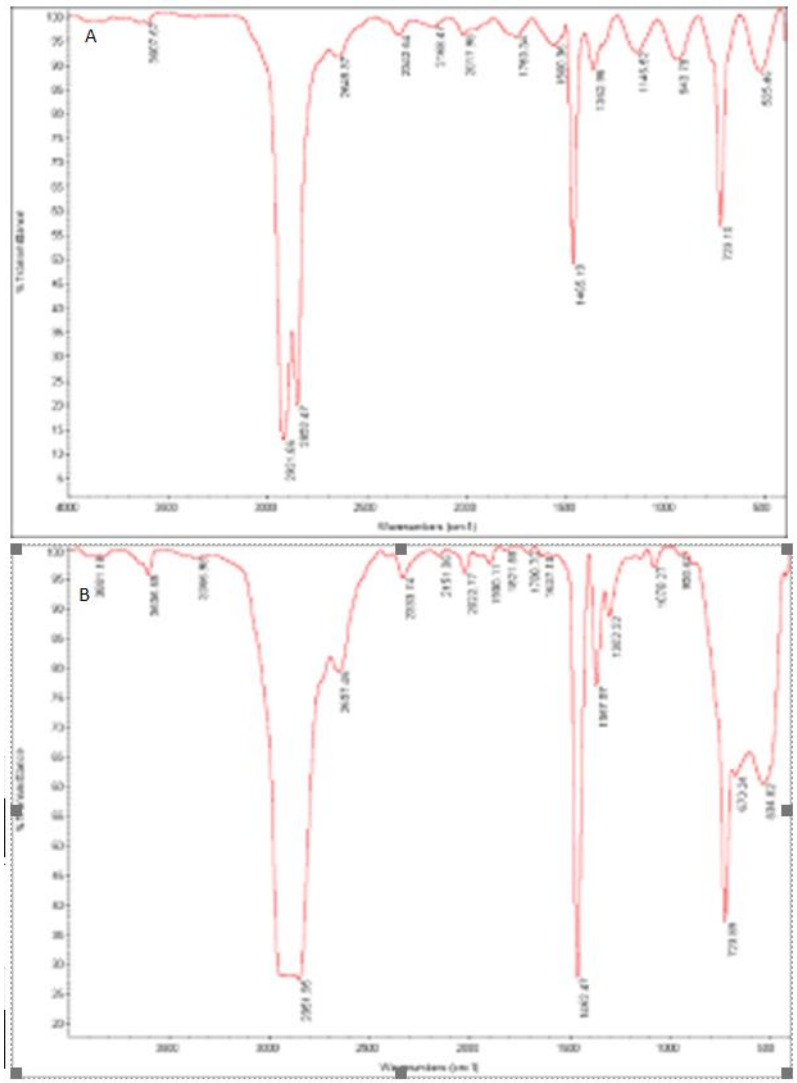

Figure 2. FTIR spectra of reference (A) and Ag and Cu nanoparticles (B).

Migration measurement

To define the range of migration threat of nanoparticles from packaging to the caviar, an inductively coupled plasma mass spectrometer (ICP-MS) instrument was applied according to the procedure (Fabricius *et al.*, 2014). To prepare each sample, 2 gr of each caviar sample was weighed, dissolved in 10 mL nitric acid and heated in a microwave for digestion at 200ºC. The digested samples were then diluted to 50 mL using pure water and analysed by ICP-MS apparatus (Li *et al.*, 2017).

Evaluation of chemical parameters

Measuring water percentage

Initially, one empty crucible was put in oven at 100ºC for 1 h, it was cooled, a sample placed on it and weighed, followed by adding 20 mL deionised water and placed in a water-bath to evaporate. Later, to dry sample, crucible was returned to oven at 100ºC for 4 h, let to be cooled, weighed, and placed in an oven for more than 1 hand weighed again for the last time. The

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percentage of water was obtained using the equation 1 (Hilderbrand, 1991):

$$\text{Moisture\%} = \frac{(A-B) \times 100}{W} \quad \text{(equation 1)}$$

Where A is the weight of each sample (g) plus the crucible, B is the final weight of each sample (g) plus the crucible (the last weight) and W is the sample weight (g).

