J. Microbiol. Biotechnol. (2016), 26(2), 408–420
http://dx.doi.org/10.4014/jmb.1506.06048

jmb

Hydrogel Dressing with a Nano-Formula against Methicillin-Resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* Diabetic Foot Bacteria

Moustafa Y. El-Naggar[1]*, Yousry M. Gohar[1], Magdy A. Sorour[2], and Marian G. Waheeb[1]

[1]*Botany and Microbiology Department, Faculty of Science, Alexandria University, Alexandria, Egypt*
[2]*General Surgery Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt*

Received: June 19, 2015
Revised: November 13, 2015
Accepted: November 16, 2015

First published online
November 24, 2015

**Corresponding author*
Phone: +2-3-392 2316;
Fax: +2-3-391-1794;
E-mail: elnaggar801@gmail.com

pISSN 1017-7825, eISSN 1738-8872

This study proposes an alternative approach for the use of chitosan silver-based dressing for the control of foot infection with multidrug-resistant bacteria. Sixty-five bacterial isolates were isolated from 40 diabetic patients. *Staphylococcus aureus* (37%) and *Pseudomonas aeruginosa* (18.5%) were the predominant isolates in the ulcer samples. Ten antibiotics were in vitro tested against diabetic foot clinical bacterial isolates. The most resistant *S. aureus* and *P. aeruginosa* isolates were then selected for further study. Three chitosan sources were tested individually for chelating silver nanoparticles. *Squilla* chitosan silver nanoparticles (Sq. Cs-Ag^0) showed the maximum activity against the resistant bacteria when mixed with amikacin that showed the maximum synergetic index. This, in turn, resulted in the reduction of the amikacin MIC value by 95%. For evaluation of the effectiveness of the prepared dressing using *Artemia salina* as the toxicity biomarker, the LC_{50} was found to be 549.5, 18,000, and 10,000 μg/ml for amikacin, Sq. Cs-Ag^0, and dressing matrix, respectively. Loading the formula onto chitosan hydrogel dressing showed promising antibacterial activities, with responsive healing properties for the wounds in normal rats of those diabetic rats (polymicrobial infection). It is quite interesting to note that no emergence of any side effect on either kidney or liver biomedical functions was noticed.

Keywords: Chitosan, diabetic foot, hydrogel, multidrug resistance, nanosilver

Introduction

Diabetic neuropathy, peripheral vascular diseases, and ischemia are usually the major factors involved in diabetic foot ulceration (DFU), which represents one of the major complications for diabetic patients [42]. Upon a polymicrobial infection, these ulcers heal slowly and patients may suffer more complication of the heavy exudate formation that may lead to limb loss [48, 20]. Better understanding of the pathophysiology and molecular biology of the bacteria infecting these diabetic wounds may help for finding the most efficient solutions for curing the diabetic ulcers and for enhancing the healing process, and much more attention must be paid to actively promoting the expression of biological factors [38].

Egypt has more diabetic individuals then before [60] and the amputation incidences accounted for up to 20% of hospital admissions. This may relate to barefoot walking, inadequate facilities for diabetes care, low socio-economic status, and illiteracy [50]. Patients became more vulnerable to the acquisition of the methicillin-resistant *Staphylococcus aureus* (MRSA) owing to long hospital stay and excessive use of antibiotic therapy [28]. Infection with the multidrug-resistant (MDR) *Pseudomonas aeruginosa* is increasing at an alarming rate worldwide, especially through nosocomial infections [31]. Moreover, about 30% of the DFUs are associated with MRSA. DFUs with MDR bacterial infection may result in worse consequences, which ultimately lead to mortality [15].

Bacterial resistance to antibiotics poses a huge concern

Table 1. Specific primers used to identify certain genes in MRSA and *P. aeruginosa*.

Strain	Primer	Sequence	Gene	Amplicon size (bp)	Reference
S. aureus	MecA-F	GTA GAA ATG ACT GAA CGT CCG ATAA	*mecA*	310	[9]
	MecA-R	CCA ATT CCA CAT TGT TTC GGTCTAA			
	FemB-F	TTA CAG AGT TAA CTG TTA CC	*femB*	365	[13]
	FemB-R	ATA CAA ATC CAGCAC GCT CT			
P. aeruginosa	gyrB-F	CCTGACCATCCGTCGCCACAAC	*gyrB*	222	[23]
	gyrB-R	CGCAGCAGGATGCCGACGCC			
	ETA-F	GACAACGCCCTCAGCATCACCA	*ETA*	397	[35]
	ETA-R	CGCTGGCCCATTCGCTCCAGCG			
	oprL-F	ATGGAAATGCTGAAATTCGGC	*oprL*	504	[22]
	oprL-R	CTTCTTCAGCTCGACGCGACG			
	Pa16S-F	GGGGGATCTTCGGACCTCA	*16S rDNA*	618	[53]
	Pa16S-R	TCCTTAGAGTGCCCACCCG			

and treatment with any new antibiotic will eventually result in the emergence of resistant bacterial isolates [58]. Chitosan has been successfully included in a wide variety of pharmaceutical applications, such as transdermal drug delivery carriers [10] and especially those used for wound dressing [4]. Chitosan films also can be used as a proper candidate in healing DFUs, which are common in patients with diabetes mellitus. It works efficiently and in a faster manner for combating long-term complications [32]. Chitosan-containing metal nanoparticles can significantly overcome multiple drug resistance mechanisms, such as uptake decrease and efflux increase of drug from the microbial cell, and inhibit biofilm formation. Moreover, nanoparticles target antimicrobial agents to the infected site where higher doses of drug are successfully delivered [41].

Treatment of DFUs represents a challenge in the development of new and widely satisfying wound dressings [36]. Polysaccharide hydrogels are very convenient for producing effective dressings. Hydrogel wound dressings are available in different forms or as a spreadable viscous gel of three-dimensional polymeric networks. They are semipermeable to gases and water vapor and can provide a moist and hydrated environment for the wound area [45, 49, 17]. Hydrogels can be the most effective form of chitosan for chronic wound dressings that can provide completion of the healing process for wounds of all requirements [54].

The present study aimed at finding a suitable and effective formula for a potent antibacterial activity with an effective healing of the infected diabetic ulcers.

Materials and Methods

Isolation of Clinical Samples

Bacterial samples were collected over a period of 6 months (July to December), 2012, from forty diabetic patients admitted to the Vascular Surgery Unit and the Diabetic Foot Unit, Alexandria Main University Hospital, Faculty of Medicine, Egypt. Bacterial samples were isolated from heel, dorsum, and amputation sites. Bacterial isolation from the samples was performed by swabbing [12], aspiration, and tissue biopsy [39]. All samples were immediately transferred within 1–2 h to the laboratory, incubated at 37°C for 24 h in sterile nutrient broth tubes, vortexed, and then inoculated onto sterile blood agar plates using sterile swabs. All bacterial samples were initially identified by gram-staining and the application of routine biochemical tests. Selected bacterial isolates of *S. aureus* and *P. aeruginosa* were also identified with the API NE system (bioMerieux, Paris, France). The results obtained were interpreted using the APILAB PLUS software (ver. 3.3.3, France).

Identification of Bacterial Isolates by Polymerase Chain Reaction (PCR)

Bacterial isolates were finally identified by PCR technique using specific primers purchased from Sigma Aldrich, Germany (Table 1). Genomic DNA was extracted according to the procedure given in the protocol of the Gene Jet Genomic DNA Purification Kit (Thermo Scientific) (Sigma Aldrich, Germany).

