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Abstract

Chitin membranes containing nanosilver were evaluated for use as antimicrobial wound dressings. Chitin at a concentration of 0.25% dissolved in 5% lithium chloride-dimethylacetamide and nanosilver synthesized by gamma irradiation were used for fabrication of chitin-nanosilver membranes. UV–vis spectroscopy and energy dispersive x-ray (EDX) analysis with scanning electron microscopy (SEM) were used to confirm the presence of silver nanoparticles. Fluid absorption capacity, moisture vapour transmission rate, antimicrobial activity, effect on cell viability, *in vitro* wound healing property and the silver elution profile were determined, to assess the wound dressing properties of the chitin-nanosilver membranes. The antimicrobial efficacy of chitin membranes containing silver nanoparticles was observed against a broad range of microbes such as *Acinetobacter baumanii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Candida albicans*. The chitin-nanosilver membranes prepared with 100 ppm silver resulted in 6-log to 8-log reduction in viable counts after 24 h and had a positive impact on fibroblast proliferation. The fluid handling capacity, cell viability test and silver elution profile indicate that the chitin-nanosilver dressing can contribute to effective management of infected wounds. *In vitro* studies have demonstrated the antimicrobial activity and wound-healing properties of chitin membranes containing nanosilver.

1. Introduction

Chitin is a biopolymer endowed with excellent biological properties and has been widely explored for use as a wound dressing. Its major attractions are biocompatibility, a non-immunogenic nature, antimicrobial activity, pain-killing properties, hemostatic effect, and acceptable biodegradation products by virtue of biopolymer origin [1, 2]. N-acetylglucosamine and glucosamine with a significant role in wound repair are formed on cleavage by lysozyme. Chitin is reported to promote rapid dermal regeneration and accelerate the healing of wounds. It can be fabricated as gels, films, fibres, beads, support matrices, and in blends as well. A number of studies have demonstrated the role of chitin and its derivatives in inducing wound healing and reconstruction of connective tissue [3, 4]. The wound-healing properties of chitin and its capability to form a film make this biopolymer suitable for development of wound dressings [5].

Wound dressings with antimicrobial activity are required for management of wounds infected with microorganisms that delay the wound-healing process. Silver is a powerful antimicrobial agent which has been used for centuries to prevent and treat various diseases, most notably infections. Various dressings based on silver are being developed in different forms, such as hydrogels, foams, hydrofibers and hydrocolloids, due to increasing antibiotic resistance threats and toxicity concerns about antiseptics being used [6, 7]. Accelerated healing with dressings containing silver has been observed for burn wounds, diabetic wounds, chronic leg ulcers, and traumatic injuries [8, 9]. Silver has been used in various forms, such as sulphadiazine, silver nitrate, and silver nanoparticles in these dressings.

Nanosilver is a broad spectrum antimicrobial agent and is reported to be effective against antibiotic-resistant strains [10, 11]. Silver in the form of nanoparticles is more effective against microbes than silver due to their unique properties. The large surface area to volume ratio of silver nanoparticles provides better contact with the microorganisms, resulting in enhanced antimicrobial activity. Reduced microbial infections with silver nanoparticles have been reported in skin and burn wounds [12]. The objective of adding nanosilver to the dressings for the treatment of burn wounds is to achieve a sustained release that can provide bactericidal levels of silver for an extended period. Frequency of dressing changes and sedative analgesia is reduced with the use of silver release dressings as compared to silver cream. These benefits make nanosilver dressings attractive in burn wound care.

Chitin dressings containing nanosilver were fabricated and characterized with the aims of protecting the wound from invasive pathogenic microorganisms and promoting wound healing. The present work describes the evaluation of a chitin-based wound dressing incorporating nanosilver as an antimicrobial agent. The present study was aimed at assessing the wound dressing properties of a chitin-nanosilver dressing in terms of fluid absorption capacity, moisture vapour transmission rate, antimicrobial activity, effect on cell viability, wound healing efficacy, and silver elution profile.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells were purchased from HiMedia (CCK005). The CHO cells were grown on Dulbecco's modified eagle medium (DMEM), (HiMedia, AL007S) with 10% fetal bovine serum (HiMedia, RM10432) and 1% antibiotic antimycotic solution (Sigma-Aldrich, A5955). The cells were cultured as per manufacturer's instructions in a 5% CO_2 incubator (Model-170S, Eppendorf) at 37 °C.

2.2. Fabrication of chitin-nanosilver dressings

Chitin at a concentration of 0.25% was suspended in 5% lithium chloride-dimethylacetamide solvent and shaken overnight. Nanosilver prepared by gamma radiation using 30, 50, 70 and 100 ppm silver ($AgNO_3$) and sodium alginate as stabilizer was added to the chitin solution. The silver nanoparticles were spherical, and of small particle size in the range 3–13 nm [13]. Dressing membranes were cast from this chitin-nanosilver solution on flat glass plates. Chitin-nanosilver membranes were washed thoroughly twice with hot water to remove the solvent. Optical transmittance of the chitin membranes with and without nanosilver was recorded using a UV–vis Dynamica Halo DB-30 spectrophotometer (Dynamica Pty. Ltd, Prahran East, Victoria, Australia). UV–vis spectra were recorded in the range 300–700 nm.

2.3. Scanning electron microscopy and EDX imaging

Scanning electron microscopy (SEM) characterization of membranes was performed on a Carl Zeiss EVO MA 15 (Carl Zeiss NTS GmbH, Germany) scanning electron microscope at an accelerating voltage of 20 kV. Dry chitin membranes and chitin-nanosilver membranes were placed on double-sided carbon adhesive tape onto an aluminium SEM stub. An Oxford INCA Energy 250 (135 eV INCA X-act Peltier cooled SDD detector) energy dispersive x-ray (EDX) was used, attached to the scanning electron microscope, to identify the elemental composition of the chitin and chitin-nanosilver membrane.