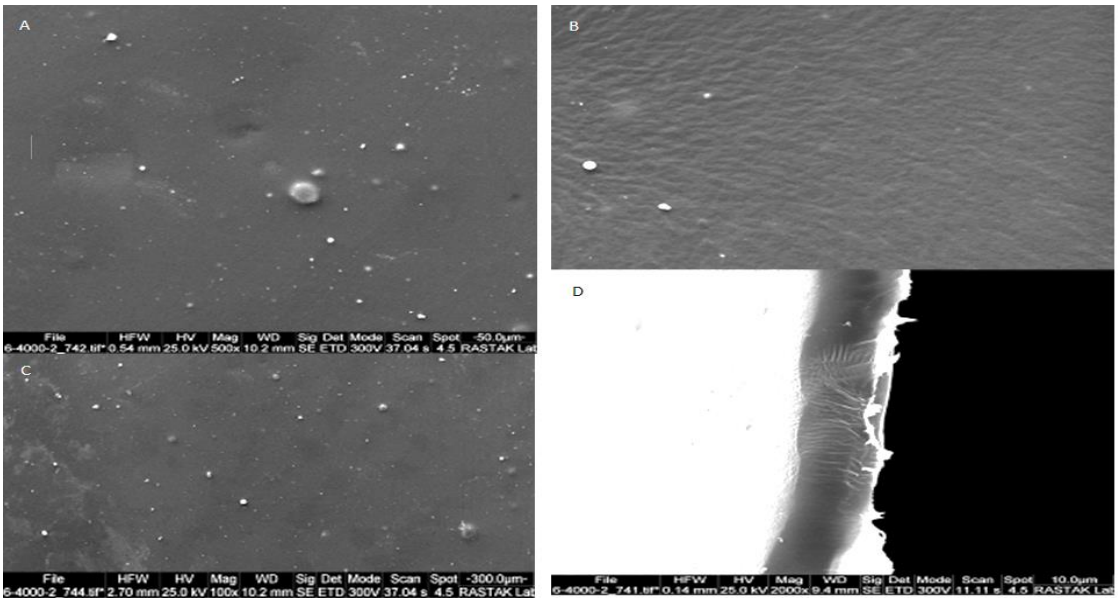

Figure 3: Field Emission Scanning Electron Microscopy with surface (A, B, C) and cross side (D) sections

Measuring water phase salt in caviar

One g of caviar accompanying with 20 mL nitric acid was added to an Erlenmeyer flask and boiled for 15 min. It was left to be cooled and 5 mL ferric ammonium sulphate was added as an indicator. The content of the flask was then titrated by 0.1 N ammonium thiocyanate. To calculate the percentage of sodium chloride (%SC) in caviar, equation 2 was used.

SC in caviar % (g) $SC\%\ (g/100g) = \frac{(A-T) \times 0.00585 \times 100}{W}$

where;

A is the consumed volume of nitrate Ag (0.1 N),

T is the consumed volume of ammonium thiocyanate, and W is the sample weight (g).

The percentage of sodium chloride in water phase salt (WPS) of caviar was determined using the equation 3 (Hilderbrand, 1991):

$$\text{WPS\%} = \frac{\text{Sodium chloride in caviar \%}}{\text{moisture \% + sodium chloride in caviar \%}} \times 100 \quad \text{(equation 3)}$$

Total volatile nitrogen (TVN) measurement

A volume of 300 mL deionized water, 5-10 gr of caviar, 2 g magnesium oxide and a couple of pieces of boiling stone were added to a distillation balloon. Then, 25 mL boric acid (2%) and a few drops of methyl red indicator were added to an Erlenmeyer flask and put

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under the cooling section in the distillation device. Subsequently, the distillation balloon was heated to a boil condition and then left to be condensed. The distilled solution was then titrated with 0.1 N sulfuric acid to measure TVN (Goulas and Kontominas, 2005; Maghami *et al.*, 2019).