The PCR amplification process was carried out (using Maxima hot start green PCR master mix kit; Sigma Aldrich) for 40 cycles of 95°C for 4 min, annealing at 59°C for 30 sec, and extension at 72°C for 15 sec. After PCR amplification, to estimate the sizes of the

amplification products, 5 µl was removed and subjected to agarose gel electrophoresis (2% agarose, 1× Tris-buffered-EDTA, 100 V, 90 min) and compared with a 1 KB standard ladder (Qiagen, Australia). The gel was stained with 10 µg/ml ethidium bromide, and the amplicons were visualized using a UV illuminator. MRSA ATCC 43300 was used as a positive control, whereas MSSA ATCC 25923 was used as a negative control for methicillin resistance. *P. aeruginosa* ATCC 27853 was used as a positive control.

Antimicrobial Agents and Isolates Identification

The 10 antibiotics used in this study represent five antibiotic groups (aminoglycosides, β-lactams, chloramphenicol, macrolides, and quinolones). Antibiotics used were amikacin (30 µg/disc), meropenem (10 µg/disc), cefepime (30 µg/disc), azetronam (30 µg/disc), piperacillin (100 µg/disc), amoxicillin/clavulanic acid (30 µg/disc), vancomycin (30 µg/disc), ciprofloxacin (5 µg/disc), levofloxacin (30 µg/disc), and erythromycin (15 µg/disc). Antibiotics were purchased from Novartis Pharma, Kahira Pharma, and Amria Pharma Industrial Companies, Egypt. Antimicrobial activities of the tested antibiotics were determined by Kirby-Bauer's disc diffusion method according to Clinical Laboratory Standards Institute recommendations [7]. Inoculums sizes were adjusted to 0.5 McFarland turbidity standard for each bacterial isolate.

Chitosan Preparation

Chitosan was prepared by chitin deacetylation from crab (*Corystes cassivelaunus*), shrimp *(Palaemon serratus)*, and *Squilla* (*Squilla mantis*). It was dissolved in 1% lactic acid. The degree of deacetylation was the same for the three types of chitosan (85%). The molecular mass amounted to 5,000 g/mol [16].

Antibacterial Activity of Chitosan

Antibacterial activity was assessed by application of the disc-diffusion method [7]. Each disc received 10 µl of 6.9 mg/ml from each chitosan source and was added to a plate inoculated with tested bacteria, incubated at 37°C for 24 h, and the inhibition zone diameter was measured in millimeter unit.

Preparation of Chitosan-Based Silver Nanoparticles

Nanoparticles were prepared from three crustaceans according to Wie *et al.* [59] with some modifications. A solution of 52.0 mM $AgNO_3$ and chitosan (6.9 mg/ml) was prepared and stirred well until homogeneity. Modification was performed by adding the prepared chitosan solution in sterile test tubes (1 ml) and then different volumes of $AgNO_3$ solution were added (5, 10, 20, 30, 40, and 50 µl). The previous step was repeated at a narrower scale by adding 1, 2, 3, up to 10 µl of $AgNO_3$ to the chitosan solution. The above-mentioned mixtures were kept standing for 12 h in 95°C. The color of the solutions changed from colorless to light yellow overnight after the reaction was initiated. They contained chitosan-based silver nanoparticles, which were tested for antibacterial activity. A modification step was performed by increasing the temperature and time of the deacetylation process.

Characterization of Silver Nanoparticles

Transmission electron microscopy (TEM). Silver nanoparticles were characterized using a transmission electron microscope (Joel-100 CX, Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Alexandria, Egypt. The voltage used was 25 KeV. The samples were dissolved in deionized water and a drop was transferred onto copper grids pre-coated with carbon for microscopic observation.

UV–visible spectroscopy (UV–Vis). For tracking silver nanoparticles formation in the chitosan matrix, a UV–visible spectrophotometer was used, using 2 cm quartz cuvettes with an optical path of 1 cm.

Determination of total silver concentration. Yellow solutions of chitosan-silver nitrate solutions used in chitosan silver nanoparticles synthesis were used for determination for total silver concentration. Atomic absorption was used to determine the final concentration of silver metal in the dressing preparation.

Combinational Antibacterial Effect of Antibiotics and Chitosan-Based Silver Nanoparticles

Each antibiotic disc (6 mm inner dimension) received 10 µl of each source of the chitosan-based silver nanoparticles. Discs were then placed onto the inoculated plates (0.1 ml suspension containing 1×10^6 CFU/ml) and incubated at 37°C for 24 h, and then inhibition zone diameters if any were measured (in mm).

Synergetic/Antagonistic Indices

Synergetic/antagonistic indices were calculated according to the following equation.

$$\text{Synergetic/antagonistic index} = \frac{\text{Practical results}}{\text{Additive results}}$$

The combinatorial activity among the tested antibiotics and the three sources of chitosan-based silver nanoparticles was determined. Synergetic effect was concluded if the index <1, antagonistic effect if the index >1, and additive effect if the index =1. Practical or additive results are expressed by the inhibition zone diameter (measured in mm) [11, 21].

Minimum Inhibitory Concentration (MIC) of Amikacin

MIC values were determined by the broth-dilution method [1]. Inoculum sizes of the bacterial isolates were adjusted to 0.5 McFarland turbidity standard.

Effect of Nano-Formula on Cell Morphology of *S. aureus* and *P. aeruginosa* by Scanning Electron Microscopy (SEM)

A mixed culture of *S. aureus* and *P. aeruginosa* was incubated together with a chitosan-based silver nanoparticle formula (less than MIC dose) at 37°C for 24 h. Bacterial pellets were fixed (2.5% formaldehyde and 0.1 M sucrose) and incubated at 37°C for 1.5 h. Then, fixed cells were dehydrated through gradient concentrations of ethanol (70%, 80%, 90%, and 95%). Bacterial samples were passed through sequential steps of air-drying, mounted on SEM stubs, and gold coated for 5 min, creating a 20 nm gold layer for

SEM examination (Joel-JSM-5300, Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Alexandria, Egypt.

Preparation of Starch Chitosan Hydrogel Dressing

Dressing preparation was performed according to Baran *et al*. [5] with some modifications. Soluble starch was dissolved in distilled water (5 mg/ml) and then sodium iodate (0.1 v/v) was added. Reaction was continued for 30 min at room temperature with occasional shaking. Glycerol was added (0.2 ml) for each milliliter of reaction medium and mix for 10 min. Oxidized starch (6 ml) was mixed with chitosan (20 ml, 1% (w/v)). Mixtures were cast in plastic Petri dishes (10 cm^2) and allowed to dry for several days at room temperature. After drying, carbonate buffer (0.5 M, pH 8.5) was poured over the membrane in a Petri dish and incubated for 3 h. A modification step was performed by increasing glycerol and decreasing the starch volume added to the dressing matrix. Formula was added (4 ml) before drying of the membrane. Cubes of the dressing preparation (1.5 × 1.5 cm^2) were tested for their antibacterial activity.

Biotoxicity of the Prepared Dressing

The toxicity of the dressing components was tested against *Artemia salina* as a toxicity biomarker. Different concentrations of amikacin (2, 20, 200, and 2,000 µg/ml), chitosan silver nanoparticles (6,900, 8,000, 15,000, and 18,000 µg/ml), and dressing matrix (starch (5 mg/ml) + sodium iodate (10, 100, 1,000, and 10,000 µg/ml)) were prepared. Ten milliliters of a sterile brackish water was added in 20 ml glass vials. Ten *Artemia salina* nauplii were transferred to each vial. The number of the viable biomarker was counted after 24 h of application of tested matter. The control vial contained only brackish water, with 10 nauplii. The LC_{50} of this combination (the concentration at which 50% of the biomarker nauplii individuals died) was determined from the best-fit line obtained by using the probit analysis method [30].

