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ORIGINAL ARTICLE

Effect of polymer/nanosilver composite packaging on long-term microbiological status of Iranian saffron (*Crocus sativus* L.)

Mona Eslami [a], Mansour Bayat [b,*], Amir Sasan Mozaffari Nejad [c], Azar Sabokbar [a], Amir Ali Anvar [d]

[a] *Department of Microbiology, Faculty of Science, Karaj Branch, Islamic Azad University, Karaj, Iran*
[b] *Department of Pathobiology, Faculty of Veterinary Specialized Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[c] *Department of Microbiology, Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran*
[d] *Faculty of Veterinary Medicine, Islamic Azad University, Food Hygiene Sciences, Tehran, Iran*

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Abstract *Crocus sativus* L. (saffron) is a valuable plant which is native to Iran. Saffron is the dried stigmata of the flowering part of the plant that is usually contaminated with different bacteria and fungi through production process. Antimicrobial properties of silver nanoparticles are well recognized. To survey the effects of nanosilver packaging on microbiological status of spiked, saffron samples over a six month period were chosen. Saffron samples from five regions of Khorasan province were purchased and de novo frequencies of microbial contaminants were determined using standard procedures. Totally 35 g of saffron was spiked with known numbers of four bacterial and two fungal species and packaged into one gram packets. The packaging materials consisted of polyethylene polymers containing 0, 400, 800, 1200 or 4000 ppm nanosilver (as Ag). Total and differential numbers of spiked microorganisms in the packaged saffrons were enumerated at initial and at six time points of seven, 14, 28, 64, 90 and 180 days. Baird-Parker agar (BP agar), Kenner Fecal (KF), Salmonella–Shigella agar (SS agar), Violet Red Bile Glucose Agar (VRBGA), and Sabouraud Dextrose agar (SD agar) media were used for enumeration of the six spiked microorganisms including *Staphylococcus aureus*, *Enterococcus faecalis*, *Salmonella* Enteritidis, *Enterobacter* species and *Escherichia coli*, *Fusarium oxysporum* and *Aspergillus flavus*, respectively. Direct

* Corresponding author. Tel.: +98 2144817170; fax: +98 2144817175.
E-mail address: dr_mansour_bayat@yahoo.com (M. Bayat).
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antibacterial activity of the composites was also determined. De novo frequencies of microorganisms in five saffron samples were at acceptable levels with dominance of fungi species. Nanosilver embedded packages accelerated the reduction in live microbial numbers in saffron samples and the efficacy was the best in packages containing 4000 ppm nanosilver particles. Nanosilver packaging can significantly reduce microbial burden of saffron.

1. Introduction

Saffron is a valuable spice with high added value among agricultural crops. The spice is, in fact, a dried stigmata obtained from the flowering part of the *Crocus sativus* plant (Abdullaev, 2002). It is native to Iran and farmers from Khorasan province are the major suppliers of the spice in the world (Maggi et al., 2011). The mechanical way of stigmata harvesting is not introduced yet (Asimopoulos et al., 2013), therefore, human contribution to the harvesting process is associated with high contamination rate of the product with different microorganisms, including bacteria and fungi from human origin. Microorganisms, especially spore-forming species, living in the soil and water resources, are the other contaminants of the spice (Hamid Sales et al., 2012). Saffron, as a spice, is often used in a very little amount and the presence of any living contaminants will inevitably be eliminated during cooking process, therefore, the main problem is the contribution of the contaminants to spoilage of the product, that leads to undesirable changes in flavor and taste characteristics of the product and lowers the acceptability of the spice for customers. Saffron is usually packaged in well sealed containers and stored in cool places, however, long-term storage, especially in moisture and warm places may make the product prone to reabsorption of water and regeneration of the contaminating microorganisms and finally putrefaction of the spice. Saffron should be properly packaged and stored far from moisture and light at temperatures between 5 and 25 °C. Iranian national standards organization has regulated permissible limits for the presence of some common microorganisms which may be found in saffron. According to the ISIRI: 5689 document enterococcus species and *Escherichia coli* should not be present in the saffron products at all, and the maximum permissive levels of spore-forming sulfite-reducing clostridia and molds in one gram of saffron have been limited to less than 10^2 and 10^3 microorganisms, respectively. European regulatory bodies, in addition to above mentioned microorganisms, have strictly recommended analysis of saffron samples for identification of other species, including *Bacillus cereus*, *Salmonella* species and *Clostridium perfringens* (Cosano et al., 2009).

