(REFEREED RESEARCH)

ASSESSMENT OF THE ANTIFUNGAL ACTIVITY OF LINING LEATHER TREATED WITH SILVER DOPED HYDROXYAPATITE

GÜMÜŞ KATKILI HİDROKSİAPATİT İLE MUAMELE EDİLEN ASTARLIK DERİLERİN ANTİFUNGAL AKTİVİTESİNİN DEĞERLENDİRİLMESİ

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ABSTRACT

In this study, silver doped hydroxyapatite (Ag-HA) prepared by the microwave method was applied to leather in the finishing composition and its antifungal effect was investigated. The surface morphology of treated leathers was observed using Scanning Electron Microscopy (SEM). The Ag content in leather samples was identified with inductively coupled plasma optical emission spectrometry (ICP-OES). The antifungal activities of the control leather and the 0% – 5% Ag doped hydroxyapatite treated leather samples on the test fungi Aspergillus niger TEM and Trichoderma viride TEM were evaluated according to ASTM D 4576-08:2008 Standard Test Method for Mold Growth Resistance of Wet-Blue Leather. According to the results of the study, the antifungal performances of the treated leather were increased by increasing the concentration of Ag in hydroxyapatite. Moreover, the leather treated with Ag doped hydroxyapatite containing 2% or more of silver showed strong antifungal activity and it was decided that Ag doped hydroxyapatite could be used as an antifungal agent on leathers.

Keywords: Silver doped hydroxyapatite, Leather, Antifungal activity, Mold resistance.

ÖZET

Bu çalışmada, mikrodalga yöntemi ile hazırlanan gümüş katkılı hidroksiapatit (Ag-HA) finisaj kompozisyonu içinde deriye uygulanmış ve antifungal etkisi araştırılmıştır. Muamele edilen derilerin yüzey morfolojisi Taramalı Elektron Mikroskobu (SEM) kullanılarak gözlenmiştir. Deri örneklerindeki Ag miktarı İndüktif Eşleşmiş Plazma Optik Emisyon Spektrometresi (ICP-OES) ile tespit edilmiştir. Kontrol deri ve %0 -%5 Ag katkılı hidroksiapatit ile işlenmiş deri örneklerinin Aspergillus niger TEM ve Trichoderma viride TEM mantarlarına karşı antifungal aktiviteleri ASTM D 4576-08:2008 "Wet-blue Derilerin Mantar Gelişimine Karşı Direnci Standart Test Yöntemi"ne göre değerlendirilmiştir. Çalışma sonuçlarına göre, muamele edilen derilerin antifungal performansları hidroksiapatit içindeki Ag konsantrasyonunun artması ile artmıştır. Ayrıca, %2 veya daha fazla Ag içeren gümüş katkılı hidroksiapatit ile muamele edilen deriler daha güçlü bir antifungal aktivite göstermiş ve Ag katkılı hidroksiapatitin deri üzerine bir antifungal madde olarak kullanılabilirliği düşünülmüştür.

Anahtar Kelimeler: Gümüş katkılı hidroksiapatit, Deri, Antifungal aktivite, Mantara karşı direnç.

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1. INTRODUCTION

Leather is an ideal nutrient source for molds because as a natural product it contains grease, dyes and tanning agents. The risk of mold growth is present in nearly all process stages of leather production as well as in its subsequent processing and use (1). Mold not only has a negative effect on the appearance and odor of the goods attacked; it can also cause considerable material damage or even render articles unfit for use. Under some circumstances a health hazard may exist for persons coming into contact with the affected goods (2).

More stringent hygiene demands have also led to the development and widespread use of materials and components with antimicrobial finishes in the leather industry. Antimicrobial finishes are used to avoid microbial colonization and its consequences as well as to prevent the spread of infection in the case of various shoe components or in certain areas such as orthopaedics, work shoes, and the like (3). Currently, adequate protection of leather is ensured solely by using toxic and ecologically critical preservatives such as benzothiazoles or phenol derivatives. However, Gu et al. noted that because of the disparate antimicrobial spectrum and the problem of toxicity, normal leather fungicides such as 2-(thiocyanomethylthio) benzothiazole are not appropriate for use in shoe lining leather (4).