SEM of Cross-Section of Chitosan Hydrogel

Morphologies of the surface and the cross-section of the hydrogel samples were visualized using a Joel JSM-5300 scanning electron microscope (JEOL Technics Ltd., Japan) at the Electron Microscope Unit, Faculty of Science, Alexandria University, Alexandria, Egypt. The microscope had an accelerating voltage of 25 KeV after gold coating for SEM observations at 5,000× and 15,000× magnification powers.

In Vivo Potency of the Prepared Dressing

Forty-eight male albino rats (*Rattus norvegicus albinus*) weighing 150–180 g were used. All rats were adults of 4 months of age, kept at 23–25°C and 10–14 h light and dark cycles. This experiment was performed in an approved animal care center in accordance with the rules of the ethics committee at Alexandria University and the corresponding animal care protocol. Twenty-four rats were normal non-diabetic (group 1), eight replicates for negative and positive controls and treatment. For induction of diabetes mellitus, another set of 24 (group 2) were injected with alloxan (40 mg per kg of body weight) (Sigma Aldrich, Germany) in their penial vein [8]. After 48 h, blood sugar was analyzed with a blood glucose monitoring system (On Call Plus, China). After confirmation that group 2 was diabetic, rats were anesthetized with anesthetic ether. For both groups, the dorsum surface was shaved of body hair, the skin was disinfected with 70% isopropyl alcohol, and a 1 cm wound was made in the middle of the dorsum. Bacterial suspensions of *S. aureus* (0.1 ml) and *P. aeruginosa* (0.1 ml) were adjusted (0.5 McFarland turbidity standard) to correspond to approximately 10^8 CFU/ml [6] and then vortexed and dropped on the wounds. Negative controls from both groups were not infected and not treated with dressing. Positive controls from both groups were infected by the mixed culture of *S. aureus* and *P. aeruginosa* and were not treated with dressing. Rats were previously infected with mixed culture and treated with the prepared dressing. It is worthy to mention that the change of the dressing was on a daily basis. The wound healing rate (WHR) was then calculated as follows: WHR (%) = $A_o - A_n(100)/A_o$, where A_o is the wound area on day 0; n = day 2, 7, or 14 [29].

Effect of the Prepared Dressing on Some Parameters of Kidney and Liver Functions

Blood samples were collected from the dorsal pedal vein into clean tubes before and after 12 days of the dressing treatment [40]. Samples were stored in an icebox at −4°C until transferring to the analysis laboratory. Samples were centrifuged for 3 min at 1,500 rpm until clear sera were obtained. Analysis was performed within 24 h of blood collection using kits from Diasys Diagnostic Systems for biomedical analysis (Star Dust FC, Germany) (n = eight for each group).

Statistical Analyses of the Data

Data were analyzed using IBM (SPSS, 2012) software package ver. 20.0. Qualitative data were described using number and percent. Comparison between different groups regarding categorical variables was tested using the Chi-square test. Statistical significance was set at $p < 0.05$.

Results

Bacterial Isolates

Sixty-five clinical bacterial isolates were obtained from 40 patients. Thirty-seven isolates (56.9%) were proved to be gram-positive bacteria and 28 isolates (43.1%) were gram-negative bacteria. There were 24 isolates (37%) of S. *aureus*, 10 isolates (15%) of MRSA, 12 isolates (18%) of *P. aeruginosa*, 7 isolates (11%) of *E. coli*, 5 isolates (8%) of *Klebsiella pneumoniae*, 4 isolates (6%) of *Proteus mirabilis*, and 3 isolates (5%) of *S. pyogenes*. Of the cases, 62.5% were polymicrobial

infections and 37.5% were mono-microbial infections.

Antibiotic Sensitivity Pattern of the Bacterial Isolates

A sensitivity profile was determined for the most six resistant isolates of *S. aureus* (MRSA) and MDR *P. aeruginosa* (three isolates each). The most resistant *S. aureus* and *P. aeruginosa* isolated were selected for further study. Vancomycin recorded the maximum average inhibition zone diameter (16 mm), whereas cefepime and erythromycin failed to show any antibacterial activity. All *S. aureus* isolates were methicillin resistant by testing sensitivity to methicillin and oxacillin. For *P. aeruginosa*, imipenem recorded the maximum average inhibition (15.67 mm), and there was complete resistance to cefepime, azetronam, and erythromycin as no inhibition was recorded. All *P. aeruginosa* isolates were multidrug resistant. Vancomycin and amikacin had a significant effect over the rest of the antibiotics against *S. aureus* and *P. aeruginosa* (p = 0.0253 and 0.0251), respectively.

Molecular Identification of the Bacterial Isolates

Selected bacterial isolates were further identified by PCR using specific primers for each (Fig. 1). All *Staphylococcus* isolates in lanes 1–5 were proven to be *S. aureus* and methicillin resistant, showing amplicon sizes of 310 bp and 651 bp (for *mecA* and *femB* primers, respectively), except MSSA ATCC 25923, which showed the absence of *mecA* gene. For *P. aeruginosa*, four primers were used to amplify four highly specific gene fragments of *P. aeruginosa* (*16S rDNA*, *gyrB*, *oprL*, and *ETA*), giving amplicons with sizes of 618, 222, 504, and 397 bp, respectively in lanes 1–4.

Synthesis and Characterization of Chitosan-Based Silver Nanoparticles

Silver nanoparticles were synthesized at 5 µl of silver nitrate (golden color solution). Dark color solutions appeared upon addition of 10 µl of $AgNO_3$, which indicated the formation of silver oxide in nanoparticles synthesis. When silver nitrate was added in a narrower scale, silver oxide was formed at 6 µl of silver nitrate, which indicated that 5 µl of silver nitrate was the optimum volume for silver nanoparticle synthesis. The size of particles was determined for 100 particles as shown in the histogram, and ranged from 6 to 9 nm in the chitosan solution (Fig. 2A). They were also characterized by UV- Vis absorption, showing maximum peak λ_{max} = 420 nm, indicating the presence of silver nanoparticles with a, spherical structure. There were not any detected impurities, as there were clear sharp peaks at different time intervals, which confirmed the stability of the synthesized nanoparticles (Fig. 2B).

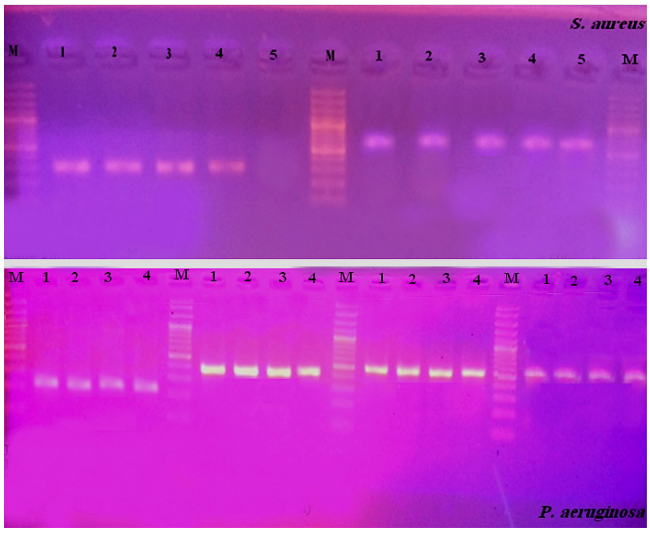

Fig. 1. Agarose gel electrophoresis patterns showing PCR amplification products from different *S. aureus* and *P. aeruginosa* isolates.