2.4. Fluid absorption capacity of chitin-nanosilver dressings

The absorption capacity of chitin membranes with nanosilver was determined by weighing the membranes periodically and for up to 96 h after immersion in the test fluid consisting of sodium chloride and calcium chloride solution, similar to wound fluid and serum (BP, 1995). Absorption was determined according to the following equation (equation (1)).

$$\text{Fluid absorption capacity (\%)} = (W_s - W_d)/W_d \times 100 \quad (1)$$

where W_d and W_s represent the weights of the dressings in the dry and swollen states.

2.5. Moisture vapour transmission rate of chitin-nanosilver dressings

The moisture vapor transmission rate (MVTR) of chitin membranes containing nanosilver was measured as per the standard test method for vapour transmission of materials, ASTM E96 [14]. Chitin-nanosilver membranes were placed tightly over the test solution in a container [15] and reweighed periodically up to 96 h. The MVTR ($g/m^2/h$) was determined by the decrease in weight of a container covered by a chitin membrane by the following equation (equation (2)).

$$\text{MVTR} = (G/t)/A \quad (2)$$

where G is the weight loss of the samples (g), t is the test time (hours), and A is the effective membrane area (m^2).

2.6. Evaluation of antimicrobial activity of chitin-nanosilver dressings

The antimicrobial activity of chitin-nanosilver dressings was evaluated *in vitro* using microbial strains *Acinetobacter baumanii* (MTCC 1920), *Enterobacter cloacae* (MTCC 509), *Escherichia coli* (MTCC 1687), *Klebsiella pneumoniae* (MTCC 432), *Proteus vulgaris*

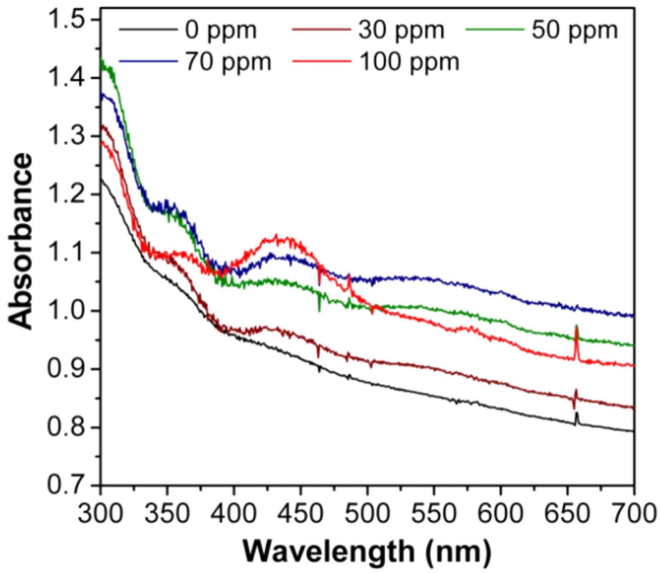

Figure 1. UV-vis absorption spectra of chitin membranes.

(MTCC 426) and *Candida albicans* (MTCC 227). The chitin-nanosilver membranes were immersed in tryptone soya broth inoculated with 10^4 to 10^5 CFU/ml of the test organism and incubated. The samples were serially diluted and plated for viable counts at periodic intervals up to 168 h, using a soyabean casein digest agar medium.

2.7. Silver elution profile

The release of silver from chitin membranes containing 100 ppm nanosilver was estimated in sterilized distilled water. The samples of size 10 cm × 10 cm were incubated in 100 ml distilled water for 1, 2, 3, 4, 5, 6 and 7 days at 37 °C. The samples were filtered through an 0.22 μm filter after the removal of membranes and stored at 4 °C for silver quantification. The silver concentrations were measured by atomic absorption spectrophotometer novAA 400 (Analytic Jena, Germany).

2.8. Cytotoxicity test

The cell cytotoxicity of chitin-nanosilver membranes was evaluated by the MTT (3-[4,5-methylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. CHO cells were seeded at a density of 10 000 cells per well in a 96-well plate. Circular sections of chitin membrane prepared with different concentrations of 0, 100, 200, and 300 ppm nanosilver and 1 μl each of dimethylacetamide (DMAC), 5% lithium chloride-dimethylacetamide solvent (DMAC + LiCl), and silver nanoparticles (AgNP) were added in respective wells for 6 h and 24 h. After incubation, the media was removed carefully from the microplate. Next, 100 μl of fresh serum free media and 10 μl of MTT solution (M2128, Sigma) was added to each well. The plate was incubated at 5% CO_2 in the dark for 3 h. After incubation, 100 μl of acid/alcohol solution was added per well. The absorbance at 570 nm was recorded by multi-mode microplate reader (Synergy H1 Hybrid, Biotek Instruments Inc).

2.9. Wound healing assay

In vitro wound healing scratch assaying of chitin-nanosilver membranes was performed on fibroblast cells. CHO cells were seeded in 6-well plates at a cell density of 1 × 10^6 cells/well. A scratch was made using a 200 μl microtip across the diameter of the well after overnight incubation. Chitin membranes with different nanosilver concentrations (0, 100, 200, and 300 ppm) were placed at the center of each scratch. The migrated cells were observed at 0 h, 2 h, 4 h, 6 h and 24 h time points using a FLoid™ cell imaging station. The area covered by the migrated cells was calculated by ImageJ software measuring the mean gray area of the images.

2.10. Statistical analysis

Data are presented as mean ± standard error mean. A student's t-test was used to statistically evaluate significant differences. Differences were considered statistically significant if $p < 0.05$.