One of the biomaterials most studied for its extraordinary biocompatibility, bioactivity, and osteoconductivity is hydroxyapatite (HA), a bioceramic material from the family of apatites with the general formula $Ca_{10}(PO_4)_6(OH)_2$. HA is the main inorganic constituent in human bones and teeth, and is widely used in medical applications such as implants, coatings and prostheses (5). With its ion-exchange capabilities, HA can be effective in controlling microorganisms by the introduction of transient metal ions such as silver (6).

Silver has been known as a disinfectant for many years and has a broad spectrum of antimicrobial activity while exhibiting low toxicity. Several *in vitro* studies have reported that the silver ions in HA coatings play an important role in preventing or minimizing initial microbial development (5). Recently, the antimicrobial effects of silver-substituted HA have been studied in different materials, including textiles (7).

Ag doped HA is a new antimicrobial agent used for enhancing antimicrobial performance. To the best of our knowledge it has not so far been used for the leather industry. Antimicrobial treatment can be applied in a number of ways including coating to the finished leather. The purpose of our work was to evaluate the antifungal property of leathers treated with silver doped hydroxyapatite prepared by the microwave method.

2. MATERIALS AND METHODS

2.1 Preparation and application of Ag-HA solutions to lining leather

Analytical grade calcium hydroxide ($Ca(OH)_2$, Merck), diammonium hydrogen phosphate (DAP, $(NH_4)_2HPO_4$, Merck), and silver nitrate ($Ag(NO)_3$, Fluka) were used for the preparation of Ag-HA by the microwave method according to the literature (8).

The shoe lining leathers (goat crust) used in this study were produced by a conventional process using neither bactericide nor fungicide. Leather samples were cut under sterile conditions and, except the control sample (without any treatment), 0% - 5% Ag-HA solutions (Ag-HA: lacquer: water in proportions of 1:1:1, with the addition of penetrator at 5% by total volume) were applied in the finishing composition to the grain side of these samples (0% here and after means pure HA solution without Ag). After application, the leather specimens were passed through a drying process at 105°C for 15 min and ironed at 100°C. In this way, a thin film containing Ag-HA was formed on the leather samples.

2.2 Characterization of Ag-HA treated lining leather

The surface morphology of treated leathers was observed using Scanning Electron Microscopy (FEI Quanta 250 FEG, accelerating voltage 20.0 kV, USA). Leather samples were coated with a thin film of gold using an Emitech K550X ion sputtering device at 15 milliamperes in an 8×10^{-2} mbar vacuum. Later, images were taken in a scanning electron microscope.

The Ag contents of the leather samples were determined using inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 2100 DV, USA). For this purpose the test samples were cut into small pieces and these pieces were ground with a Restch SKI mill (Germany) in order to make analyses according to SLC 2. The mill was thoroughly cleaned before processing each sample (9). To detect the Ag contents of the finished leathers and to reveal whether they were in accordance with the counted content, the leathers were digested according to modified EPA 3050B (10). 20 ml aqua Regis (a 3:1 mixture of concentrated HNO_3 (65%, Merck) and HCl (37%, Merck) was added to a 0.5 ± 0.001 g dry weight ground sample, then the tubes were covered with a ribbed watch glass and placed in a digestion vessel (Heating digester DK 6, VELP Scientifica, Italy) 180°C for 2 hours. After the wet burn operation was completed and the contents of the tube had completely dissolved, the contents were filtered through a filter with 0.45 µm diameter pores into 25 ml volumetric flasks, and made up to 25 ml with ultra-pure water. Tests were carried out in triplicate.

2.3 Antifungal activity assessment

The antifungal activities of the control leather and the 0% – 5% Ag doped hydroxyapatite treated leather samples were evaluated according to the ASTM D 4576-08:2008 Standard Test Method for Mold Growth Resistance of Wet-Blue Leather (11). The fungi *Aspergillus niger* TEM and *Trichoderma viride* TEM were investigated in the experiments.

