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Efficacy of Silvernano Bacterial Cellulose Insole on *Micrococcus sedentarius* Growth

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ABSTRACT

Silver nanoparticles (Ag^0) impregnated into bacterial cellulose (BC) by immersion in an antimicrobial concentration of 1,700 µg/ml (10 mM) $AgNO_3$ solution were used to synthesize silver nanoparticle-impregnated bacterial cellulose (Ag^0BC) composites. Ag^0BC incorporated into insertable footpad was developed for reduction of foot odor associated with pitted keratolysis, primarily caused by *Micrococcus sedentarius*. A scanning electron microscope (SEM) revealed the porous structure of Ag^0BC fibers impregnated with Ag^0 particles. The antibacterial activity of Ag^0BC for *Micrococcus sedentarius* revealed a growth inhibition zone of 30 mm in diameter by disc diffusion method. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of $AgNO_3$ solution against *Micrococcus sedentarius* were 0.42 µg/ml and 0.83 µg/ml, respectively. The effficiency of an Ag^0BC footpad applied to shoe samples was measured via total bacterial counts over a time period of 14 days. As expected, the percentage of surviving bacteria in three different types of shoe samples was gradually decreased in the period beginning on the 2nd day. Moreover, no bacterial growth was found in any shoe sample within 8–14 days. However, the cytotoxicity of 5.31–1,700 µg/ml $AgNO_3$ concentrations revealed 50% growth inhibition (IC_{50}) on human dermal fibroblast cells by resazurin microplate assay (REMA). Thus, it can be concluded that Ag^0BC, synthesized with lower doses of $AgNO_3$ concentration (< 1,700 µg/ml), may be able to apply in Ag^0BC footpad production for inhibition of *Micrococcus sedentarius* growth and prevention of foot odour in different types of footwears.

Keywords: Bacterial cellulose, foot odour, footpad, *micrococcus sedentarius*, silver nanoparticles

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INTRODUCTION

Silver (Ag) is a well-known metal that can be used to inhibit the growth of both Gram-positive and -negative microorganisms (Klasen, 2000a; Klasen, 200b; Morones et al., 2005; Barud et al., 2011). The effectiveness of silver nanoparticles (Ag^0), generated on bacterial cellulose (BC), in the antimicrobial agents is due to their large surface area which can effectively interact with microbes. The inhibitory processes involve the penetration of Ag particles into the bacterial cell wall, followed by the binding of Ag ions to SH (sulfhydryl) groups of metabolic enzymes and proteins. The cytotoxic mechanism of Ag particles affects cellular metabolism which leads to inhibition of cell growth by the destruction of both the electron transport system in the respiratory chain and substantial movement across cell membranes (Cho et al., 2005). Today, silvernano technology is currently used as antibacterial additives in many health care products (Vance et al., 2015).

Bacterial cellulose (BC) was produced from *Gluconacetobacter xylinus* (*Acetobacter aceti* subsp. *xylinus* or *A. xylinus*). BC is an alternative material in the production of shoe pads due to its many advantageous properties. According to these properties, BC is different from plant cellulose due to being composed of lignin and hemicellulose as its major components. BC structure is a 3-dimensional network composing of cellulose fibers, which are relatively well absorbed and store any liquid due to its porous nature. The structure exhibits high water permeability which drains an adhesive irrigation fluid over the dry surfaces (Czaja et al., 2007). BC has been used extensively in food applications and also successfully applied to the medical field, such as for artificial skins and wound dressing (Hutchens et al., 2006; Czaja et al., 2007; Shahbazzadeh et al., 2011; Wu et al., 2014). However, BC does not have the ability to kill or inhibit the growth of microorganisms (Maneerung et al., 2008).