3. Results

Chitin membranes containing nanosilver were characterized using UV–vis spectroscopy. Figure 1 presents the UV-visible absorption spectra of chitin and chitin-nanosilver membranes prepared with concentrations of 0, 30, 50, 70 and 100 ppm silver. No absorption peak was observed for the chitin membranes without nanosilver. Surface plasmon absorption centering around 420–430 nm was observed for the chitin membranes impregnated with different

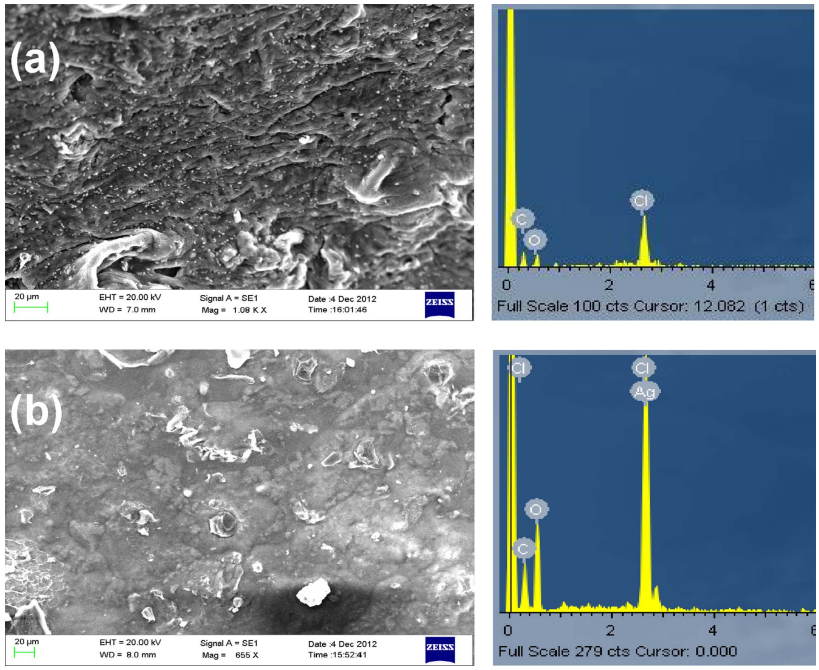

Figure 2. SEM and EDX images of: (a) chitin membrane without nanosilver; (b) chitin membrane with nanosilver.

Table 1. Fluid absorption capacity of chitin membranes containing nanosilver.

	Fluid Absorption Capacity (%)				
Time (h)	0 ppm	30 ppm	50 ppm	70 ppm	100 ppm
2	2.31 ± 0.78	2.72 ± 0.73	2.16 ± 0.31	2.19 ± 0.03	2.15 ± 0.72
6	2.79 ± 0.96	3.45 ± 0.81	2.70 ± 0.11	2.77 ± 0.02	2.91 ± 0.98
24	2.88 ± 1.02	3.46 ± 0.79	3.01 ± 0.08	2.89 ± 0.02	2.97 ± 0.99
48	2.93 ± 1.08	3.68 ± 0.91	3.22 ± 0.17	2.97 ± 0.01	3.08 ± 1.09
96	2.91 ± 1.12	3.75 ± 0.94	3.32 ± 0.23	3.01 ± 0.01	3.13 ± 1.15

Values are mean ± SE ($n = 4$); $p > 0.05$.

concentrations of nanosilver. The peak at about 420–430 nm for the chitin-nanosilver membranes reflects the surface plasmon resonance absorption of silver nanoparticles.

The surface microstructure of the chitin and chitin-nanosilver membranes was studied using SEM (figure 2). The surface morphology of chitin-nanosilver composite membranes shows the presence of silver nanoparticles that are dispersed. The elemental analysis of the chitin and chitin-nanosilver membranes was done through EDX spectroscopy. Representative EDX spectra are shown for chitin membranes with and without nanosilver. EDX spectra (figure 2(b)) confirm the presence of silver nanoparticles in the chitin-nanosilver membranes.

The fluid uptake of chitin membranes with different concentrations of nanosilver was recorded. The degree of absorption of chitin-nanosilver membranes with increasing immersion time in test fluid is presented in table 1. The fluid absorption capacity of chitin membranes was 2.70% ± 0.11% to 3.45% ± 0.81% at 6 h. No significant increase in absorption capacity was observed at 24 h. The absorption capacity ranged from 2.88% ± 1.02% to 3.46% ± 0.79%. The rate of fluid absorption was found to decrease significantly with time. Change in the percentage absorption of the chitin films was not significant up to 96 h. No effect was evident of nanosilver concentration on the fluid absorption by chitin membranes. The fluid absorption capacities of membranes with and without nanosilver were comparable and the difference was not significant ($p > 0.05$) between the two types of membranes.

The MVTR ($g/m^2/24$ h) of chitin membranes is presented in figure 3. The MVTR for chitin membranes both with and without nanosilver was found to decrease with time. At 48 h, the MVTR was 3975.5 ± 480.7 $g/m^2/h$ for chitin membranes without nanosilver and in the range of 3900.7 ± 492.7 to 4786.31 ± 284.7 $g/m^2/24$ h for chitin membranes with nanosilver ($p > 0.05$). With time, a further decrease in MVTR was observed. After 96 h, the MVTR of chitin-nanosilver membranes was 2693.6 ± 225.2 to 3323.9 ± 136.7 $g/m^2/24$ h.

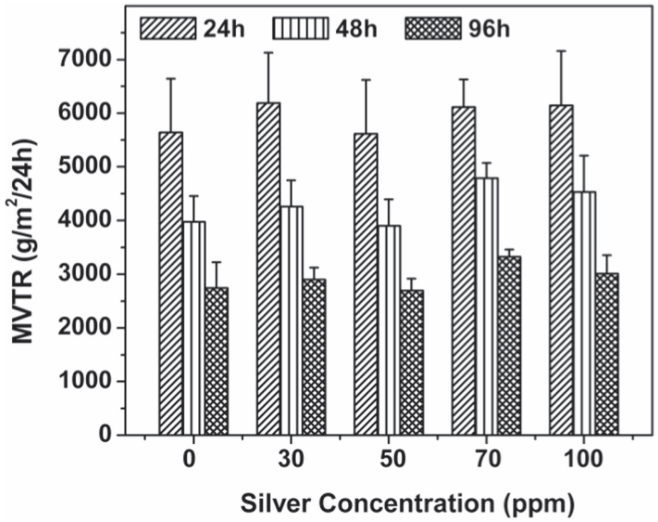

Figure 3. Moisture vapor transmission rate of chitin membranes containing nanosilver.