For *S. aureus*; M: (Marker) 1KB DNA ladder; Lane 1: MRSA 1; Lane 2: MRSA 2; Lane 3: MRSA 3; Lane 4: MRSA ATCC 43300 (positive control); Lane 5: MSSA ATCC 25923 (negative control). In the first five lanes, the amplicon products of *mecA* are at 310 bp, which disappeared in the fifth lane. The other five lanes; the amplicon products of *femB* at 651 bp. In the case of *P. aeruginosa*; Lane 1: *P. aeruginosa* 1; Lane 2: *P. aeruginosa* 2; Lane 3: *P. aeruginosa* 3; Lane 4: *P. aeruginosa* ATCC 27853 (positive control). The first four lanes represent the amplicon gene products of *gyrB* at 222 bp and the second four lanes represent the amplicon gene products of *ETA* at 397 bp. The third and fourth four lanes are the amplicon gene products of *oprL* at 504 bp and *16S rDNA* at 618 bp, respectively.

Antibacterial Activity of Chitosan and Chitosan-Based Silver Nanoparticles

The total average inhibition zone diameter of bulk chitosan for *S. aureus* and *P. aeruginosa* was 11.88 and 14.11 mm, respectively. The total average inhibition of chitosan-based silver nanoparticles against *S. aureus* was 14.67 mm, and for *P. aeruginosa* was 15.67 mm. For both *S. aureus* and *P. aeruginosa*, *Squilla* chitosan and *Squilla* chitosan-based silver nanoparticles exhibited the maximum average inhibition. Chitosan silver nanoparticles showed more activity than bulk chitosan. *S. aureus* showed more resistance towards chitosan and chitosan-based silver nanoparticles than *P. aeruginosa* (Fig. 3).

Determination of the Total Concentration of Silver Metal

Yellow chitosan-silver nitrate solution (Cs Ag^0) gave a

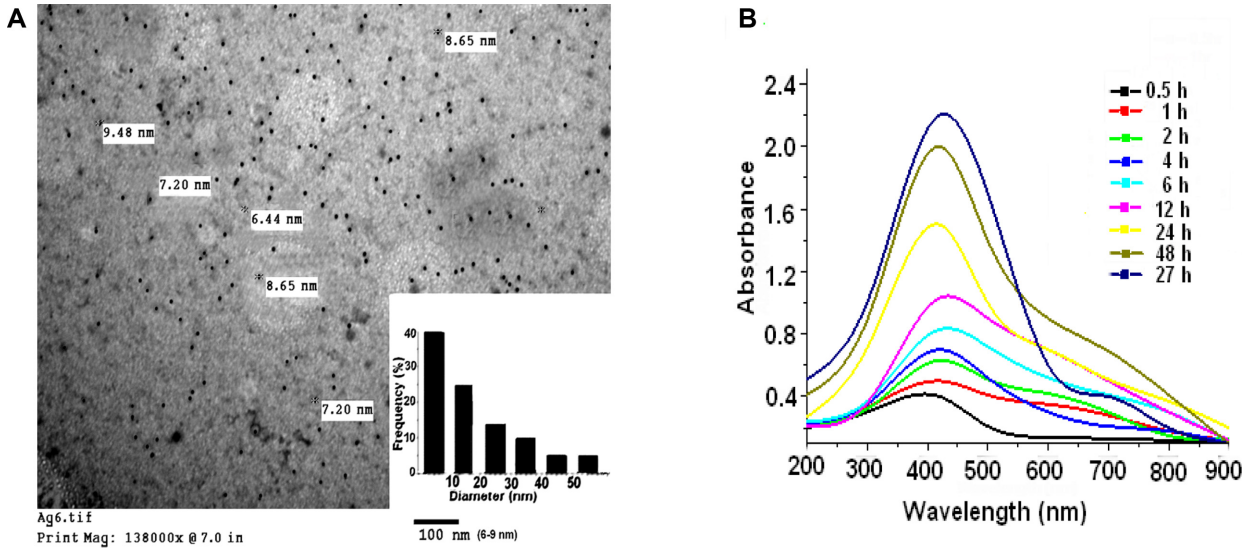

Fig. 2. Characterization of chitosan-based silver nanoparticles.
(**A**) TEM of silver nanoparticles in chitosan matrix; the range of diameter range is illustrated in white boxes. (**B**) UV-visible absorption spectra at different time intervals for chitosan-based silver nanoparticles for the detection of the maximum wavelength and stability.

concentration of silver equal to 5 ppm to be the total concentration of silver in prepared dressing. The silver metal concentration was measured in the chitosan silver nanoparticle solution where 5 µl of silver nitrate was added to chitosan.

Synergetic/Antagonistic Indices

Synergetic/antagonistic indices for evaluation of the combinatorial activity among antibiotics and the three sources of chitosan-based silver nanoparticles were determined. Upon combination of the tested antibiotics, individually and with each source of chitosan based-silver nanoparticles, amikacin had the maximum average synergetic/antagonistic indices with all sources of chitosan nanoparticles for six isolates of both *S. aureus* and *P. aeruginosa,* which were 1.460 and 1.523, respectively. Aztreonam was antagonistic with all sources of chitosan silver nanoparticles, giving an average index of 0.414 for *S. aureus* and 0.801 for *P. aeruginosa* isolates, forming the worst combinations (Fig. 4A). *Squilla* chitosan silver nanoparticles showed maximum average synergetic/antagonistic indices when combined with tested antibiotics for *S. aureus* (1.225) and *P. aeruginosa* (1.321), with highest synergy, followed by shrimp and then crab chitosan-based silver nanoparticles (Fig. 4B). *Squilla* chitosan nanosilver showed maximum activity and synergy with amikacin (1.57 and 1.6) for *S. aureus* and *P. aeruginosa*, respectively, than shrimp and crab chitosan silver nanoparticles. This combination was chosen as a potential formula (Fig. 4C).

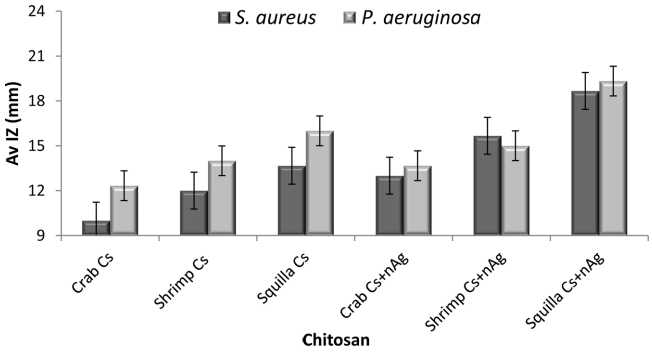

Fig. 3. Comparison of chitosan-based silver nanoparticles efficiency to the bulk chitosan against both *S. aureus* and *P. aeruginosa.*
Cs: Chitosan. Av IZ: average inhibition zone diameter for most resistant tested bacterial strains.