Micrococcus sedentarius, a Gram-positive bacterium, is a major cause of pitted keratolysis in tropical regions (Almeida Jr et al., 2016). Pitted keratolysis is caused by cutaneous infection of the stratum corneum by *Micrococcus sedentarius*, although *Dermatophilus congolensis* and *Corynebacterium* species have also been implicated. Pitted keratolysis has a worldwide distribution and commonly affects people wearing occlusive footwear for prolonged periods (Kaptanoglu et al., 2012). Under appropriate conditions such as hyperhidrosis in heat and humid environments, these bacteria produce proteolytic enzymes which digest the stratum corneum of the sole's surface (Vlahovic, 2016). Plantar hyperhidrosis predisposes to foot odour associated with pitted keratolysis and pitted lesions, affecting quality of life and emotional well-being. The treatment of pitted keratolysis is based on topical antibiotics including natural antimicrobial agents; such as Gallnut (Yamuna & Sudha, 2013), fragrant agents; such as citral, citronellal and geraniol (Ara et al., 2006) and avoiding foot humidity; however, resistant cases have been reported by diverse patients (Singh & Naik, 2005; Blaise et al., 2008; Makhecha et al., 2017).

Thus, this study aims to create a silvernano bacterial cellulose (Ag^0BC) which is applied to the footpad, for inhibition of *Micrococcus sedentarius* growth, a major factor associated with foot odour (smelly feet) in tropical countries (Sokolnicki et al., 2006). There are currently many products that claim to reduce undesirable smells; for example, deodourant sprays, antiperspirant products and copper-infused socks (Quaranta et al., 2011). However, some of these products can cause allergies, irritation to the skin and increased antimicrobial resistance (Singh & Naik, 2005). Therefore, the antibacterial efficiency of Ag^0BC incorporated into an insertable footpad on shoe samples is investigated in this research to develop the current and future of quality footpad production for preventing foot odour.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

M. sedentarius (strain DMST 9365) was purchased from the Department of Medical Sciences (DMSC, Thailand). Cells were maintained on nutrient broth (NB) and transfered into the nutrient agar (NA) plates. The inoculated plates were further incubated at 37 ˚C for 24 hours (Piumnoppakun, 2009).

Acetobacter xylinum (strain TISTR 975) was obtained from the Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR, Thailand). Cells were grown in glucose yeast extract (GYE) broth at 30°C for 3–5 days. The culture was spread uniformly and futher incubated on GYE agar plates.

Antibacterial Activities of Silver Nitrate by Disc Diffusion Method

M. sedentarius was grown in NB until the optical density (OD_{625}) reached 0.08–0.1 (approximately of 10^8 cfu/ml) and spread onto the NA agar plate. Then, 1,700 µg/ml (10 mM), 170 µg/ml (1 mM) and 17 µg/ml (0.1 mM) of $AgNO_3$ solutions was dropped on the the disc which was placed on a cultured agar plate. After this plate was incubated at 37°C for 24 h, the diameter of inhibition zones was examined by comparing with a control disc (without $AgNO_3$ solution).

Production and Purification of Bacterial Cellulose

A single colony of *Acetobacter xylinum* grown on a GYE agar plate was transfered into 50 ml GYE broth. A BC pellicle was produced on the surface of the culture medium after incubation at 30°C for 24 h. The pellicle emerged with a diameter of 8.5 cm and a wall thickness of 0.6 cm; pellicles were harvested and purified in 1% NaOH and repeatly washed in distilled water followed by the method of Maneerung et al. (2008), before use in further assays.

Synthesis of Silvernano Bacterial Cellulose (Ag^0BC)

Ag^0BC was produced according to the Maneerung et al. (2008) protocol. Briefly, silver nanoparticles were impregnated into a BC pellicle by immersing in 1,700 µg/ml $AgNO_3$ solution for one hour. The BC was then washed with ethanol for 30 sec, followed by soaking in sodium boro-hydride ($NaBH_4$) solution at a proportion of 1 $AgNO_3$: 100 $NaBH_4$ for 10 min. Ag^0BC were rinsed in ultra-pure water, frozen at -20°C and freeze-dried before use in the following steps.