The antimicrobial efficacy of chitin membranes containing silver nanoparticles was tested against different microbial strains. The effect of chitin-nanosilver dressings on *Acinetobacter baumanii* is presented in figure 4(a). A bactericidal effect was evident on exposure to chitin-nanosilver dressings. However, the bacterial counts were found to increase with time in the absence of a chitin-nanosilver dressing. After 6 h of exposure, about a 5-log reduction in counts was detected with the dressing of 30 ppm nanosilver as compared to the dressing without nanosilver. Maximum cell reduction was detected in the presence of a chitin-nanosilver dressing of 100 ppm. No cell viability was detected in the presence of 70 and 100 ppm nanosilver at 24 to 168 h.

The antimicrobial efficacy against *Enterobacter cloacae* is presented in figure 4(b). The initial counts of *Enterobacter cloacae* in the broth were in the range of 4.88 to 5.27 log CFU/ml. There was 2-log reduction in the cells after 3 h of exposure to chitin-nanosilver membranes of 30 and 50 ppm. The reduction was not significant with time in the presence of 30 and 50 ppm chitin-nanosilver membranes. About a 3-log reduction in count was observed with nanosilver dressings of 70 and 100 ppm after 3 h of exposure. Complete killing was observed with the 70 and 100 ppm nanosilver dressings after 6 h. No cell viability was detected with the 70 and 100 ppm chitin-nanosilver membranes up to 168 h of exposure.

Figure 4(c) represents the killing effect of chitin-nanosilver membranes against *Escherichia coli* with an initial count of log 4.83 $\pm$ 0.09 CFU/ml. Continuous retardation of growth was seen, with complete killing over time. No viable counts were detected in chitin-nanosilver dressings with 50 ppm silver after 72 h. Complete cell death was observed after 24 h with 70 ppm and after 6 h in the presence of the 100 ppm chitin-nanosilver membrane. No bacterial growth was observed with 50 to 100 ppm nanosilver dressings up to 168 h.

The antimicrobial effect of chitin-nanosilver dressing on *Klebsiella pneumoniae* is presented in figure 4(d). The initial bacterial load was in the range of 4.75 to 5.08 log CFU/ml. A bactericidal effect was observed with chitin-nanosilver membranes and the effect was found to increase with the silver concentration. Significant reductions in viable counts were recorded with the 30, 50 and 70 ppm nanosilver membranes up to 168 h of treatment as compared to the absence of chitin-nanosilver membranes. Complete killing was observed after 3 h with 100 ppm nanosilver. No cell viability was detected after 24 to 168 h in the presence of chitin-nanosilver membranes containing 100 ppm nanosilver.

Initial contamination of *Proteus vulgaris* was in the range of 6.56×10^4 to 1.52×10^5 CFU/ml. Viable cell counts were reduced in the presence of 30, 50, 70 and 100 ppm nanosilver-containing chitin membranes (figure 4(e)). About a 2-log reduction in the viable cells was observed after 3 h in the presence of 30 to 100 ppm nanosilver dressings. Cell counts in the presence of the 100 ppm nanosilver dressing were recorded after 6 h of treatment to be 0.97 $\pm$ 0.37 log CFU/ml. However, counts in the absence of a chitin-nanosilver dressing were 6.90 $\pm$ 0.18 log CFU/ml. A bactericidal effect was observed with the 100 ppm nanosilver membrane after 24 h of incubation. No cell viability was detected up to 168 h in the case of treatment with a 100 ppm chitin-nanosilver membrane.

Antimicrobial activity of chitin-nanosilver membranes against *Candida albicans* is shown in figure 4(f). The antifungal activity of the membrane containing silver nanoparticles against *Candida albicans* was observed. The increase in the killing effect was found to be proportional to the concentration of nanosilver and the time over which the strain was exposed to