Samples of the leather (2x2 cm^2) were placed at the center of the Petri dishes and then 20 ml of the growing medium (potato dextrose agar - PDA) was added to the upper level of the leather samples. After allowing the agar solution to solidify for approximately 20 minutes, a suspension of $1x10^5$ spores/ml of spores of the test fungus was spotted on to three points on the leather sample, on the left, on the right, and at the top. The Petri dishes were incubated for two weeks at a temperature of 26°C. After 3, 7 and 14 days of incubation periods, the degrees of growth observed on the specimens were visually assessed using the rating scale according to ASTM standard (11). Evaluation was performed by rating the percentage of the area of the 2x2 cm^2 leather samples covered by mold with a value of 0-4. In this way, a mold development of 0% was rated as 0, a mold development of 12% was rated as 0.5, a mold development of 25% was rated as 1, a mold development of 50% was

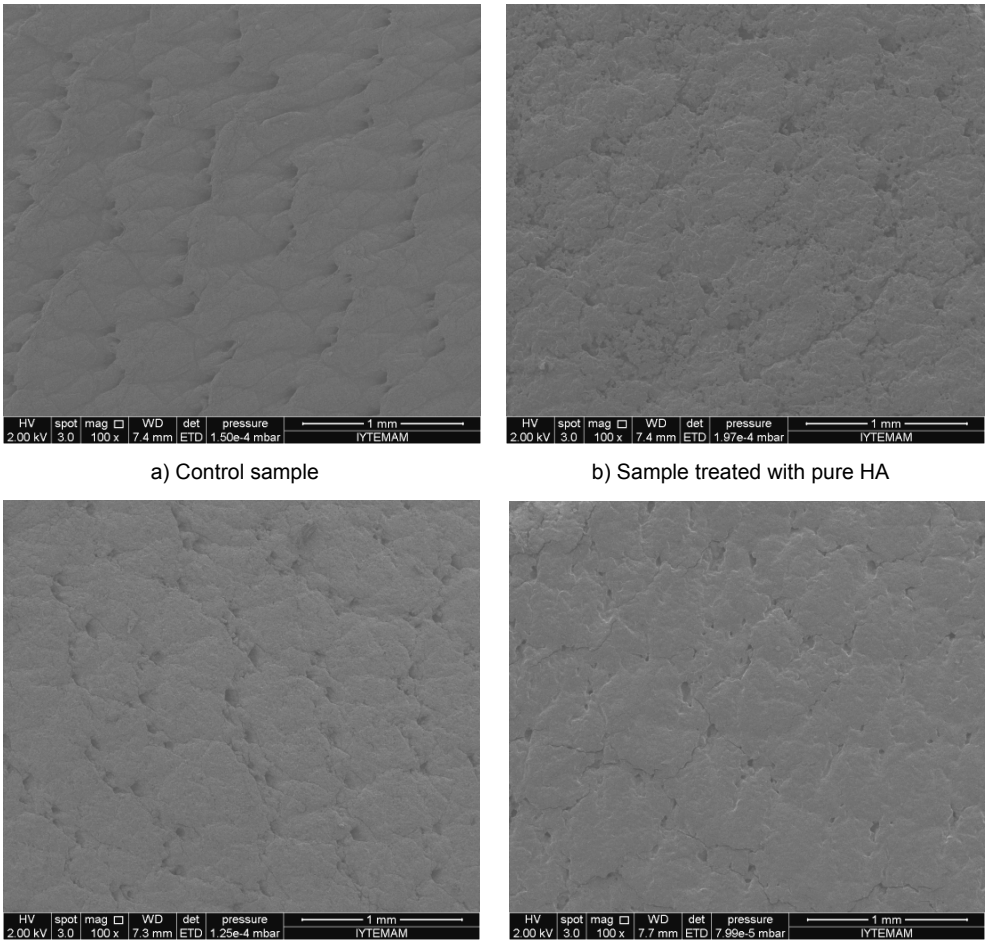
rated as 2, a mold development of 75% was rated as 3, and a mold development of 100% was rated as 4.