Current advances in nanotechnology have brought about new facilities for food industry through introducing numerous targeted packaging techniques. A package is a manufactured product consisting of any material or a combination of materials which can be used to present, contain, protect, handle and distribute the goods from raw materials to finished products, in every phase of the distribution chain. Numerous plastic polymers with different barrier properties are commercially available. There are also many additive materials with antimicrobial or sensory properties which can be easily added to plastic before polymerization without any changes in physical properties of the final composite. Flexibility, clarity, low cost, ease of transport, storage and use are well known attributes of plastic (Mills et al., 2012).

Antibacterial and antifungal properties of silver ions have already been recognized (Feng et al., 2000; Yamanaka et al., 2005). Bulk silver is chemically inert, and rarely releases $Ag+$ ions into the solution. On the contrary, nano particles have shown notable chemical activity and studies have shown, after a while, 11–49% of total Ag content of silver nanoparticles have been released, as $Ag+$ ions, into the solution (Lee et al., 2012; Reidy et al., 2013; Echegoyen and Nerin, 2013). Temperature, pH, presence of O_2 or other oxido-reductants can heavily affect these phenomena.

The aim of this survey was to investigate the antimicrobial effects of nanosilver packaging on microbiological status of spiked saffron samples over a six month period.

2. Materials and methods

2.1. Materials

This is an experimental interventional study that was carried out in the microbiology department of Islamic Azad University of Karaj Branch during 2013. Our goal was to evaluate antimicrobial properties of nanosilver particles embedded to polyethylene composites on microbiological status of long-term stored saffron samples. Saffron samples were directly prepared from the trusted producers of Khorasan province. Totally 50 g of newly dried and packaged saffron samples were purchased from five production centers located in four different cities of the province (R1–R5) (ten packages of one gram saffron from each production center). Polyethylene, as granules, was purchased from a specialized market place. All culture and isolation media and related reagents were from the university resources.

2.2. Microbiology of collected samples

The collected saffron samples were first analyzed using standard procedures in order to determine de novo frequencies of bacterial and fungal species. All bacterial and fungal isolation and identification processes were according to the recommendations prepared by the Institute of Standards and Industrial Research of Iran (ISIRI). ISIRI: 9433 for sulfite-reducing bacteria under anaerobic conditions, ISIRI: 1810 for *Salmonella* species identification, ISIRI: 10899-2 for fungi species, ISIRI: 2198 for enterococcus species, ISIRI: 2946 for *E. coli*, ISIRI: 10530 for *B. cereus*, ISIRI: 2197 for *C. perfringens* and ISIRI: 6806-3 for coagulase positive staphylococci were used.

2.3. Spiked samples

Primary experiments showed that de novo frequencies and types of microorganisms in collected samples were not high enough to clearly demonstrate antimicrobial properties of nanosilver, and therefore, could not help to discriminate any changes in bacterial or fungal counts between different storage times. For achieving high sensitivity in identification processes, spiked saffron samples were prepared. A saffron sample (35 g pooled from previous stage) was prepared and homogeneously spiked with standard species of 4 types of bacteria and 2 types of fungi. For the purpose, suspensions containing 0.5 McFarland turbidity from each of the six microorganisms were prepared and were equally mixed. Several drops from the mixture were added to pooled saffron and homogenized to yield 10^6 live cells per one gram sample. Spiked microbial species were as follows: *Salmonella* Enteritidis, *Staphylococcus aureus*, *Enterococcus faecalis*, *E. coli*, *Fusarium oxysporum* and *Aspergillus flavus*. Spiked sample was divided into 35 parts and packaged into 35 packets of one gram saffron using the composite films.

2.4. Polyethylene polymer-nanosilver composite

For preparation of composite films, silver nanoparticles were chemically synthesized using AgNO3 as source materials and according to the detailed protocol reported by Darroudi et al. (2010). Polyethylene-nanosilver composite films containing different silver concentrations were produced through the extrusion method.