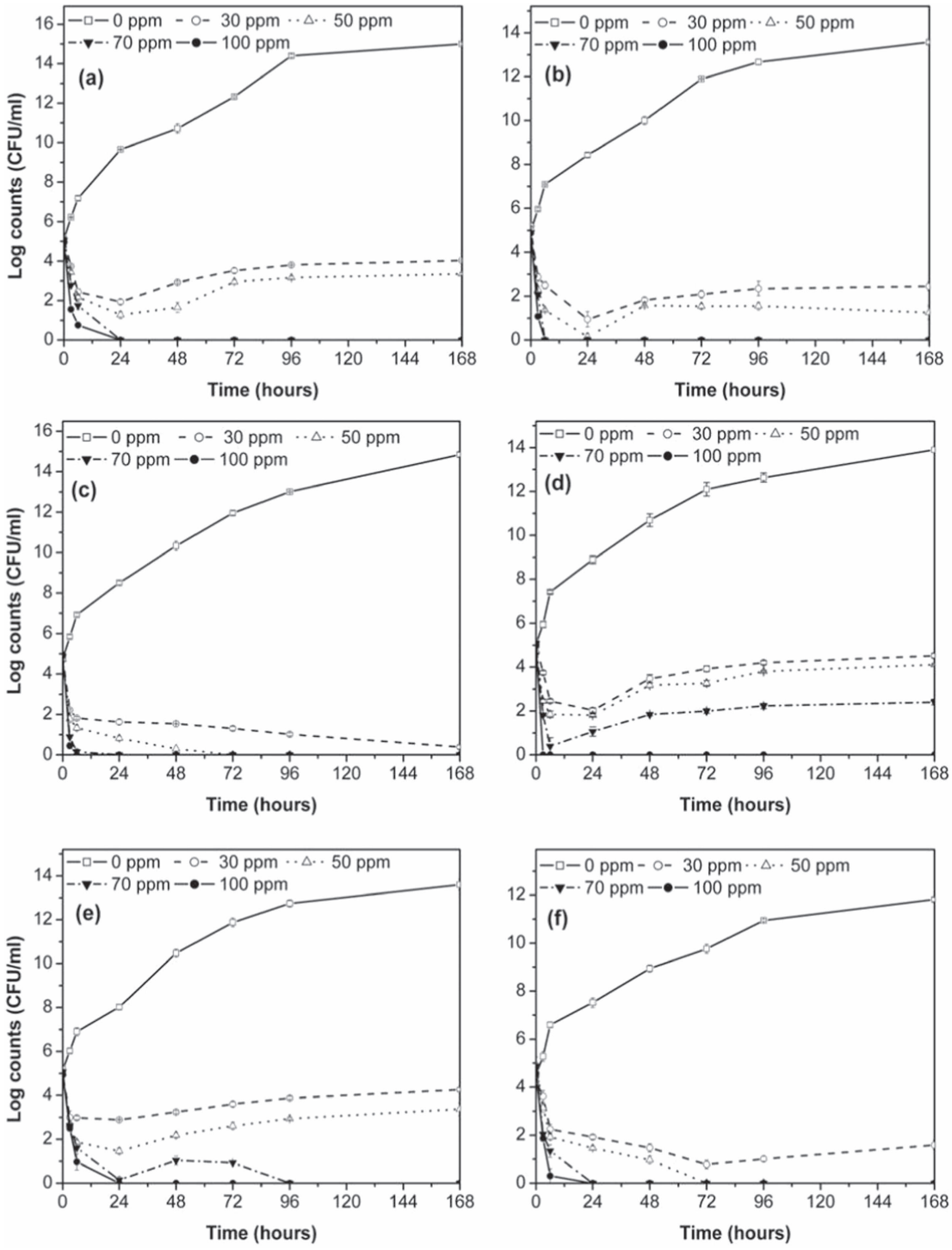

Figure 4. Antimicrobial effect of chitin membranes containing nanosilver on: (a) *Acinetobacter baumanii*; (b) *Enterobacter cloacae;* (c) *Escherichia coli;* (d) *Klebsiella pneumoniae*; (e) *Proteus vulgaris,* and; (f) *Candida albicans*. Data presented are mean ± SE, n = 4.

nanosilver. A 4-log reduction in viable cell count was observed for *Candida albicans* with 100 ppm nanosilver chitin membranes treated for 6 h. No cell viability was detected with the 70 and 100 ppm silver nanoparticles membranes after 24 h exposure. No growth or proliferation was observed with time up to 168 h in the presence of 70 and 100 ppm chitin-nanosilver membranes.

Silver release from chitin-nanosilver membranes was measured periodically. Release of silver as observed up to 7 days is presented in figure 5. Silver release from the chitin-nanosilver membranes prepared with 100 ppm nanosilver was 23 ppm on day 1 and was found to increase with time. A maximum release of 40 ppm was recorded at 7 days. The chitin-nanosilver membranes prepared with 100 ppm silver were effective against the wound pathogens tested and the silver release was recorded in the range reported for clinical use. Membranes with 100 ppm (NS-1) and higher concentrations of 200 ppm (NS-2) and 300

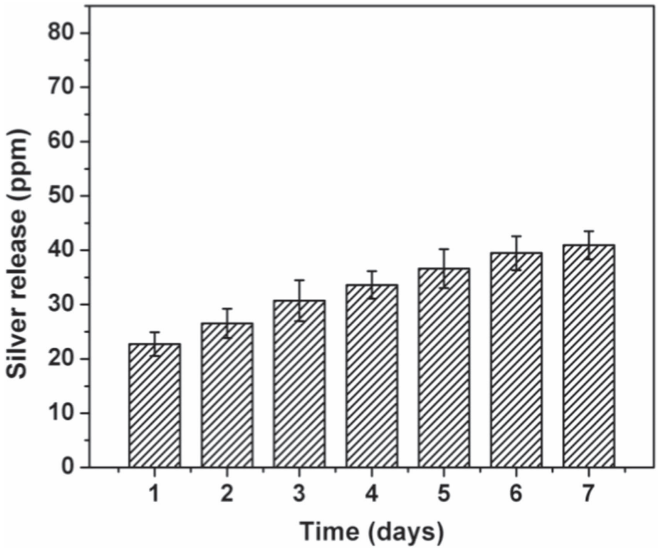

Figure 5. Silver elution profile of chitin-nanosilver membranes. Data presented are mean ± SE, n = 4.

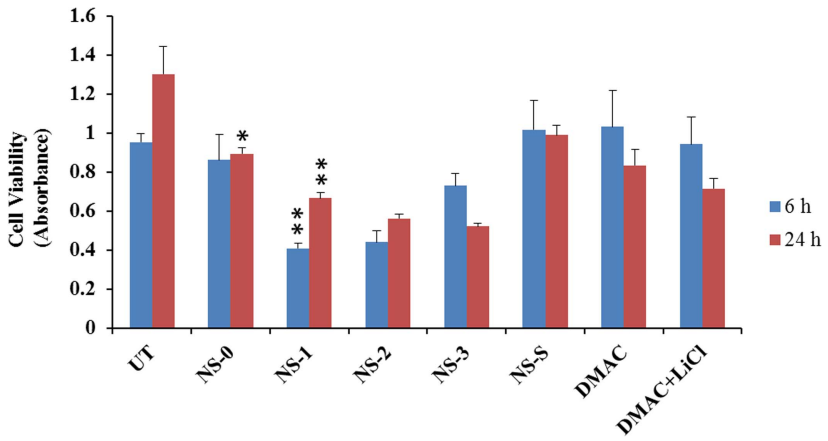

Figure 6. Cell viability of fibroblast cells in the presence of chitin membranes containing different concentrations of nanosilver and solvents. Data presented are mean ± SE (n = 4). $^{*}p < 0.05$, $^{**}p < 0.005$. (UT: untreated; NS-0: 0 ppm; NS-1: 100 ppm; NS-2: 200 ppm; NS-3: 300 ppm; NS-S: nanosilver; DMAC: dimethylacetamide; LiCl: lithium chloride).

ppm (NS-3) nanosilver were used for MTT and wound-healing assay.