MIC Values of Amikacin Against *S. aureus* and *P. aeruginosa*

The MIC values (Fig. 5) of amikacin for all bacterial isolates decreased, after the combination of amikacin and *Squilla* chitosan-based silver nanoparticles, from the total average of 48 µg/ml to 2 µg/ml. The MIC for all bacterial isolates decreased by 95% after the combination between amikacin and *Squilla* chitosan-based silver nanoparticles,

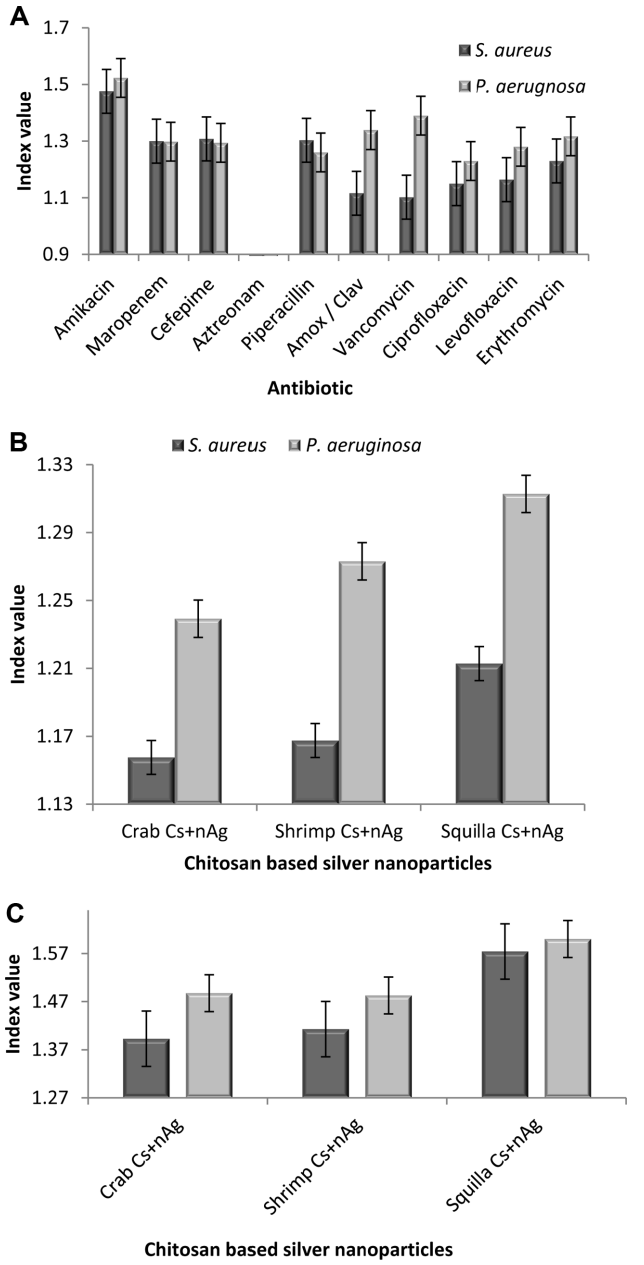

Fig. 4. Synergetic/antagonistic indices of (**A**) tested antibiotics upon combination with chitosan-based silver nanoparticles, (**B**) three sources of chitosan silver nanoparticles upon combination with chitosan, and (**C**) amikacin and chitosan-based silver nanoparticles.
Amox/Clav: Amoxycillin/Clavulanic acid.

confirming that the antibacterial activity is synergetic.

Impact of the Suggested Formula on Bacterial Cell Morphology

After formula addition in a concentration less than the

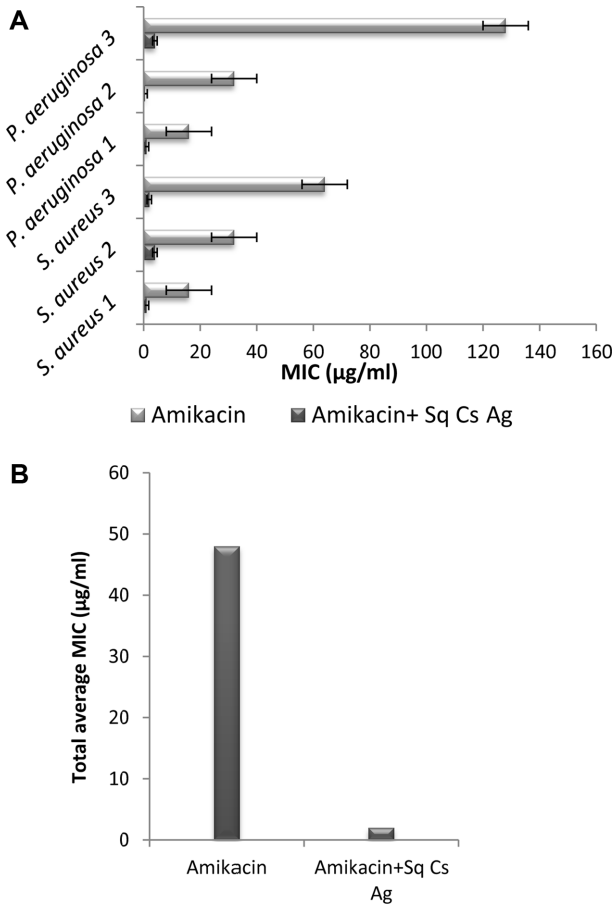

Fig. 5. MIC of amikacin before and after the combination with Squilla silver nanoparticles against *S. aureus* and *P. aeruginosa* (**A**) and the decrease of total average of MIC values after formula treatment with formula (**B**).

MIC dose, the bacterial cells (*S. aureus* and *P. aeruginosa*) were completely lysed and lost their structural entity (Fig. 6B). For *S. aureus*, the cell wall seemed rough and shrinking (arrow) at 15,000× magnification (Fig. 6C). In the case of *P. aeruginosa*, the cell wall was ruptured, forming holes (arrow), with magnification of 35,000× (Fig. 6D), compared with the intact control cells (Fig. 6A).

Evaluation of Hydrogel Dressing Preparation

The prepared hydrogel dressing (flexible and semi-transparent) was assessed for its antibacterial activity against the bacterial isolates. The inhibition zone diameter (in mm) for *P. aeruginosa* 1 amounted to 65 mm, *P. aeruginosa* 2 was 56 mm, *S. aureus* 1 was 59 mm, and *S. aureus* 2 was 50 mm. Regarding the inhibitory activity of the hydrogel dressing, the application of dressing treatment resulted in an increase in the activity by 3.9-fold compared with the