Antimicrobial Activity of Silvernano Bacterial Cellulose

Freeze-dried Ag^0BC was cut into disc-shapes of 6 and 15 mm and placed on an *M. sedentarius*-culture agar plate ($1x10^8$ cfu/ml). The plates were incubated at 37°C for 24 hours and the diameter of inhibition zone was measured by comparing with pure BC.

Antimicrobial Activity of Ag^0BC Footpad

Ag^0BC was cut into a foot-shape and placed on three different types of shoe samples: A, B and C. The bacterial cells were collected with sterile cotton swabs on the shoe-surface interface ($2x2$ inch^2). Each swab sample were placed and diluted in 10 ml of 0.85% NaCl solution. The bacterial suspensions were transferred to agar plates and incubated at 37°C for 24 h. Then, the total number of surviving cells were monitored by counting the number of colony-forming units (CFUs) every 2 days for 14 days, comparing with a control shoe (without Ag^0BC footpad).

Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assay

Stock solutions of 100 mM $AgNO_3$ were prepared by dissolving 17,000 µg of $AgNO_3$ in 1 ml (17 mg/ml) of distilled water. The solutions were diluted in NB to obtain an $AgNO_3$ concentration of 3,400 µg/ml. Then, serial dilutions of $AgNO_3$ solution were prepared in NB with concentrations ranging between 0.0033 and 1,700 µg/ml (20 dilutions). Fifty µl of each dilution was added to 50 µl of bacterial suspension in a 96-well microtitre plate. The plates were incubated at 37°C for 24 hours and examined for bacterial growth by comparing to the controls. MIC was defined as the lowest $AgNO_3$ concentration for inhibiting visible growth of *M. sedentarius*. The macroscopically clear wells were selected as the MIC value. MBC was determined by transferring culture from the wells observing no visible growth to NA plates. The plates were incubated at 37°C for 24 hours. The lowest $AgNO_3$ concentration showing no visible growth on NA plates was considered to be the MBC value. The experiment for each dilution was performed in triplicate and was compared with control wells, culture medium for sterility testing (well 21) and culture medium mixed with microorganism for bacterial growth (well 22).

SEM Characterization of Ag⁰BC

Scanning electron microscopy was used to observe the formation of silver nanoparticles on BC pellicles before and after exposure to $AgNO_3$. The surface images of freeze-dried BC membranes were observed and taken by SEM (JEOL model JSM-5410LV, Scientific and technological research equipment centre [STREC], Chulalongkorn University, Thailand).

Cytoxicity Assay

A Resazurin Microplate Assay (REMA) was used to evaluate the toxicity effect of $AgNO_3$ solution on human dermal fibroblast, neonatal (HDFn, C-004-5C). The assay was performed by the National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand), according to the method of O′Brien et al. (2000). Briefly, 10^4 cells were treated with various $AgNO_3$ concentrations, followed by addition of 10% Resazurin solution and measurement of fluorescence signals. Cell viability and 50% inhibitory concentration (IC_{50}) values were calculated using SOFTMax Pro software (Molecular Devices, USA). Ellipticine and 1% DMSO were used as a positive and a negative control, respectively.

RESULTS AND DISCUSSIONS

Determination of $AgNO_3$ Concentration in *M. sedentarius* Growth Inhibition

Antibacterial activity of three different $AgNO_3$ concentrations (1,700, 170 and 17 µg/ml) against *M. sedentarius* was determined by measuring the diameter of inhibition zones. It was found that the $AgNO_3$ solution exhibited zones of inhibition at least 15 mm in diameter. In the presence of 1,700 and 170 µg/ml $AgNO_3$ solution, the diameter of growth inhibition rings against *M. sedentarius* was 21 and 15 mm, respectively. In constrast, no inhibition zone was observed with 17 µg/ml $AgNO_3$ solution (Figure 1). The results demonstrated that $AgNO_3$ solution has the ability to inhibit the growth of *M. sedentarius*. The concentration of 1,700 µg/ml $AgNO_3$ solution was used for production of Ag⁰BC in the following step.