2.5. Packaging and analysis of spiked samples

Five types of films containing five distinct concentrations of nanosilver (0, 400, 800, 1200 and 4000 ppm) were used for packaging of the spiked samples. Storage times were proposed to be up to 180 days. All spiked samples were kept at cold room (10 °C) in a dark and dry place. Total and differential numbers of spiked microorganisms in the packaged saffron were enumerated at the initial and at 6 time points of 0, 7, 14, 28, 64, 90 and 180 days. The following media were used for plating and enumeration of the 6 spiked strains: Baird-Parker agar (BP agar) for *S. aureus*, Kenner Fecal (KF) for *E. faecalis*, Salmonella–Shigella agar (SS agar) for *S.* Enteritidis, Violet Red Bile Glucose Agar (VRBGA) for *E. coli*, Sabouraud Dextrose agar with chloramphenicol (Sc) for 2 types of fungi and Plate Count Agar (PCA) for total estimation of live microorganisms in the spiked saffron samples.

2.5.1. Colony count

All colonies grown on PCA were counted. On BP plate only colonies with black center and clear zone in the margin were enumerated. On KF plate purple to red colonies were considered. On VRBGA plate dark purple colonies with 1–2 mm in diameter and purple diffuse zone in the margin were enumerated. On SS plate colorless colonies with or without black center were considered as *S.* Enteritidis. Sc plate was selective for fungi and all grown colonies on Sc were considered as fungi species.

2.6. Direct antibacterial effect of the composites

For evaluation of the antibacterial properties of PEP–AgNP composites in liquid media, we exposed *E. coli* bacteria to the composite in tube-based broth culture (Umar et al., 2013). Briefly, the composite layers (1 control and 5 tests) were sterilized with 70% ethanol and squares of 1 × 1 mm in diameter were cut and immersed in Mueller–Hinton broth in tubes (10 tubes for each composite type). After 6 h of incubation in 4 °C (for $Ag+$ release), the tubes were placed at 37 °C and 10 min later 50 µl from *E. coli* suspension (0.5 McFarland) was added. At 10 time points of 1, 5, 10, 20, 30, 40, 50, 60, 90, 120 min 30 µl samples were taken and counted using colony-forming units (CFU) assay.

2.7. Statistical analysis

The data were analyzed by SPSS version 16.0 IBM statistical software. According to the nature of data, two independent samples *T*-test or paired samples were used. *T*-test was used for statistical comparisons. The differences among the mean values were found to be significant at $P \leqslant 0.05$.

3. Results

3.1. Microbiology of collected samples

De novo frequencies of microorganisms isolated from saffron samples collected from five main producers are presented in Table 1. Ten packages of one gram saffron were purchased from each producer, aseptically pooled and analyzed for the presence of any bacteria or fungi.

3.2. Analysis of spiked saffron samples

Totally 35 × 1 g packages had been spiked with the six microorganisms. At each proposed time point, 5 samples (one from each group) were taken and they underwent extensive analysis for isolation and enumeration of the 6 species by the CFU method. In addition to total colony counts on PCA plates, differential enumeration of each of the six species at 7 time points was done.

3.2.1. Effect of strain

Our results showed that nanosilver (embedded in the polymers) has almost the same antimicrobial effects on the six spiked species upon storage. Data related to polymers containing 0 and 4000 ppm nanosilver are presented in Fig. 1A and B, respectively. Results for polymers containing 400, 800 and 1200 ppm nanosilver were between the two values, and therefore, not presented here.

Lower section (B) represents colony counts for saffron samples spiked with 10^6 cells of 6 species and packaged in PEP–AgNP composites containing 4000 ppm Ag. Seven day preservation of saffron samples inside packages containing 4000 ppm AgNP led to statistically significant reduction in colony counts for all strains ($P < 0.01$). For 14 days, the reduction was continued but the changes only for *E. coli* and *S.* Enteritidis species were statistically significant ($P < 0.05$). *Salmonella* species at 64 days, *S. aureus* and *E. faecalis* species at 90 days of

Table 1 De novo frequencies of isolated microorganisms (CFU/g) in saffron samples purchased from 5 regions of Khorasan province (Mean ± SE).