Statistical analysis

Data analysis was performed using SPSS 20.0 software. The ANOVA test was used to compare the difference between groups ($p<0.05$) followed by Duncan's multiple range to pair-wise test.

Results

Scanning electron microscope (SEM)

The structure of Ag and Cu nanoparticles in TiO_2 nanocomposite was observed using a SEM instrument with a magnification of 15 and 30 KX on the scale of 1000-2000 nm. The morphology of Ag and Cu nanoparticles is shown in Fig. 3. The homogenous distribution of nanoparticles on the polymer is clearly illustrated in Fig. 4 showing different metal ions of nanopackaging. In addition, as the nanoparticles size decreased, the antibacterial activity improved. It appears that Ag and Cu nanoparticles to be incorporated into the TiO_2 nanocomposite while Ag average particle sizes was ranging between 13.5 – 31.7 nm (200 nm) and that of Cu-NPs ranged from 19.9 to 26.8 nm (200 nm) in size (Fig. 3). EDX result (Fig. 4) contained large amounts of nano-ions clearly showing different metal ions included in nanopackaging.

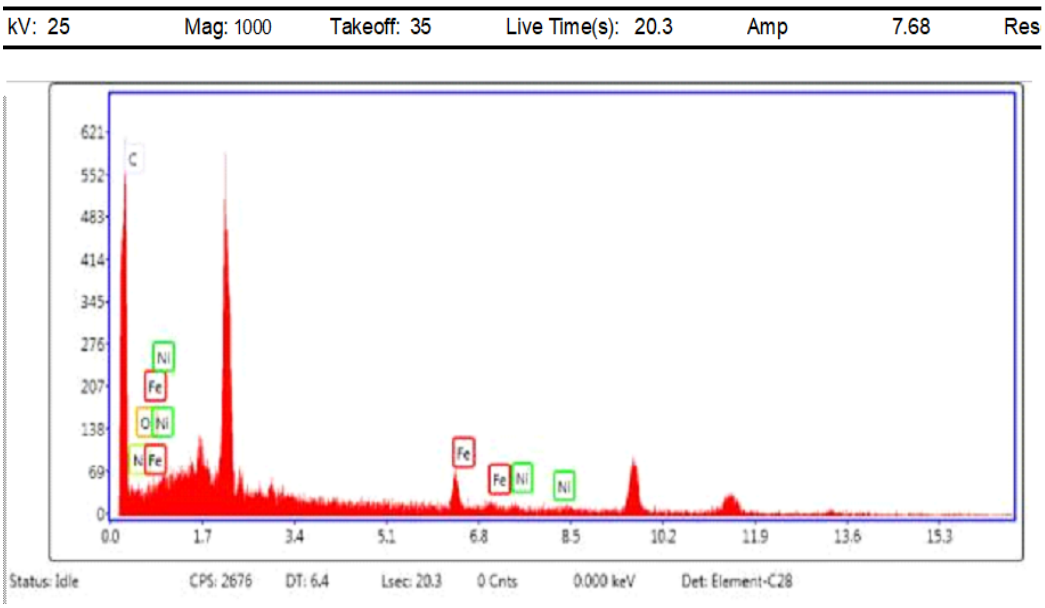

Figure 4: Energy-dispersive X-ray spectroscopy including constituent elements of nanopackaging (Ni, Fe,…)

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Antimicrobial and chemical values

The result of colony count on the aforementioned interval days is given in Figure 5. The results of the microorganisms' colony count are presented in Table 1 and Figure 5.