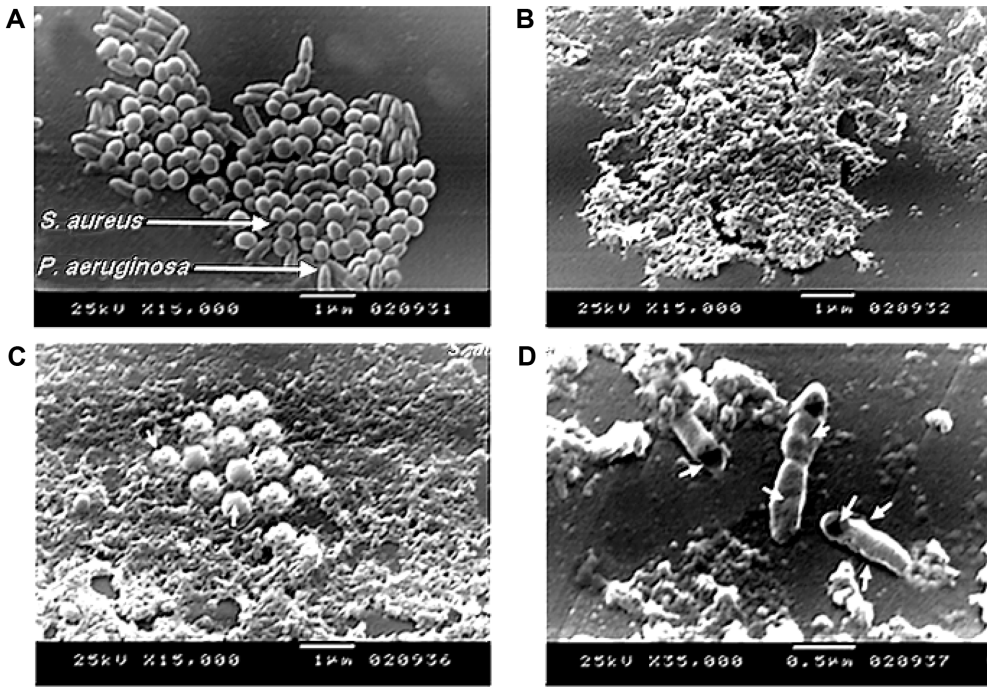

Fig. 6. Scanning electron micrographs of (**A**) mixed culture of *S. aureus* and *P. aeruginosa* (control), (**B**) after treatment for 24 h with the suggested formula, (**C**) treated *S. aureus* cells, and (**D**) treated *P. aeruginosa* cells.

application of amikacin alone.

Toxicity Test on *Artemia salina* Biomarkers

The LC_{50} was found to be 2.74, 4.255, and 4 for amikacin, *Squilla* chitosan-based silver nanoparticles + 5 ppm Ag^0, and dressing matrix (sodium iodate + starch), which were equivalent to the concentration of 549.5, 18,000, and 10,000 μg/ml, respectively. All used concentrations were quiet safe and much lower than the $LC_{50.}$

Morphology of Hydrogel Dressing

Scanning electron micrographs of the hydrogel exhibited silver nanoparticles embedded into a smooth porous surface. A cross-section of the sheet showed large pores and tubules (arrows) with spongy appearance, which showed the porosity of the dressing and the cross-linking between main composing polymers (chitosan and starch).

Response of Wound Healing Rates to the Prepared Dressing Application

After bacterial infection, symptoms developed, such as swelling in skin around the wound area, appearance of green exudates, pus formation, and skin dehydration. Prepared dressing had antibacterial and healing effects on the infected wounds (with mixed bacterial culture) for both non-diabetic and diabetic rats. There was no sign of skin redness (flushing) and irritation. Dressing treatment showed significant wound healing rates (p = 0.0042, 0.001) compared with negative and positive control non-diabetic groups and diabetic ones (p = 0.0021, 0.001), respectively. Diabetic rats showed slower impaired wound healing than normal ones. Treated, and negative and positive control diabetic rats had lower wound healing rates compared with the normal ones, by 26.8%, 32.5%, and 49.4%, respectively, on the third day. However, on the sixth day of follow-up, the percentage decreases were 16.7%, 22.8%, and 50.1%, and were 3.5%, 11%, and 10.2% on the eleventh day of follow-up (Figs. 7 and 8).

Effect of Dressing Application on Kidney and Liver Functions

All parameter levels of blood urea nitrogen, ALT (alanine aminotransferase), AST (aspartate aminotransferase), serum creatinine, serum uric acid, and total bilirubin for the experimental rats were found to be within the normal range. There was no significant change in the tested parameters before and after treatment for non-diabetic and diabetic rats ($p < 0.05$) at 0.05 level and there was no sign of nephrotoxicity, which confirmed the safety of the dressing treatment on kidney functions. Moreover, the dressing

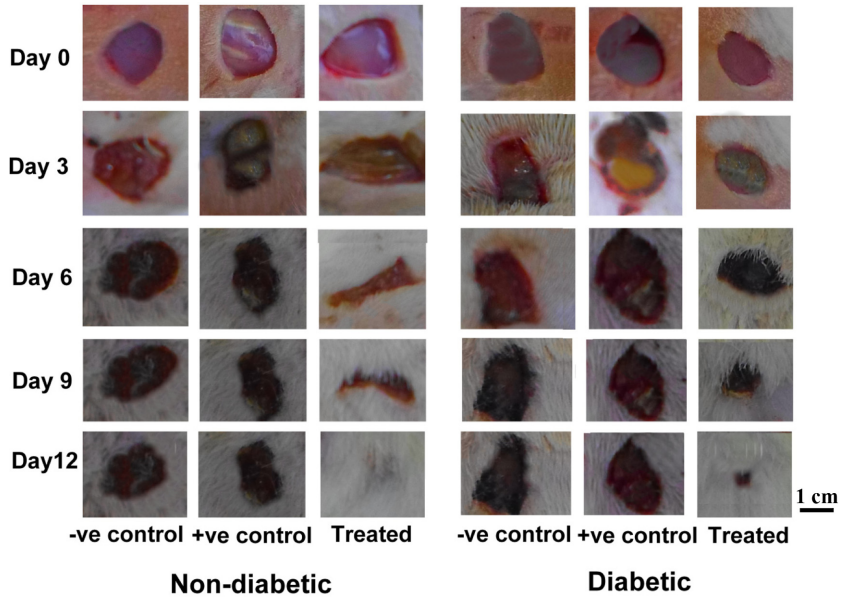

Fig. 7. In vivo evaluation of the application of the topical hydrogel dressing on wound healing of normal and induced diabetic experimental rats.
Wounds were about 1 cm in diameter and photographed with a digital camera.

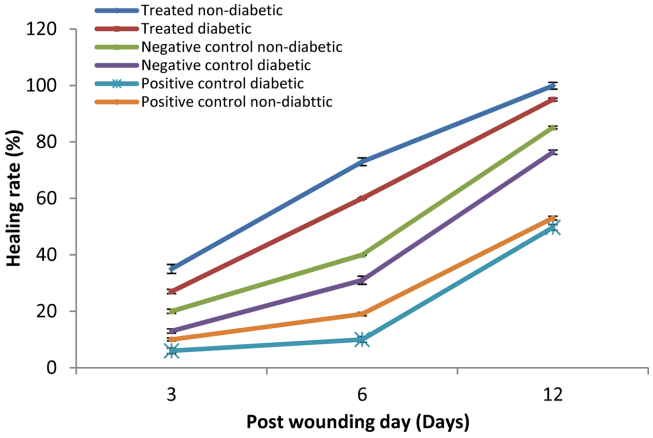

Fig. 8. Response of wound healing rates to the topical dressing treatments in the treated and control groups.
Results are presented as the mean ± SD (n = 8). Each point represents the mean percentage of wound healing rates.

preparation had no adverse side effects on liver biomedical functions (Table 2).

Discussion

Combinational therapy and nano-dressing are the most safe alternative approaches than conventional antimicrobial agents. This approach holds promise in that it seeks complete infection recovery without selection pressure that leads to the emergence of resistant strains and for not forcing a patient to lose a part of the body. Both MRSA and *P. aeruginosa* were the most distributed and the main causative agents of the infected ulcers. Similarly, Sugandhi and Prasanth [55] recorded that out of 51 bacterial isolates, *S. aureus* (41%) and *P. aeruginosa* (35%) remained the causative agents of diabetic foot ulceration compared with the other species such as *Enterococcus* spp. (4%), *E. coli* (4%), *Salmonella* spp. (4%), *Bacillus* spp. (4%), *Micrococcus* spp. (2%), *Listeria* spp. (2%), *Shigella* spp. (2%), and *Proteus* spp. (2%). The antibiotic sensitivity pattern was similar to the present study in choosing amikacin as one of the most effective antimicrobial agents against different bacterial species.