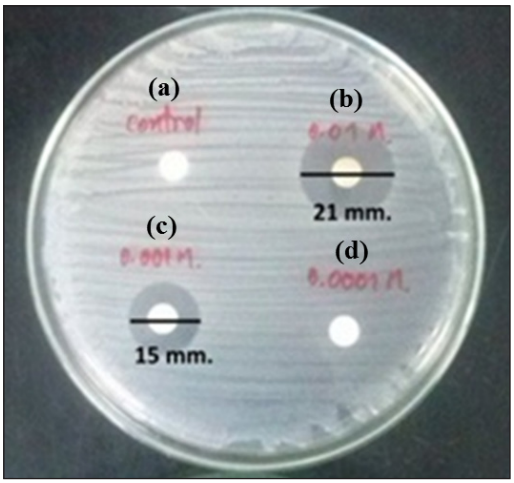
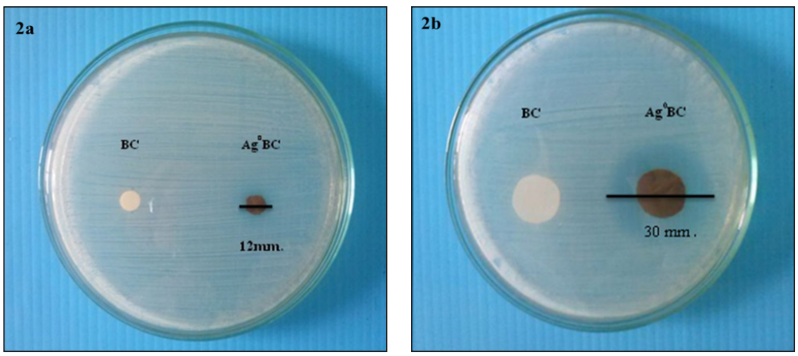


Figure 1. Three different concentrations of $AgNO_3$ solution in inhibiting *M. sedentarius* growth. Agar plate shows the zones of inhibition generated by (b) 1,700 µg/ml [0.01 M] and (c) 170 µg/ml [0.001 M] $AgNO_3$ solution, comparing with (a) control (without $AgNO_3$ solution) and (d) 17 µg/ml [0.0001 M]. Long straight lines indicate the diameter of the zones of inhibition.

Antimicrobial Activity of Silvernano Bacterial Cellulose in the Inhibition of *M. sedentarius* Growth

Freeze-dried Ag^0BC pellicles, with diameters of 6 and 15 mm, were placed on *M. sedentarius* agar-plate culture for determination of bacterial growth inhibition. The diameter of inhibition zone on Ag^0BC pellicles was 12 and 30 mm around 6 and 15 mm discs, respectively (Figure 2), comparing with the BC without Ag^0. However, the zone of inhibition exhibited on Ag^0BC around the 6 mm disc was smaller than that on the agar diffusion test around the 21 mm disc, with the same concentration of 1,700 µg/ml $AgNO_3$. These differences may result from the distribution of Ag^0 particles which are not thoroughly absorbed in BC pellicle pores during the Ag^0BC production process. Yang et al. (2012) has reported that the most typical carbon source in fermentation processes affected the structure of the BC pellicle; such as surface, pore size and shape. Thus, the BC structural changes have an effect on the amount of Ag^0 particle size distribution in BC pellicles during the Ag^0BC production process. However, the results indicated that the Ag^0BC was able to inhibit the growth of *M. sedentarius* in the presence of Ag^0 particles, while the individual BC alone expressed an undetectable antimicrobial activity.