On each specific day (3, 5, 7, 8, 9 and 10) of the experiment, the colony count of test samples was determined and compared with the control samples on the same day. A significant difference between the colony counts of the two pathogenic agents (*E. coli* and *A. niger*) was observed on the third, fifth, seventh, eighth, ninth and tenth days. The results indicated a lower of colony counts for the test samples in comparison to their corresponding controls. Among them, the lowest amount of *E. coli* bacteria was found on days 5 and 10, while for *A. niger* the lowest amount was measured on the fifth day.

In addition, there was a gradually decreasing number of colony counts for *S. aureus* bacteria on all interval days except on the third and eighth days, with the highest amount of colony growth occurring on the third day for this bacterium. Precisely, based on a comparison between the test and control samples on each day, the lowest amount of *E. coli* and *S. aureus* bacteria were observed on the tenth day, while there was a significant difference between their colony count with the minimum amount for *E. coli* bacteria growth.

Table 1: Growth of selected bacteria affected by Ag/Cu/TiO_2 nanocomposite

Microorganisms	Days	Treatment	Control
E. coli	3	3.0×10^6	4.0×10^6
	5	1.1×10^6	4.1×10^8
	7	1×10^6	3×10^8
	8	1×10^6	3×10^8
	9	2×10^6	8×10^8
	10	1×10^6	8×10^8
S. aureus	3	3.0×10^7	1.1×10^7
	5	1.4×10^7	1.6×10^7
	7	1.9×10^7	2.1×10^7
	8	2×10^7	1.1×10^7
	9	3×10^7	2×10^6
	10	1×10^7	4×10^7
A. niger	3	5.0×10^6	6.0×10^6
	5	1×10^6	4×10^6
	7	1×10^6	2×10^6
	8	6×10^6	7×10^6
	9	4×10^6	8×10^6
	10	5×10^6	9×10^6

The results of the minimum inhibitory concentrations (MIC) test demonstrated that among different concentrations of serially diluted antimicrobial solutions, 1/4 mg/mL was the lowest concentration with antibacterial activity

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against *E. coli* and *S. aureus* growth. In presence of two pathogenic bacteria (*E. coli* and *S. aureus*), the results of the agar diffusion test exhibited no halo growth around the discs of any of the four nanocomposite samples. This result indicates the perfect inhibitory effect of synthesized Ag/Cu/TiO_2 nanocomposites against *E. coli* and *S. aureus* bacteria. On the other hand, a significant antimicrobial activity against the growth of *A. niger* was found on the fifth day while a significant inhibitory effect was defined against *S. aureus* on all interval days except the third and eighth days. The results of the microorganism colony count demonstrated that the nanocomposite containing Ag/Cu and TiO_2 had the highest antibacterial activity against *E.coli* and *S. aureus* on the tenth day, at which it was more effective in preventing the growth of *E. coli* than *S. aureus.*