The identification of *S. aureus* (MRSA) was carried out by the PCR technique using specific primers to certain genes for *S. aureus* species (*femB*) and for the identification of methicillin resistance (*mecA* gene). As approved by Jonas *et al.* [19], were confirmed reliable results for MRSA identification within 18 h with the application of duplex PCR of *mecA* and *femB*. Lin *et al.* [26] also developed a duplex regime for the PCR assay for rapid microbial detection of *S. aureus* and *P. aeruginosa*, using the *femB* gene of *S. aureus* and the DNA gyrase subunit B gene of *P. aeruginosa*. Moreover, *P. aeruginosa* isolates were identified in this study via specific gene fragments; namely, *16S rDNA*, *gyrB*, *oprL*, and *ETA*. Anuj *et al.* [3] proved that the

Table 2. Kidney and liver function parameters for both non-diabetic and diabetic rats before and after dressing application.

		-ve control	+ve control	Before treatment	-ve control	+ve control	After Treatment
Non-diabetic	BUN (mg/dl)	15.6 ± 0.7	16.5 ± 1.0	15.8 ± 1.19	16.0 ± 1.0	16.9 ± 0.8	16.6 ± 1.18
	Serum creatinine (mg/dl)	0.43 ± 0.05	0.48 ± .062	0.47 ± 0.05	0.44 ± 0.05	0.49 ± 0.06	0.49 ± 0.06
	Serum uric acid (mg/dl)	1.63 ± 0.14	1.5 ± 0.1	1.56 ± 0.2	1.6 ± 0.1	1.65 ± .0.11	1.7 ± 0.1
	Total bilirubin (mg/dl)	0.24 ± 0.02	0.25 ± .04	0.24 ± .03	0.24 ± 0.04	0.26 ± .04	0.25 ± .03
	ALT (U/l)	18.76 ± 1.3	18.6 ± 1.2	19.48 ± 0.99	19.1 ± 1.34	19.6 ± 1.33	19.3 ± 1.3
	AST (U/l)	49.6 ± 1.7	52 ± 1.0	46.7 ± 1.2	52 ± 2.0	50 ± 1.0	47.25 ± 1.8
Diabetic	BUN	16 ± 1.0	15.3 ± 0.6	15.7 ± 0.57	15.7 ± 1.15	15.6 ± 1.15	17 ± 1.7
	Serum creatinine	0.58 ± 0.06	0.61 ± 0.07	0.65 ± 0.04	0.59 ± 0.07	0.63 ± 0.8	0.66 ± .02
	Serum uric acid	1.64 ± 0.11	1.57 ± 0.09	1.6 ± 0.12	1.75 ± 0.07	1.6 ± 1	1.63 ± 0.14
	Total bilirubin	0.26 ± .02	0.26 ± 0.03	0.25 ± 0.04	0.24 ± 0.03	0.28 ± 0.03	0.26 ± 0.04
	ALT	18.93 ± 1.2	19.1 ± 1.8	19.63 ± 1.3	19.5 ± 1.8	18.9 ± 1.2	19.5 ± 1.3
	AST	50.2 ± 1.6	51.2 ± 0.5	53 ± 1.7	51.2 ± 1.16	50.7 ± 0.65	47.8 ± 2.4

BUN = blood urea nitrogen.
ALT = alanine aminotransferase.
AST = aspartate aminotransferase.
N.S.: Nonsignifican.
All values are presented as the mean ± SD (standard deviation).

gyrB gene coding a type II topoisomerase is a better candidate for bacterial species identification. De Vos *et al*. [10] concluded that the specificity of the *oprL* gene-based PCR for *P. aeruginosa* eliminated the chances for the emerging of false-positive results of closely related species. Hussien *et al*. [18] evaluated the specificity of the *ETA* gene in the identification of *P. aeruginosa* and the results yielded 96% to 100% specificity using specific primers to certain *ETA* gene fragments.

Exhibition of chitosan antibacterial activity against six resistant bacterial isolates is due to attraction to negative cell-walled bacterial cells adsorbing bacteria on their surface, as it is the only cationic natural polymer. This can be further explained on the grounds proposed by Lee *et al*. [24] who stated that the positive charge of chitosan allows for electrostatic interactions between positively charged polymer and carboxyl-negative charge on the bacterial cell. The activity of chitosan molecules was enhanced by their chelation to silver nanoparticles. Mock *et al*. [33] stated the synergetic effect between chitosan acetate and nanoparticle silver on MRSA, *A. baumannii*, *P. mirabilis*, and *P. aeruginosa* by enhancing bacterial cell wall permeability. Silver nanoparticles could also increase the activity of antibiotics in a similar study by Mohammed [34], who studied gentamicin (an aminoglycoside with a very similar structure and same active groups as amikacin) with silver nanoparticles against the MRSA isolate. The antimicrobial activity of gentamicin increased because of its combination with silver nanoparticles, which may be due to interaction of the active groups in the antibiotic molecules such as hydroxyl and amide groups present, which chelate silver nanoparticles. In a similar study by Gohar *et al*. [14], combination of *Squilla* chitosan as a capping agent to silver nanoparticles showed the highest synergetic indices with cephradin and piperacillin, separately against diabetic foot bacteria. The reduction in the MIC value by 94% for gram-positive bacteria and 88% for gram-negative bacteria was attained. In this study, the synergy between the formula components resulted in the decline of MIC value by 95% against all clinical isolates.

As evidenced by the SEM study, bacterial cells from both species lost their structural entity when exposed to the proposed formula for 24 h, leading to cell wall roughness, shrinkage, and disaggregation for *S. aureus* clusters, and forming large holes in the outer wall layer for *P. aeruginosa*. These results matched the observations reported by Tao *et al*. [57] about bacterial cells rupturing after the exposure to chitosan for 20 min. On the other hand, Regiel *et al*. [43] observed an increased roughness of the cell surface, appearance of filaments, presence of pits in the cell wall, and complete lysis of cells for *S. aureus* and *E. coli* upon exposure to chitosan films containing silver. Sondi and Salopek-Sondi [52] have explained that the antimicrobial inhibitory effect of silver nanoparticles resulted in the

formation of ''pits'' in the cell wall of bacteria and this is concentration dependent.

Amikacin is considered the most potent aminoglycoside with broad-spectrum activity, especially against gram-negative rods. Amikacin is proved as an effective antibiotic [44, 47, 56, 61] for the treatment of patients infected with gram-negative bacteria, even for those suffering bacteremia caused by gentamicin-resistant bacteria. Combinations of amikacin with doripenem, colistin, or levofloxacin were synergistic against 100 *P. aeruginosa* isolates [56]. Savov *et al.* [47] demonstrated that there was a significant decrease in MICs for amikacin and ampicillin/sulbactam compared with the MIC values of each antibiotic alone against 52 strains of multi-resistant *A. baumannii,* showing a synergistic effect against 98% of the isolated strains. Rey-Jurado *et al*. [44] stated that the drug combination of amikacin, levofloxacin, and ethambutol was the best choice over all the tested combinations, with a mean decrease of log 5.84 CFU/ml against 12 isolates of *Mycobacterium tuberculosis*. In vitro synergy of bactericidal activities upon combination of amikacin with a Chinese drug was beneficial for the combinatorial therapy of MRSA infection [61].