3. RESULTS AND DISCUSSION

3.1 Leather surface morphology

The colloidal solutions of Ag-HA which were obtained were applied to the surface of lining leather. Later, the leather samples were subjected to drying and heat treatment so that a film would be formed. Figure 1 shows SEM images of the surface morphology of the control (the untreated sample), and the samples treated with pure HA and 1% and 5% Ag-HA. It can be seen in the SEM images that the surface of the sample leather is rough and the pores are wide (Figure 1a), the samples treated with pure HA were slightly rough because of the tendency to flocculation of the HA particles in the film which formed on the surface (Figure 1b), while a very thin, light-colored matt film had formed on the surface of the leathers treated with the HA solution with the addition of Ag (Figures 1c and 1d). It was seen that because the Ag-HA film covering the lining leather was very thin, it generally took the form of the leather surface and in comparison with the control sample, the leather pores were closed. It could be clearly seen that the Ag-HA particles were closing the leather pores and that the thin film on the leather surface was more homogeneous, completely covering the leather surface. Changes in micro-structure result from the leather pores, color and shine.

Authors investigated SEM surface scanning SEM images of a thin film of an Ag-HA/Ti nanocomposite covering titanium material (12). They reported that this showed a homogeneous distribution of Ti and Ag in the thin film, and that this gave the film an antibacterial effect. Other author used a wet chemical method to synthesize an antimicrobial powder based on calcium phosphate with added metal ions (5% Ag^{+} and 14% Zn^{2+}) and applied it to fabric, which was then examined with SEM imaging (13). It was reported that the antimicrobial powder showed a homogeneous distribution on every type of cloth used in the experiment. It was found that the antimicrobial dust formed agglomerations in various places on fabric in connection with a tendency to come together to reduce surface energies. In our study, pure HA particles in the film on the surface of the leather samples showed a tendency to agglomerate, but the film layer on leathers treated with Ag-HA was more homogeneous, and this supports the above-mentioned studies.



a) Control sample b) Sample treated with pure HA

c) Sample treated with 1% solution of Ag-HA d) Sample treated with 5% solution of Ag-HA

Figure 1. SEM images of the control sample (a), and samples treated with pure HA (b), 1% Ag-HA (c), and 5% Ag-HA (d).

3.2 Silver content of leathers

Table 1 and Figure 2 show the findings regarding silver content as obtained by ICP-OES analysis. As seen in the Table, a very low silver content was found in the control sample of well below 1 ppm. In fact, in the same way, silver was found in the leather samples treated with pure HA in ICP-OES analysis. Silver is not used directly in the stages of leather processing, but it is thought that it may enter the structure of the leather as a result of impurities in chemicals used in the production process. It was seen that the amounts of silver in the leather samples treated with Ag-HA were close to the calculated values, and as the Ag concentration increased, the amount found increased. While there was a fall in the amount of silver in the leathers treated with 1-2% Ag-HA, in leathers treated with 3% and 5% Ag-HA an increase in the amount of Ag was found. However, this difference was slight and did not reach the level of significance.

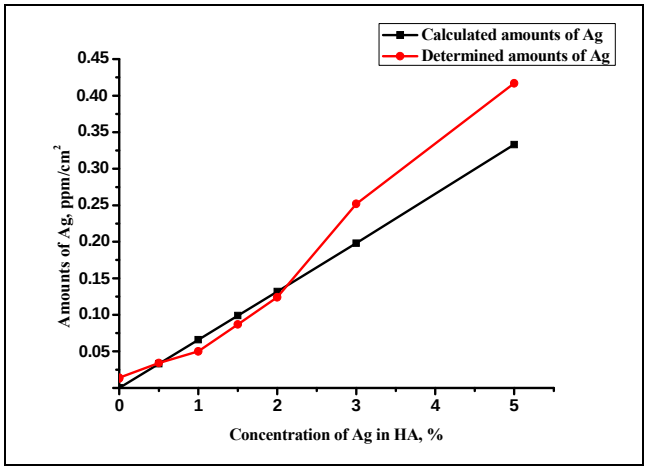


Figure 2. Amounts of Ag in Ag-HA treated leathers.