(a) (b)

Figure 2. Antimicrobial activity of Ag^0BC in 6 mm (a) and 15 mm (b) diameter discs against *M. sedentarius*, comparing with pure BC. Black straight-lines indicate inhibition zone diameters of 12 and 30 mm around 6 and 15 mm Ag^0BC pellicles, respectively.

Efficiency of Silvernano Bacterial Cellulose Footpad for Inhibition of Bacterial Growth in the Shoe Samples

Foot-shaped Ag^0BC was placed in three different types of shoe samples: A, B and C. The inhibition of bacterial growth among all shoe samples was determined by counting the number of total bacteria every 2 days for 14 days, comparing with the control shoe (without Ag^0BC footpad). After the Ag^0BC footpad was applied to shoe samples for two days, the percentage of bacterial survival was shown to rapidly decrease to 14.7%, 0.82% and 1.67% from A, B and C shoe samples, respectively, comparing with the control shoe (84.37%)

(Figure 3). Moreover, no viable cells were found in the shoe samples A, B and C from 8 to 14 days, comparing with the control shoe (84.37%). These results indicated that the Ag^0BC footpad has additional effective inhibition of bacterial growth in all shoe samples.

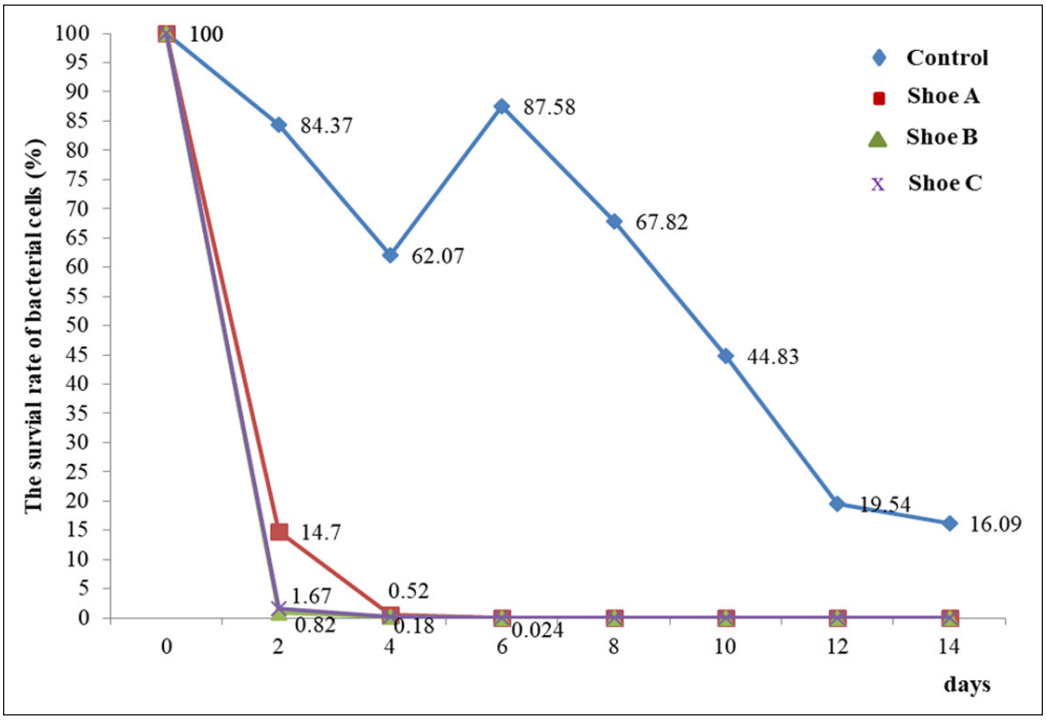


Figure 3. Percentage of bacterial survival in three types of shoe samples: A, B and C, supplied with Ag^0BC footpads for 0–14 days. From 8 to 14 days, the results demonstrated a 0% cell survival rate from all shoe samples, A(■) B(▲) and C(x), compared with 67.82–16.09% of cell survival rates in control shoes (♦).