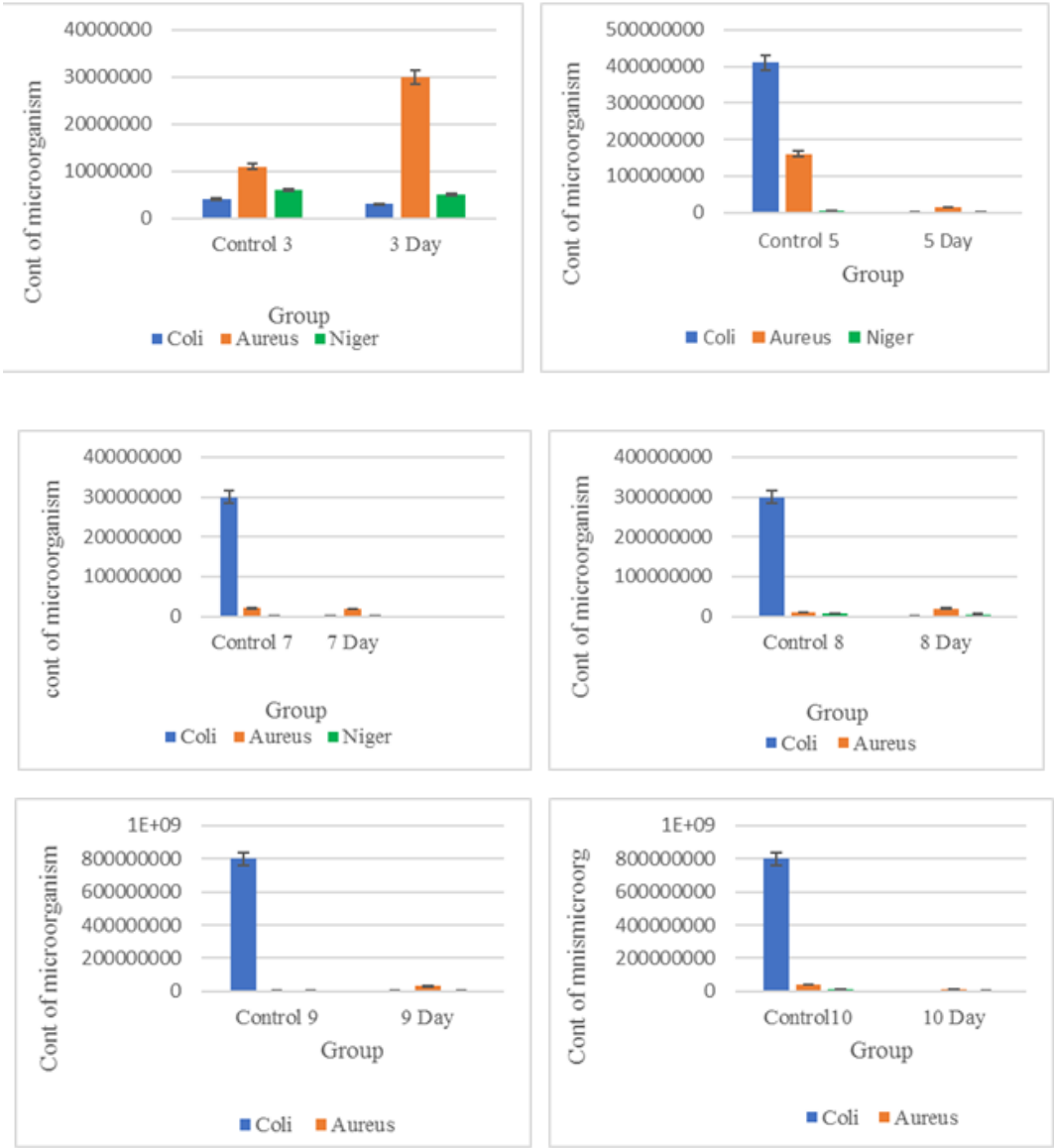

Figure 5: The results of colonies count on interval days (3, 5, 7, 8, 9, and 10).

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Table 2: The values of chemical criteria.

Experiment	Standard	Test
water %	*Huso huso* 55-45 wild caviar 45-35	*Huso huso* 47 wild caviar 41
water phase salt %	< 5	3
total volatile nitrogen (TVN)	< 30	28

Chemical analysis (Table 2) showed the caviar covered with nano-packaging was in the range of standard values. As such, the water content of caviar was about 3% less than the standard limitation (<5%) and TVN also decreased to 28 mg/100 mg, less than the determined limitation (<30 mg/100 mg).

AAS results showed no migration of nanoparticles from nano-packaging to caviar. ICP-MS results showed that 0.168 ppm of silver was released to caviar samples on 32nd and 62nd days of the experiment.

The results of the FTIR test on the synthesized Ag/Cu/TiO_2-nanocomposite were analyzed by employing infrared radiation program analysis software (Figure 3). The peaks at 2980 cm^{-1} and 2940 cm^{-1} are related to the symmetric and asymmetric C-H bond from aliphatic CH_2. Meanwhile, peaks 901 cm^{-1} and 600 cm^{-1} are shown for the CH bond of ring and OH for out of plane, respectively. The results of chemical tests including tests for water percentage, water phase salt percentage and TVN are presented in Table 2.