Effects of chitin membranes, chitin-nanosilver membranes, nanosilver and solvents on the cell viability of fibroblast cells were investigated using an MTT test (figure 6). The results show no adverse effects of DMAC, DMAC + LiCl, and nanosilver synthesized by gamma radiation on the cell viability. Lower cell viability was recorded for chitin membranes containing nanosilver as compared to chitin membranes without nanosilver. There was an increase in cell viability after 24 h in the presence of chitin membranes containing nanosilver (NS-1 and NS-2). However, cell viability was found to decrease after 24 h of incubation with a 3X nanosilver membrane.

To assess the wound-healing effects of chitin membranes containing nanosilver, a scratch assay was performed. Representative images of *in vitro* wound healing scratch assays performed on fibroblast cells (CHO cell line) following the treatment with chitin membranes with and without nanosilver after 0, 2, 4, 6 and 24 h are presented in figure 7. The mean gray area representing the cell-free area is presented in figure 8. To evaluate the wound closure, the residual cell-free area measured was calculated as a percentage of the initial cell-free area. There were no significant differences in the size of the cell-free area in the presence of chitin membranes with different concentrations of nanosilver after 6 h (NS-0: 75.5%, NS-1: 77.8%, NS-3: 78.1%, and NS-3: 80.9%). The residual cell-free area was reduced to 59.4% after 24 h of treatment in the presence of chitin membranes without nanosilver. Influenced by nanosilver, the cell-free area measured 69.1% (NS-1) and 60.4% (NS-2). However, no significant decrease in the cell-free area was observed in the presence of chitin membranes containing the highest concentration of 3X nanosilver. Maximum cell death was observed in the wells containing 3X

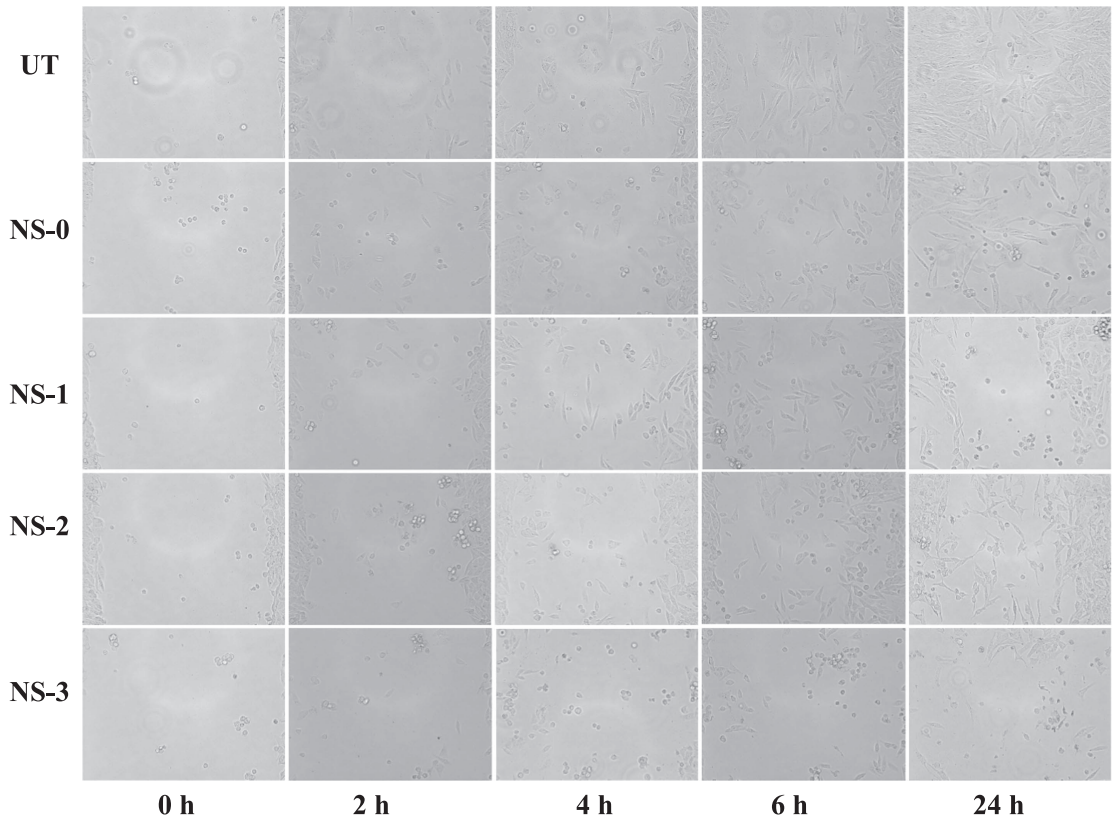

Figure 7. Representative images of *in vitro* wound-healing scratch assays performed on fibroblast cells (CHO cell line) following treatment with chitin membranes containing different concentrations of nanosilver for 0, 2, 4, 6 and 24 h. (UT: untreated; NS-0: 0 ppm; NS-1: 100 ppm; NS-2: 200 ppm; NS-3: 300 ppm).