3.3 Antifungal activities

Assessment of antifungal activity against *T. viride* of the 0%-5% Ag-HA treated leathers and the control leather specimens is given in Table 2. According to the results obtained, there was intensive growth of fungi on the control leather specimens (85%) and on the leathers treated with pure HA (55%) after three days of incubation, and this reached 100% of growth after seven days on the control leathers and after 14 days on the pure HA treated leather samples. A slight growth of *T. viride* was observed on the 0.5% – 1.5% Ag-HA treated samples after three days but there was no growth on the leather samples treated with 2% – 5% of Ag-HA. After seven days of incubation, the slight growth of *T. viride* was observed on the 2% Ag-HA treated leather sample whereas no growth was determined on the 3% and 5% Ag-HA treated leather samples. After an incubation period of two weeks, the leather specimens treated with 3% and 5% of Ag-HA showed the highest antifungal activity against *T. viride* (Table 2).

Assessment of antifungal activity against *A. niger* of the leather specimens treated with 0%-5% Ag-HA and the control leather is given in Table 3. Visual examination showed that after three days of incubation the growth of *A. niger* on the control leather specimens was about 75% and 65% on the leathers treated with pure HA; after seven days of incubation it reached 100% on the control leathers and 90% on the pure HA treated leather samples; after 14 days of incubation the control sample and the pure HA and 0.5% and 1% Ag-HA treated leather samples were 100% covered by *A. niger*. A slight growth of *A. niger* was observed on the 0.5% – 1.5% Ag-HA treated samples after 3 days of incubation but there was no growth on the leather samples treated with 2% – 5% of Ag-HA. A slight growth of *A. niger* was observed on the 2% and 3% Ag-HA treated leather samples after seven days of incubation whereas such growth was determined on the 5% Ag-HA treated leather samples only after 14 days of incubation (Table 3).

The control leather samples and those treated with pure HA did not show resistance to fungi. It was observed when examining the mean protected areas that performance against both fungi increased with an increase in the silver content of the HA. While the leather samples treated with 2% and 3% Ag-HA performed well, the best antifungal performance was shown by the samples treated with 5% Ag-HA. Thus, the test results clearly show that Ag-HA can be used as an antifungal coating material on leather.

Table 1. Calculated and measured amounts of Ag in leathers treated with HA with 0-5% added Ag

Leather samples	Calculated value (ppm/cm^2)	ICP-OES value (ppm/cm^2)	Difference (ppm/cm^2)
Control	0	0.013	0.013
Pure HA	0	0.014	0.014
0.5% Ag-HA	0.033	0.034	0.001
1.0% Ag-HA	0.066	0.050	-0.016
1.5% Ag-HA	0.099	0.087	-0.012
2.0% Ag-HA	0.132	0.124	-0.008
3.0% Ag-HA	0.198	0.252	0.054
5.0% Ag-HA	0.333	0.417	0.084

Table 2. Antifungal activity against *T. viride* of 0% – 5% Ag-HA treated leather specimens

Treatment	Surface overgrown by mold in 3 days (%)	Rate	Surface overgrown by mold in 7 days (%)	Rate	Surface overgrown by mold in 14 days (%)	Rate
Control	85	3	100	4	100	4
Pure HA	55	2	90	3	100	4
0.5% Ag-HA	12	0.5	50	2	100	4
1.0% Ag-HA	5	0	50	2	100	4
1.5% Ag-HA	5	0	25	1	75	3
2.0% Ag-HA	0	0	12	0.5	50	2
3.0% Ag-HA	0	0	0	0	25	1
5.0% Ag-HA	0	0	0	0	25	1

Table 3. Antifungal activity against *A. niger* of 0% – 5% Ag-HA treated leather specimens

Treatment	Surface overgrown by mold in 3 days (%)	Rate	Surface overgrown by mold in 7 days (%)	Rate	Surface overgrown by mold in 14 days (%)	Rate
Control	75	3	100	4	100	4
Pure HA	65	2	90	3	100	4
0.5% Ag-HA	50	2	85	3	100	4
1.0% Ag-HA	25	1	65	2	100	4
1.5% Ag-HA	12	0.5	50	2	100	4
2.0% Ag-HA	12	0.5	25	1	75	3
3.0% Ag-HA	12	0.5	25	1	50	2
5.0% Ag-HA	5	0	12	0.5	25	1