Measurement of Minimal Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Different concentrations of $AgNO_3$ solution, 0.0033–1,700 µg/ml, were used to determine the growth inhibition effect on *M. sedentarius* by MIC and MBC evaluations. From the MIC result, the growth of *M. sedentarius* was found to completely inhibit in the number (No.) of wells 1 through 13 (Table 1), comparing with the wells of controls (wells No.21 and 22). Therefore, the MIC of $AgNO_3$ solution against *M. sedentarius* was 0.42 µg/ml (well No.13). However, $AgNO_3$ concentration at the MIC level (0.42 µg/ml) did not completely inhibit the growth of *M. sedentarius* on NA medium (Figure 4, sector 5, well No.13). The concentration of 0.83 µg/ml $AgNO_3$ solution (Figure 4, sector 4, well No.12) significantly prevented growth of *M. sedentarius* on agar medium. Therefore, for the MBC result, the lowest concentration of $AgNO_3$ solution that inhibited the visible growth of *M. sedentarius* was 0.83 µg/ml.

Table 1
Effect of silver nitrate concentrations on the growth of M. sedentarius *at the minimum inhibitory concentration (MIC) level*

Number of Wells	$AgNO_3$ Concentrations (μg/ml)	*M. sedentarius* Growth*
1-13	1,700-0.42	-
14	0.21	+
15	0.10	+
16	0.05	+
17	0.026	+
18	0.013	+
19	0.0065	+
20	0.0033	+
21	Agar	-
22	*M. sedentarius* culture	+

* Plus (+) and minus (-) symbols define as the presence and absence of *M. sedentarius* growth, respectively

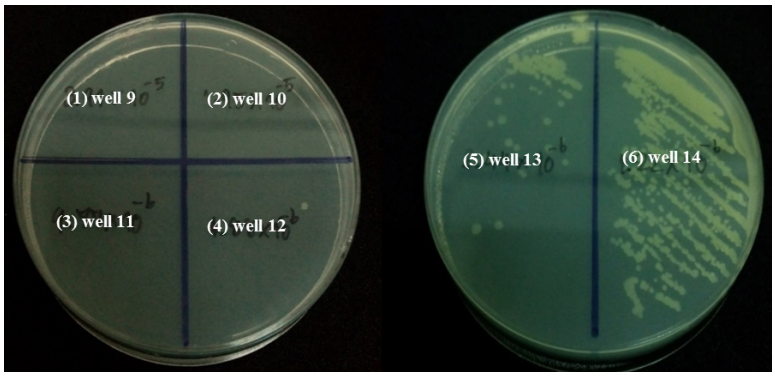
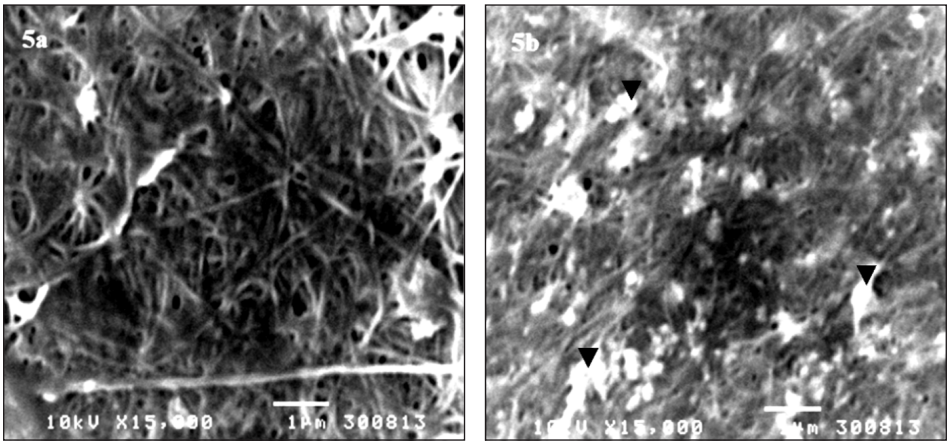