Discussion

In this study, a TiO_2 nanocomposite based on Ag-NPs and Cu-NPs (Ag/Cu/TiO_2 nanocomposite) was prepared. Its antimicrobial activity against three food borne pathogens (*E. coli*, *S. aureus* and *A. niger*) and its ability to extend shelf-life of caviar were evaluated. The SEM analysis exhibited that Ag/Cu nanoparticles were consistently spread onto the TiO_2-nanocomposite. Our results also indicated that the combined usage of Ag/Cu/TiO_2 nanoparticles could provide all desirable objects that we expected such as, increasing antimicrobial activity, reducing the release of nanoparticles into the food (caviar) and lowering the price compare to the other synthesized nanocomposite (due to the use of Cu). Safari and Yosefian (2006) showed raw, processed caviar and product stored at -3°C were about 9, 11 and 25 mg/100 g, which was similar to those in this study. The use of various nanoparticles (organic and inorganic) in food packaging is growing. While, interest in nanoparticles of metals and metal oxides such as Cu, Ag, Zn, Ti, Au, ZnO, TiO_2 and MgO is growing because they can endure severe administration conditions. Among them, Ag, Cu, and TiO_2 nanoparticles have been utilized because of their desired characteristics. Silver

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nanoparticles have been proven to possess potent antipathogenic properties against a broad range of microorganisms, including bacteria, viruses and fungi (Martinez-Abad *et al.*, 2012). The improved antimicrobial activities of Ag-NPs have been proved compared to the metallic Ag with larger size considering that, as much as the size of particles are smaller, the surface area is larger and consequently, better interaction with the microorganisms (Toker *et al.*, 2013). In addition, Ag-NPs can be integrated into the various matrices through different approaches and they showed high stability at high temperatures (Toker *et al.*, 2013). Copper nanoparticles have been employed due to their unique properties including antiviral and antibacterial activities, along with catalytic and surface properties and because of their low cost when compared to the other nanoparticles such as Ag (Khan *et al.*, 2016; Gondwal and Joshi Nee Pant, 2018). Titanium dioxide (TiO_2) nanomaterials have been known due to their great chemical constancy and biocompatibility along with their high photocatalysis, which provides a potent antimicrobial capacity to eliminate various microorganisms (Zhang *et al.*, 2013; Ma *et al.*, 2016).

Food-borne diseases are caused by various microorganisms, including bacteria, viruses, and parasites. *E. coli* and *S. aureus* are among the most infectious bacteria that contribute to food poisoning. *E. coli* is considered the most infectious bacteria in human feces, and *S. aureus* is one of the most predominant pathogens on humans' skin and causes 89% of all disease outbreaks caused by food contamination (Baysal and Çelik, 2019). Moreover, *A. niger* is an opportunistic pathogen that causes disease by producing aflatoxins in contaminated foods (Paterson and Lima, 2017). The risk of illness associated with these food-borne pathogens can be partially controlled by using proper food packaging.

Previous studies employed Ag, Cu and /or TiO_2 nanoparticles in combination with other materials and investigated their antipathogenic effect on different microorganisms along with their potential to prolong the shelf life of various foods. For instance, the results of a study demonstrated that using LDPE/Ag/TiO_2 nanocomposites (consisting of Ag 3%) inhibited the growth of *Candida albicans*, *S. aureus*, *E. coli* and *A. niger*, thereby increasing the shelf life of pikeperch (*Sander lucioperca*) fillets (Barani *et al.*, 2018). Furthermore, Zhang *et al*. (2017) have reported that the synthesized Chitosan-TiO_2 nanocomposites prevented the growth of *E. coli*, *S. aureus*, *Candida albicans* and *A. niger*, and increased the shelf life of food. In other research, evaluation of the antibacterial effects of Ag-TiO_2 nanocomposite film to increase the shelf life of Iranian caviar was performed (Anvar *et al.*, 2019). They found that precise concentrations of Ag-NPs could prevent microorganism' growth in caviar, thus prolonging its shelf life. In a study, a