It was reported that very low concentrations of ionic silver (Ag^+) were effective against many microorganisms and that concentrations of 10^{-6} – 10^{-9} M were sufficient against bacterial, fungal and viral pathogens (14). Shibata et al. obtained acrylic resin with 0, 1, 5 and 10% TiO_2-HA added, and evaluated its antifungal activity against *C. albicans* ATCC 1002 (15). It was reported that after exposure to ultraviolet light, the number of fungi on the acrylic resin with 1% TiO_2-HA added was less than that on the resin containing 0% TiO_2-HA, but the difference did not reach statistical significance; however, after exposure to ultraviolet light for 2, 4 and 6 hours, the number of fungi in the resin with 5% and 10% added TiO_2-HA showed a significant reduction. In other study, the antimicrobial characteristics of a ceramic powder of calcium phosphate with added metal ions were examined (16). The antimicrobial powder was added at a proportion of 3-5% of solid matter to the upper layer of bandages, shoe felt, tiles, paints used to cover wall surfaces, and plastic materials, and it was found that these materials gained a good antimicrobial character.

No study was found in the literature relating to the application of Ag-HA to leather and evaluation of its antifungal activity, and therefore comparisons were made with studies on the antifungal effects of silver and other metal solutions on leather. Gaidau *et al.* (17) treated wet-blue leather and metal-free leathers by immersion in nano-Ag and Ag-TiO_2 based colloidal solutions prepared by the electrochemical method. The authors found that chromium-tanned leather treated with a colloidal silver solution exhibited antifungal activity up to seven days of fungal exposure, and chromium-tanned leather treated with Ag-TiO_2 solution presented a good antifungal activity up to 28 days. The antimicrobial performance of nano-Ag coatings on leather materials against the test fungi *C. albicans* and *A. niger* was evaluated by the application of the Agar overlay method. According to the test results it was found that 20 g/cm^2 and higher concentrations of nano-Ag on the leather samples were effective against the microorganisms tested, as pronounced and clear inhibition zones were seen around these samples (18). The authors found that compared to chrome tanning, leather tanned with oxazolidine-nano-SiO_2 demonstrated a higher resistance to mold than conventional wet blue. The latter began to grow mildew when incubated for three days, whilst the oxazolidine-nano-SiO_2 tanned leather which was incubated for six days had no mildew, showing a good antifungal effect (19). In this study, the leather specimens treated with 2% - 5% of Ag-HA showed the highest antifungal activity against both fungi species for the entire testing period.

Some authors state that the broad antimicrobial spectrum of Ag-HA is similar and has the same width as the spectrum of silver ions, including bacteria, viruses, and fungi (20). While others claim that the inhibition of microorganisms is due to the release of silver ions from HA lattices into the surrounding medium (21). Nevertheless, a high concentration of silver nanoparticles may cause adverse health effects (22). Thus, a novel agent based on silver-doped hydroxyapatite would diminish the adverse effects of silver.

4. CONCLUSION

It was determined from SEM images in the study that pure HA particles in the surface film formed on leather samples had a tendency to flocculate, but that the film on samples treated with Ag-HA was more homogeneous. It was seen from the results of ICP-OES analysis that the amounts of silver observed on the leather samples treated with Ag-HA were close to the calculated amounts, and that as the Ag concentration increased, the amount found increased.

According to the results of the mold resistance test, it was found that the Ag-HA provided effective antifungal properties to the leather material. The antifungal characteristics of the Ag-HA treated leathers against the test fungi *A. niger* and *T. viride* were observed to increase along with increases in the amount of silver in the HA; leather samples treated with 2% and 3% Ag-HA showed good characteristics, but the best antifungal characteristics were shown by the samples treated with 5% Ag-HA. The results are promising for the use of Ag-HA as an antifungal coating on leather.

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