	E. coli	*Enterobacteria*	*Salmonella*	*Enterococcus faecalis*	*Clostridium perfringens*	*Bacillus cereus*	*Staphylococcus aureus*	*Fusarium oxysporum*	*Aspergillus flavus*	Total count
R1	0 ± 0	20 ± 5	0 ± 0	0 ± 0	20 ± 5	0 ± 0	100 ± 19	500 ± 88	0 ± 0	450 ± 66
R2	10 ± 1.5	800 ± 110	0 ± 0	500 ± 88	0 ± 0	100 ± 23	0 ± 0	200 ± 44	50 ± 8	2400 ± 330
R3	0 ± 0	0 ± 0	0 ± 0	30 ± 4	50 ± 11	30 ± 5	0 ± 0	10 ± 2	10 ± 2	150 ± 39
R4	22 ± 4.5	200 ± 35	0 ± 0	0 ± 0	0 ± 0	80 ± 18	100 ± 18	0 ± 0	0 ± 0	200 ± 61
R5	0 ± 0	0 ± 0	0 ± 0	56 ± 9	30 ± 5	0 ± 0	0 ± 0	300 ± 55	0 ± 0	700 ± 101

Collected saffron samples did not have any *Salmonella* species. Enterobacter species were seen only in 3 samples (R1, R2, R4) that *E. coli* were only present in two of them with very low frequencies (R2: 10 ± 1.5 and R4: 22 ± 4.5). The right column shows total colony counts related to each producer that obtained from PCA plating. It is worth to note that several dilutions of samples were prepared for plating and all tests were done in duplicates. PCA plating results demonstrate that immediately after production and packaging, all saffron samples had notable amount of living microorganisms. R1 to R5 abbreviates five regions of Khorasan province that the samples were prepared from.
R1: Torbat-e-Heydariyeh, R2: Torbat-e Jam, R3: Qaen County (1), R4: Qaen County (2), R5: Gonabad.

preservation have completely been eliminated from the saffron samples.

3.2.2. Effect of nanosilver concentration

In addition to control packages (0 ppm nanosilver), 4 types of packages with 4 distinct concentrations of 400, 800, 1200 and 4000 ppm with embedded nanosilver were used. The comparison of total colony counts at 7 time points showed that antimicrobial effects of nanosilver composites were directly related to their silver content (Fig. 2).

3.3. Direct antibacterial activity of the composites

Known numbers of *E. coli* cells were exposed to 1 × 1 mm polyethylene–nanosilver composites in broth culture media. Temporal monitoring of viable cells in solution was carried out. Fig. 3 shows killing activity of the polyethylene films containing 4 concentrations of nanosilver. Composite containing 4000 ppm AgNP had the highest killing activity compared to others. Composites containing 400 ppm AgNP demonstrated two distinct phases: Growth inhibitory or killing effect on the first phase and no effect on cell growth on the second phase.

4. Discussion

The current study was designed to evaluate application of silver nanoparticles, as an antimicrobial agent, for packaging the Iranian saffron which is expected to account for long-term stability of saffron quality status. Saffron was usually contaminated during production stages with different bacteria species and fungi species, and long-term storage of the contaminated saffron, especially in moisture and warm places leads to taste and flavor changes and putrefaction of the product. Thus, saffron is a valuable spice provided that it is kept in optimal conditions in order to preserve its taste, flavor and other acceptance parameters. We prepared four types of polyethylene–nanosilver composites and used them for packaging saffron samples. Then, we analyzed the microbiological status of the samples in a time dependent manner.

Primary findings showed that saffron inevitably is contaminated during production process. Collected saffron samples were positive for *Enterobacteriaceae*, *Clostridium perfringens*, *Bacillus cereus, S. aureus, F. oxysporum* and *A. flavus*. Cosano et al. (2009) have also reported the presence of *E. coli*, *Enterobacter*, spore-forming bacteria and *B. cereus* species isolated from the samples while lacking *Salmonella* species. Salari et al. (2010) have also studied the microbiology of saffron samples packaged in polyethylene polymers in a 1 year period. They also noticed, in accordance with our findings, that the gradual decline in microbial numbers of the samples, they proposed this reduction probably, is due to increasing safranal production from picrocrocin content of the saffron. Antimicrobial activity of the safranal was reported by Rezaee and Hosseinzadeh (2013).