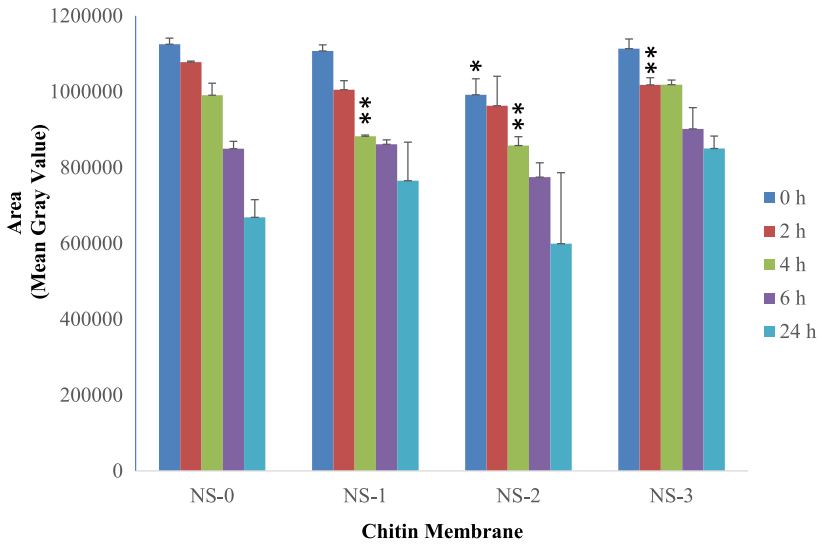

Figure 8. *In vitro* wound-healing scratch assay performed on fibroblast cells (CHO cell line). Data presented are mean ± SE (n = 3). * $p < 0.05$, ** $p < 0.005$. (NS-0: 0 ppm; NS-1: 100 ppm; NS-2: 200 ppm; NS-3: 300 ppm).

membranes. The results demonstrate that the proliferation of fibroblast cells was not affected by chitin membranes containing up to 2X nanosilver. The chitin membranes containing nanosilver (NS-1 and NS-2) favored healing. At the higher concentration of NS-3, the fibroblast proliferation was reduced.

4. Discussion

Wounds and burns are susceptible to infections by bacteria and fungi. Wound dressings containing antimicrobial agents are employed for effective healing of burn wounds and skin ulcers. The use of natural polymers such as chitin, chitosan, cellulose and

alginate for biomedical devices and materials has increased in recent years [16]. Chitin is a biomaterial abundant in nature. The monomer of chitin, N-acetylglucosamine, is present in hyaluronic acid, which has a significant role in the healing of wounds. There are a number of clinical reports on the role of chitin and its derivatives in wound healing [4, 17]. Sacchachitin membranes consisting of *in situ* chitin with wound-healing properties have also been produced from fungal mycelia *Ganoderma tsugae* [18]. Chitin nanofibrils with chitosan glycolate in the form of spray, gel and gauze preparations have been studied for wound management [19]. Electrospun chitosan-based nanocomposites have also been reported as wound dressings [20, 21]. Several reports have confirmed the efficacy of chitin in the healing of skin wounds.

The objective of a wound dressing is to support the healing of wounds. Incorporation of an antimicrobial agent in wound dressing shows several advantages over normal wound dressings. Silver with antimicrobial properties has been extensively used in wound management. A number of studies are reported on the comparative evaluation of antimicrobial activity of different methods of topical silver application. Silver-coated wound dressings have been shown to have better antimicrobial performance when compared to silver nitrate and silver sulfadiazine. Reductions in burn wound cellulites and treatment cost with silver-coated dressings as compared to silver sulphadiazine and chlorhexidine diglucamate are observed [22]. Silver-coated dressings have the advantage of reducing the frequency of changes of dressing. Silver-based dressings are reported to facilitate the early phase of wound healing and reepithelialisation [23]. Nanoparticles, due to their large surface area, have increased effectiveness against fungi and bacteria. Nanocrystalline silver dressings with antimicrobial and anti-inflammatory properties have been widely used clinically to reduce healing times compared to conventional alternatives [24].

Chitin biopolymer is reported as a promising wound dressing material. Chitin with silver nanoparticles is envisioned to play an important role in wound management. The present study involved preparation of wound dressings consisting of chitin membranes with silver nanoparticles. A lithium chloride/dimethylacetamide solvent system was used for solubilizing chitin. It has been widely used for the preparation of chitin membranes for wound dressing application [25] due to its low toxicity. Many of the other lithium chloride-tertiary amides solvent systems used for chitin dissolution, such as N,N-Dimethylpropionamide, N-Methyl-2-pyrrolidinone and 1,3-Dimethyl-2-imidazolidinone, are highly toxic [26, 27]. The UV–vis absorption spectra of chitin membranes with and without silver nanoparticles were recorded. Spectra of chitin-nanosilver membranes exhibited a plasmon resonance band of silver nanoparticles at 420–430 nm [28].

Fluid absorption and the MVTR of the chitin-nanosilver membranes were measured to evaluate the fluid handling capacity. The effectiveness of a dressing in the healing of wounds is influenced by fluid handling properties. Skin injuries result in increased loss of fluids, causing dehydration and consequently changes in body temperature equilibrium. Delays in wound healing are caused by inflammation due to accumulation of exudates. The fluid absorption of the chitin-nanosilver membranes was between 2.88% and 3.46% in 24 h. The influence of different concentrations of nanosilver on the fluid absorption of the chitin membranes was investigated. It was found that the incorporation of nanosilver in chitin does not have a significant influence on fluid absorption capacity ($p > 0.05$). The MVTR of the chitin-nanosilver membranes was in the range 2693–3323 $g/m^2/day$ after 96 h. The results are in corroboration with other studies on chitin films [25]. The absorption and moisture transmission rate show that the chitin-nanosilver membranes are capable of preventing the accumulation of fluid in the wound bed and maintaining a moist wound environment.