Figure 4. MBC level of different silver nitrate concentrations that inhibited the visible growth of *M. sedentarius* is shown in sector 4 (well No.12) of agar plate

Adsorption Efficiency of Silver Nanoparticles into Bacterial Cellulose by Scanning Electron Microscopy (SEM)

The 3-dimensional structure of Ag^0BC illustrated the impregnation of Ag^0 on BC surface pellicles and within the BC porous network by SEM (Figure 5). In contrast, the natural fibers of the BC structure demonstrated no impregnation of Ag^0 particles into BC networks. However, the distribution of silver nanoparticles on Ag^0BC pellicles was diffused with an irregular pattern, resulting in a size of Ag^0BC inhibition zone of less than the drops of silver solution on the disc. Therefore, the method of BC pellicle formation is necessary to develop and rearrange the fibrous network of the BC structure; such as the number of BC pores and the average size of BC pores that are suitable for impregnation and release of Ag^0 through BC pellicles (Kvitek et al., 2008).



(a) (b)

Figure 5. SEM of (a) naturally occurring BC pellicle and (b) impregnated BC pellicle with silver nanoparticles as indicated by the black arrows. The scale bar represents 1 μm.

Cytoxicity of Silver Nitrate Solution on Human Neonatal Dermal Fibroblasts with Resazurin Microplate Assay (REMA)

The cytotoxicity of $AgNO_3$ solution upon human neonatal dermal fibroblasts (HDFn, C-004-5C) was determined with the REMA plate method. By using the final concentration of $AgNO_3$ solution over the range of 5.31 to 1,700 μg/ml, IC_{50} values for dermal fibroblast cells were found to be 13.99 ± 2.50 μg/ml (Table 2). These results correlated with the study of nanosilver (Nano-Ag) effects against two normal human cell lines, demonstrating IC_{50} values of 6.33 and 6.68 μg/ml in mesenchymal stem cell and fibroblast HF2, respectively, with the MTT assay (Shahbazzadeh et al., 2011), suggesting the concentration-dependent toxicity effect of silver nanoparticles in human cell viability *in vitro*. However, the concentration of 1,700 μg/ml $AgNO_3$ solution selected for Ag^0BC production was toxic to the dermal cells. Therefore, the appropriately non-toxic $AgNO_3$ concentration implicated in silver nanoparticle synthesis should be less than 1,700 μg/ml for Ag^0BC footpad production.

Table 2
The IC_{50} values of silver nitrate solution on primary neonatal human dermal fibroblast (HDFn)

Sample	Final Concentrations (μg/ml)	IC_{50} ± SD (μg/ml)	Toxicity on HDFn
Cell + DMSO (Negative Control 1)	1% DMSO	-	non-cytotoxic
Cell + Distilled water (Negative Control 2)	Distilled water	-	non-cytotoxic
Ellipticine (Positive Control)	0.31-10.00	3.31 ± 0.75	cytotoxic
$AgNO_3$ solution	**5.31-1,700**	**13.99 ± 2.50**	cytotoxic

CONCLUSIONS

The present study revealed the 3-dimensional Ag^0BC structure that exhibited fibrous networks containing the impregnation of silver nanoparticles in porous BC surfaces. Ag^0BC pellicles generated from 1,700 µg/ml (10 mM) $AgNO_3$ concentration were able to completely inhibit the growth of *M. sedentarius* according to the disc diffusion method. Moreover, Ag^0BC incorporated into an insertable footpad was effective in reducing the total number of live bacteria in three different types of shoe samples from 8 to 14 days. However, further Ag^0BC analyses need to be conducted to find an appropriate $AgNO_3$ concentration for production of Ag^0BC-applied footpad which is less toxic to dermal cells (Farag et al., 2015) and eliminates bacterial growth in various types of footwears.

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