For accurate exploring of long-term impacts of nanosilver composites on contaminating microorganisms, we spiked a 35 g pooled saffron sample with 6 known strains (10^6 cells/g) and analyzed long-term viability of the organisms inside the saffron packages preserved at 10 °C in dark and dry conditions. The first question is that whether the nanosilver

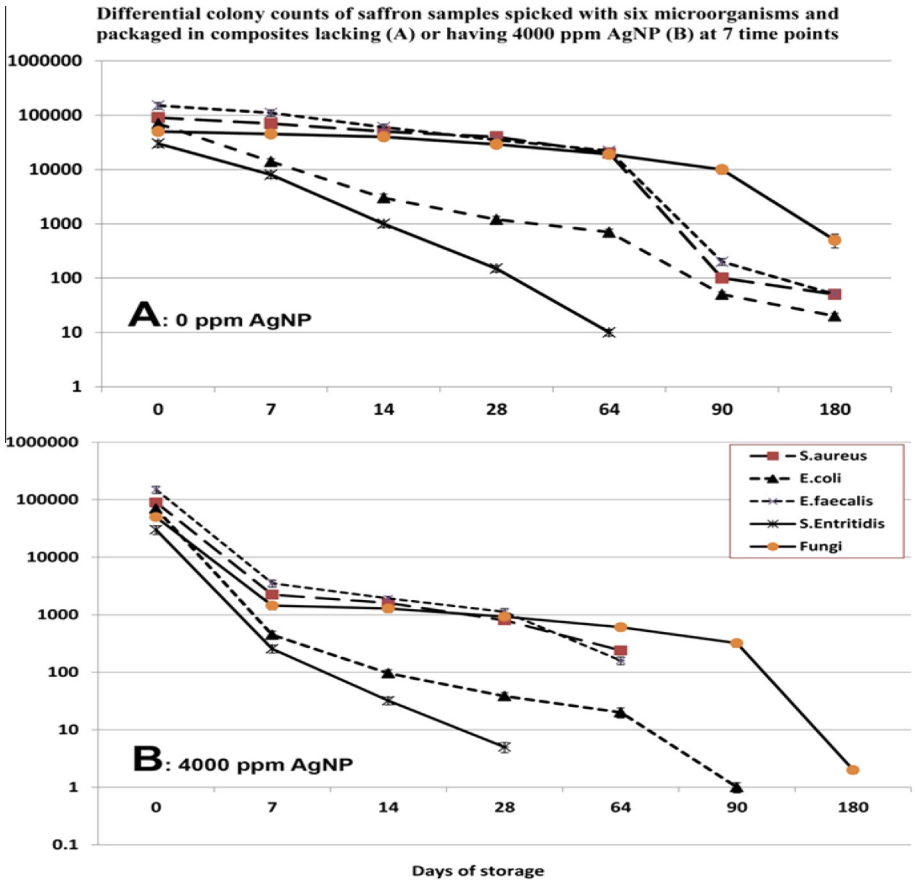

Figure 1 Differential colony counts of saffron samples, for six strains, at 7 time points, for 2 types of composites (vertical axis with logarithmic scale). Upper section (A) represents colony counts from saffron samples spiked with 10^6 cells of 6 species and packaged in PEP without silver nanoparticles. Even in the absence of AgNP, when the time of storage increases, obvious reductions in the colony counts of the six strains are apparent, but the reduction is statistically significant only for *S.* Enteritidis and *E. coli* ($P < 0.05$).

embedded in packaging materials affects the six organisms in the same manner. We observed that some strains are more susceptible than others against the antimicrobial effects of the packages, e.g., *Salmonella* Enteritidis and enterococci are more susceptible than others, while fungi are less sensitive to the agent. Similar results have been reported by Ganesh Babu and Gunasekaran (2009), Silambarasan and Abraham (2012) and Ganesh Prabu et al. (2013).

Antimicrobial activity of Ag+ ions on different microorganisms has already been reported (Feng et al., 2000; Yamanaka et al., 2005; Jung et al., 2008). It has been proposed that the antimicrobial activity of silver nanoparticles is due to the release of optimum numbers of Ag+ ions into the solution, although one report has pointed to photocatalytic activity of the nanosilver (Ahari et al., 2013) but our findings did not verify the proposal.