Bacterial species cultured from chronic wounds are commonly *Enterococcus sp., Klebsiella* sp., *Proteus sp., Enterobacter cloacae* and non-sporing Gram-negative anaerobes [29–31]. *Staphylococcus aureus, β*-haemolytic streptococci and *Pseudomonas aeruginosa* are well documented to produce destructive virulence factors [32]. *In vitro* tests of chitin-nanosilver membranes have demonstrated strong antimicrobial effects on *Acinetobacter baumanii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Candida albicans*. Reductions of more than 3-log counts were recorded with a 100 ppm chitin-nanosilver dressing after 24 h of incubation. Antimicrobial activity has been reported for silver nanoparticles against diverse microbial species. Lara *et al* [33] observed that 30 and 100 $mmol\,l^{-1}$ silver nanoparticles were effective against the erythromycin-resistant *Streptococcus pyogenes*, ampicillin-resistant *Escherichia coli*, MDR *Pseudomonas aeruginosa* and drug-susceptible strains including *Streptococcus sp*., *Escherichia coli* and *Pseudomonas aeruginosa*. Fernandez *et al* [34] tested the antimicrobial activity of silver nanoparticles using *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* and found 2.5–25 $\mu g\,ml^{-1}$ to be effective. Chitin membranes with nanosilver showed complete cell death of *Candida albicans* in 3 to 6 h. Kim *et al* [35] studied the activity of silver nanoparticles against fungi using *Candida albicans*. Silver nanoparticles disrupted the membrane potential and exhibited remarkable antifungal activity. Different wound dressing products contain and release different amounts of silver over time [36]. About a 40 ppm silver release was observed for $10 \times 10\ cm^2$ chitin-nanosilver membranes in up to 7

days. The antimicrobial efficacy of chitin membranes containing silver nanoparticles was investigated and their effective potential has been proved against a broad range of microbes such as *Acinetobacter baumanii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris*, and *Candida albicans*. Silver is incorporated in the dressings in the form of metallic silver which interacts with oxygen in wound fluid to provide a sustained release of antimicrobial silver ions. A number of studies have demonstrated the release of silver ions from wound dressings containing nanosilver [37, 38]. Silver ion release in biological media has also been studied [39].

The cytotoxicity of chitin-nanosilver membranes on fibroblast cells was assessed by carrying out MTT assay using CHO cells. Chitin-nanosilver membranes showed a reduction in cell viability. The effect of chitin-nanosilver membranes on wound healing was also demonstrated using *in vitro* scratch assays. Cell proliferation and wound healing was promoted with membranes containing up to 2X nanosilver. Results indicate a concentration- and time-dependent cytotoxicity effect of nanosilver on fibroblast cells. Our results are comparable with other studies reporting on cytotoxicity of nanosilver on fibroblast cells. Silver nanoparticles did not show a cytotoxic effect up to a concentration of 50 μg/ml on normal human dermal fibroblast (NHDF) cells [40]. Braydich-Stolle *et al* [41] showed that a 100 μg/ml concentration of silver nanoparticles resulted in necrosis of primary fibroblast cells. However, nanosilver did not produce any cytotoxic effect on mouse fibroblast cells or human osteoblasts even at a high concentration of 1% [42]. Toxicity of silver nanoparticles is suggested to be dependent on the cell type and also on the nanoparticle size [43, 44].

Chitin-nanosilver membranes NS-3 showed the highest cell viability among the three test groups after 6 h in the MTT test. Braydich-Stolle *et al* [41] reported that cellular morphology of primary fibroblast cells remained unchanged on treatment with nanosilver for 24 h, and only a slight decrease in confluence was observed. Silver nanoparticles have been reported to promote cell division at shorter durations while the cytotoxic effects are observed only after 24 h and beyond [42, 44]. As mentioned by Sadeghi *et al* [44], the human gingival fibroblast (HGF) cell line showed cytotoxicity only after 48 h. Different cell lines are affected differently in the presence of nanosilver. In the present study, the CHO fibroblast cells showed the higher cytotoxic effect at 24 h and therefore higher cell viability of NS-3 was observed at 6 h.

The CHO cells were grown on a 6-well plate with 80%–90% confluency, after which a scratch was made using a microtip. In the scratch test, NS-0 resulted in a larger cell-free zone compared to the untreated group. However, it was observed that NS-0 cells were healthy, and divided in a similar manner to the untreated cells. The difference in cell density could probably be attributed to the presence of a membrane in the treatment well as compared to observation in the absence of a membrane in the untreated well. Similar results have also been reported by You *et al* [45], where a slightly lower cell density for HaCaT cells was observed in the presence of collagen/chitosan scaffolds with nanosilver as compared to the control in a scratch assay. The authors have demonstrated in the study that silver nanoparticle-collagen/chitosan scaffolds promoted wound healing via regulating fibroblast migration and macrophage activation.

The role of silver nanoparticles in wound healing has been demonstrated by Frankova *et al* [46] using NHDFs and normal human epidermal keratinocytes (NHEKs). Silver nanoparticles were shown to inhibit production of proinflammatory cytokines, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) by these fibroblast cells which results in enhanced wound healing. A similar role of silver nanoparticles in earlier wound healing has been reported by Zhang *et al* [47]. Silver nanoparticles have also been reported to enhance the activity of cells and their proliferation [44]. The efficacy of chitin-nanosilver membranes in promoting wound healing was observed in our studies.

5. Conclusions

The results of the present study have demonstrated the antimicrobial and wound-healing properties of nanosilver incorporated chitin membranes suitable for clinical use as antimicrobial dressing. Chitin wound dressings containing 100 ppm nanosilver were found to have strong antimicrobial activity and also promoted the healing process. Chitin-nanosilver dressings possess potential clinical benefits and can be successfully employed in the management of infected wounds.

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