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green method was performed to synthesize Ag and Cu nanoparticles using the extract of *Cassia occidentalis* leaves (Gondwal and Joshi Nee Pant, 2018). Their findings demonstrated that Ag-NPs exhibited better antibacterial activity against *E. coli* while Cu-NPs were more successful against *S. typhi*. Moreover, Cu-NPs displayed higher scavenging activity than Ag-NPs. An investigation by Kumar *et al*. (2015) examined the antibacterial activity of green produced CuO nanoparticles using *Aloe vera* leaf extract against three bacterial fish pathogens (*Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flavobacterium branchiophilum*). Youssef and Abdel-Aziz (2013) reported that inserting Ag-NPs into a polystyrene (PS) matrix nanocomposite significantly suppress the growth of gram-negative bacteria (*E. coli* and *Salmonella typhimurium*), gram-positive bacteria (*Bacillus subtilis* and *Enterococcus faecalis*) and yeast. The effect of Ag and Cu NPs on the properties of fish gelatin-film was investigated and the results showed a potent antibacterial effect against gram-negative and gram-positive bacteria. They also concluded that the synthesized nanocomposite could be used as an active packaging for foodstuff by preserving food from spoilage pathogens (Arfat *et al.*, 2017)

In the present study, due to all their beneficial properties, we utilized TiO_2/Ag/Cu to produce a nanocomposite that can be used in antimicrobial packaging for Iranian caviar. The structure and morphology of nanocomposite clearly indicated the proper dispersion of Ag and Cu nanoparticles into TiO_2 nanocomposite. This leads to increase antimicrobial properties with lowering cost due to the reduced amount of Ag consumed while both Ag and Cu are homogeneously dispersed into the TiO_2 nanocomposite. Furthermore, by reducing the Ag amount, the risk of Ag ions (which have poisonous traits) being released into the food is decrease. The results of our study confirmed that Ag/Cu/TiO_2 nanocomposites have high antibacterial activity against *E. coli* and *S. aureus.* However, it is more successful in inhibiting the growth of *E. coli* than *S. aureus.* This is reasonable, as *E. coli* is a gram-negative bacterium that has a thin peptidoglycan layer along it as an outer lipid membrane whereas *S. aureus* as a gram-positive bacterium that has a thick peptidoglycan layer without an external lipid layer. Thus, antibacterial agents are more effective on gram-negative bacteria than gram-positive.

Furthermore, the colour of our synthesized nanocomposite with combination of Ag-NPs and Cu-NPs has a dimmer colour than nanocomposites with only Ag nanoparticles, which could make to bring appearance unpleasant to consumers. However, due to its advantages over nanocomposites which containing only Ag-NPs, it could be considered an ideal option for the current market. Among these benefits, the increased antimicrobial effect,

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which leads to prolonged shelf life for caviar while reducing its price could attract consumers.

The synthesized Ag/Cu/TiO_2 nanocomposite observed in this study has a higher certain surface area and consequently, exhibits more photoactivity than the other nanocomposites that have been previously examined. In addition, it demonstrated inhibitory activity against *E. coli*, *S. aureus* and *A. niger* displaying its greatest preventive effect on *E. coli* followed by *S. aureus* and *A. niger*. The colour of this synthesized nanocomposite has a dimmer colour than nanocomposites with only Ag nanoparticles, which might lead consumers to find its appearance unpleasant. However, due to the synthesized nanocomposite's advantages over nanocomposites with only Ag-NPs, it could be considered an ideal option to be used on the inner surface of food packaging specifically for caviar. However, further research is required under different conditions to verify the mechanical properties of the nanocomposite.

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