Silver is a precious metal and the second important question to be resolved was, which concentration of nanosilver is required for antimicrobial effects emerging, especially on saffron-like dry product packaging. Our results showed that 4000 ppm nanosilver composites (0.4% percentage fill rate) had the best function, but the effect was very weak and associated with a delay of several months, probably, because of lack of a suitable bed for ion migration or for AgNP movement. Composites with different nanosilver concentrations are commercially available. Cushen et al. (2014) have reported 0.1–0.5% filling rate of composites analyzed for exploring silver migration into solutions. The composites usually are consumed for packaging liquid products not for dry crops like saffron.

As mentioned above, delayed antibacterial effects of AgNP composites in our study may be due to the dry nature of our samples compared to soluble products like beverages and drinks. Ag ions and AgNP(s) may easily be released into soluble environments and its release rate is mainly under the control of several factors including moisture content of the product, time of contacts between NP and the environment, pH and temperature of the product, presence of oxygen or other oxidoreductants (Echegoyen and Nerin, 2013). That means, Ag+ release from silver nanoparticles is conversely related to pH value and high release occurs in lower pH of the solution (Cho et al., 2005; Barrena et al., 2009; Song et al., 2011; Von Goetz et al., 2013). For further elucidation of this hypothesis that the dryness of the saffron is responsible for delayed antibacterial impact of AgNP composites, we evaluated the composites antibacterial impact on well-known strain, *E. coli*, in broth media. Our findings showed 10 min exposure of *E. coli* cells to a small piece of AgNP-4000 ppm composites immersed in liquid media resulted in the complete elimination of the bacteria from the solution. That is why

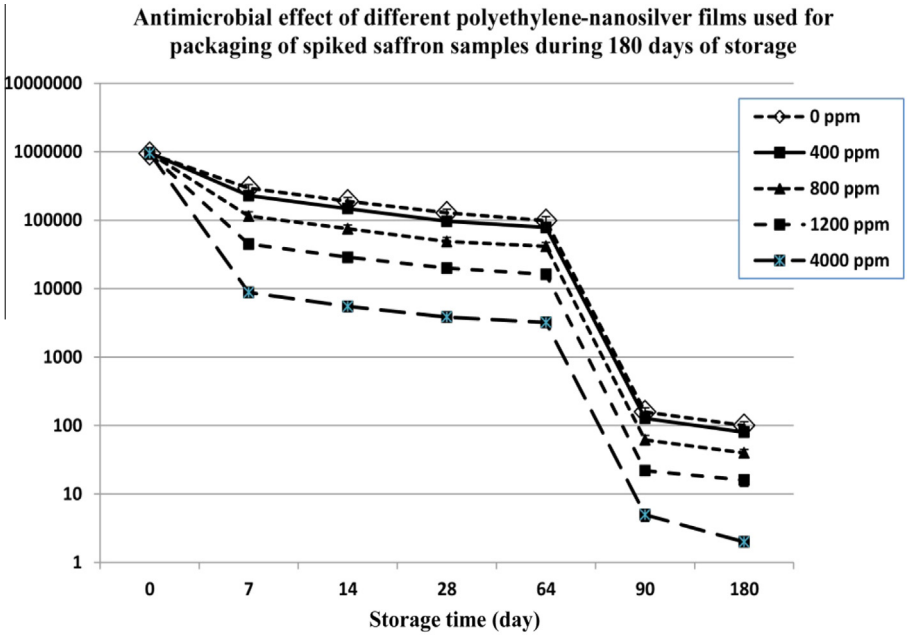

Figure 2 Effect of different silver concentrations on total colony counts at 7 time points (vertical axis with logarithmic scale). Two critical regions were observed, first between 0 and 7 days, and the second between 65 and 90 days. Seven days of storage immediately after packaging leads to significant reduction in total colony counts of all spiked species. Between 7 and 64 days a gradual decline in colony counts was seen. After 64 days significant but unexpected reductions in colony counts occur followed by a plateau region. It is worth to note that the above mentioned profile in total colony count reduction was also seen in zero concentration of silver (top line in the figure), but the reduction is statistically significant only for *S*. Enteritidis and *E. faecalis* species (see Fig. 1A).

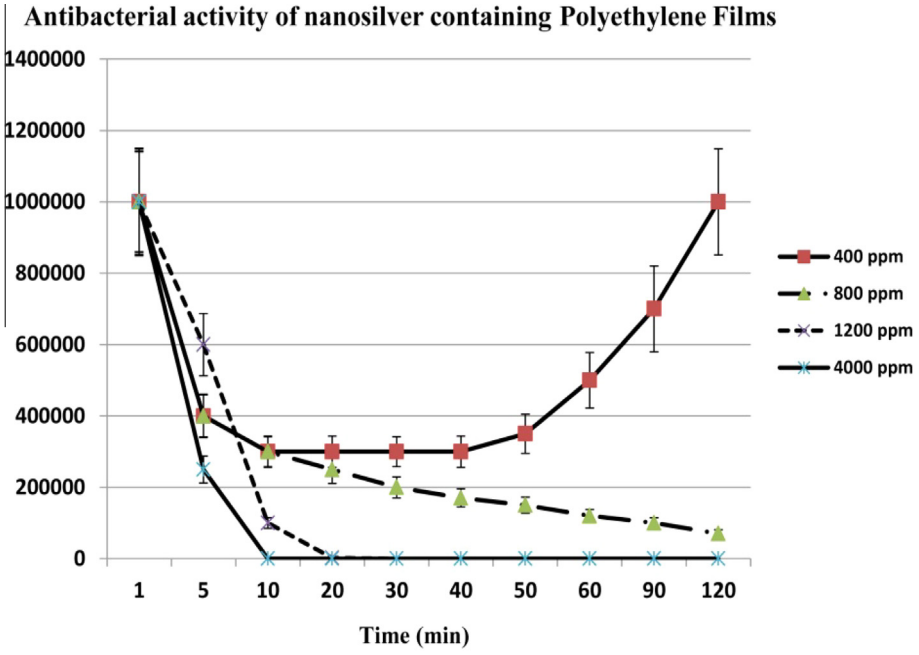

Figure 3 Antibacterial activity of nanosilver embedded polyethylene composites. A high amount of Ag+ or Ag nanoparticles release from the composite (4000 ppm) leads to increased killing effects on *E. coli* in suspension, but a lower amount of Ag release from 400 ppm films kills probably low resistant cells while keeping alive high resistance to grow later and enhances cell density in suspension.

spiked saffron samples in our study did not become sterile at all, even after 6 months of storage in relation to 4000 ppm silver composites. Ahari et al. (2013) have also reported such findings and Ag+ release in the study from composites containing 4000 ppm silver nanoparticles into products were equal to zero.

Saffron is a dry product and its microbial burden autonomously declines during storage period, but much attention should be paid to prevent the package from moisture exposure and temperature rise. Although direct exposure of microorganisms with Ag nanoparticles is deleterious (II-Hoon et al., 2006), the total numbers of released Ag nanoparticles compared to the total numbers of released Ag+ ions are very small. According to the studies of Lee and Von-Goetz only 11–12% of the released silver is in the form of nanoparticles accounting for the fact that each nanoparticle may be composed of more than 100–1000 Ag atoms, we can conclude that the total numbers of released AgNP(s) may be rare, compared to the microbial burden of the product. At least in the composite forms, silver nanoparticles are mostly present in trapped forms in the polymer matrix and their antibacterial effects are largely unfolded through substantial amounts of Ag+ ion release. Packaging dry products with polymer–AgNP composites are beneficial in order to maintain growth inhibition or killing of microorganisms, but the effect is better recognizable for liquid and semiliquid products, like beverages. Ag+ release from container walls is accelerated in acidic solutions, therefor, when acidic solutions are being packaged, silver concentration in the composite should be adjusted to follow regulatory permissive limits.

5. Conclusion

Saffron is a dry product and under optimal storage conditions, application of polymer–AgNP composites for its packaging has limited advantages but if the conditions are changed, e.g. due to any break in package or storage in high moisture place, nanosilver packaging can prevent or limit the microbial putrefaction of the product.

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