Based on the above-mentioned results, the proposed formula (amikacin (4 μg/ml) + *Squilla* chitosan-based silver nanoparticles (5 ppm Ag in 6.9 mg/ml chitosan)) is recommended for the treatment of MRSA and *P. aeruginosa* chronic wound infection. Sasikala and Durai [46] reported that hydrogel sheets produced from chitosan promote significant wound contraction and healing process in tested Wister rats. Lee *et al*. [25] showed the effectiveness of chitosan alginate hydrogels on wound healing progress in a type 1 induced diabetic rat model, which exhibited a higher wound healing rate than the conventional hydrogels. Anisha *et al*. [2] showed the potential antimicrobial activity of the wound dressing of chitosan–hyalurunic acid/nAg composite sponges for DFU infected with resistant *S. aureus*, MRSA, *P. aeruginosa*, and *K. pneumonia*. The amikacin dose used in this dressing preparation (4 μg/ml) is much lower than the prescribed dosage of the topical antibiotic (125 mg/ml). It is reported that higher doses of amikacin (as an aminoglycoside antibiotic) can lead to nephrotoxicity [37, 51]. After therapy initiation, nephrotoxicity is termed as an increase of the serum creatinine by 0.5 mg/dl or 50%, whichever is greater, [27]. In this current study, treatment by this proposed dressing preparation did not show any sign of nephrotoxicity. It was also proved safe for liver biochemical functions.

In conclusion, this study proposed a safe new formula of *Squilla* chitosan silver-based nanoparticles in hydrogel dressing form, which may have multi-functions. To the best of our knowledge, this formula has not been reported elsewhere, especially for the rare use of *Squilla* chitosan or against polymicrobial infections. It may propose a promising and successful healing regime by overcoming the resistance of MRSA and *P. aeruginosa* inhabiting diabetic foot ulcers. One more advantage to this study is the use of the lowest concentration of amikacin compared with the concentrations reported in other published studies. Although it could be applied as a promising dressing, further studies are still required to prove its clinical significance, and this dressing can be envisioned to other different wound types.

Acknowledgments

We would like to thank the technicians' team working in both the Electron Microscope Unit, Faculty of Science, and those in the Central Laboratory at the Higher Institute for Graduate Studies and Research, Alexandria University, Egypt for their technical support.

References

1. Andrews JM. 2001. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother*. **48:** 5-16.
2. Anisha BS, Biswas R, Chennazhi KP, Jayakumar R. 2013. Chitosan–hyaluronic acid/nano silver composite sponges for drug resistant bacteria infected diabetic wounds. *Int. J. Biol. Macromol.* **62:** 310-320.
3. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, Nissen MD, Sloots TP. 2009. Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the *ecfX* and the *gyrB* genes. *Diagn. Microbiol. Infect. Dis*. **63:** 127-131.
4. Archana D, Dutta J, Dutta PK. 2013. Evaluation of chitosan nano dressing for wound healing: characterization, in vitro and in vivo studies. *Int. J. Biol. Macromol*. **57:** 193-203.
5. Baran ET, Manao JF, Reis RL. 2004. Starch-chitosan hydrogels prepared by reductive alkylation cross-linking. *J. Mater. Sci. Med*. **15:** 759-765.
6. Berg JO, Mossner BK, Skov MN, Lauridsen J, Gottrup F, Kolmos HJ. 2006. Antibacterial properties of EMLAs and lidocaine in wound tissue biopsies for culturing. *Wound Repair Regen*. **14:** 581-585.
7. Clinical and Laboratory Standards Institute. 2012. Performance standards for antimicrobial susceptibility testing. *Twenty-Second Informational Supplement Document M100-S22, CLSI* **31:** 62-86.
8. De Carvalho EN, De Carvalho NAS, Ferreira LM. 2003. Experimental model of induction of diabetes mellitus in rats. *Acta Cir. Bras*. **18:** 60-64.

9. De Vos D, Lim AJ, Pirnay JP, Struelens M, Vandenvelde C, Duinslaeger L, *et al*. 1997. Detection and identification of *Pseudomonas aeruginosa* in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, *oprI* and *oprL*. *J. Clin. Microbiol*. **35:** 1295-1299.
10. Dhillon GS, Kaur S, Sarma SJ, Brar SK, Verma M, Surampalli RY. 2013. Recent development in applications of important biopolymer chitosan in biomedicine, pharmaceuticals and personal care products. *Curr. Tiss. Eng.* **2:** 20-40.
11. El-Naggar MYM. 1998. Synergistic effect of actinomycin X2 produced by *Streptomyces nasri* strain YG62 with other antibiotics. *Biomed. Lett*. **58:** 169-173.
12. Gardner SE, Frantz RA, Doebbeling BN. 2001.The validity of the clinical signs and symptoms used to identify localized chronic wound infection. *Wound Repair Regen*. **9:** 178-186.
13. Geha DJ, Uhl JR, Gustaferro CA, Persing DH. 1994. Multiplex PCR for identification of methicillin-resistant staphylococci in the clinical laboratory. *J. Clin. Microbiol*. **32:** 1768-1772.
14. Gohar YM, Barakat KM, Abu Yousef MA, Ghonaim HB. 2013. Marine chitosan-silver nano-particles as antibiotic synergizers against diabetic foot bacteria. *Arch. Clin. Microbiol*. **4:** 1-7.
15. Grimble SA, Magee TR, Galland RB. 2001. Methicillin resistant *Staphylococcus aureus* in patients undergoing major amputation. *Eur. J. Vasc. Endovasc. Surg*. **22:** 215-218.
16. Harikrishnan R, Kim J, Balasundaram C, Heo M. 2012. Dietary supplementation with chitin and chitosan on hematology and innate immune response in *Epinephelus bruneus* against *Philasterides dicentrarchi*. *Exp. Parasitol*. **131:** 116-124.
17. Himly N, Darwis D, Hardiningsih L. 1993. Poly (*N*-vinylpyrrolidone) hydrogels: hydrogel composites as wound dressing for tropical environment. *Radiat. Phys. Chem*. **42:** 911-914.
18. Hussien IA, Habeb KA, Jassim KA. 2012. Identification of *Pseudomonas aeruginosa* from burn wounds isolates by using PCR exatoxin-A specific primers. *Iraqi J. Biotechnol*. **11:** 282-291.
19. Jonas D, Grundmann H, Hartung D, Daschner FD, Towner KJ. 1999. Evaluation of the *mec*A and *fem*B duplex polymerase chain reaction for detection of methicillin-resistant *Staphylococcus aureus*. *Eur. J. Clin. Microbiol. Infect. Dis*. **18:** 643-647.
20. Jude EB, Apelqvist J, Spraul M, Martini J. 2007. Prospective randomized controlled study of Hydrofiber® dressing containing ionic silver or calcium alginate dressings in non-ischemic diabetic foot ulcers. *Diabet. Med*. **24:** 280-288.
21. Keith CT, Borisy AA, Stockwell BR. 2005. Multicomponent therapeutics for networked systems. *Nat. Rev. Drug Discov.* **4:** 71-78.
22. Khan AA, Cerniglia CE. 1994. Detection of *Pseudomonas aeruginosa* from clinical and environmental samples by amplification of the exotoxin A gene using PCR. *Appl. Environ. Microbiol*. **60:** 3739-3745.
23. Kobayashi N, Wu H, Kojima K, Taniguchi K, Urasawa S, Uehara N, *et al*. 1994. Detection of *mec*A, *fem*A, and *fem*B genes in clinical strains of staphylococci using polymerase chain reaction. *Epidemiol. Infect*. **113:** 259-266.
24. Lee DW, Lim H, Chong HN, Shim WS. 2009. Advances in chitosan material and its hybrid derivatives: a review. *Open Biomater. J.* **1:** 10-20.
25. Lee YH, Chang JJ, Yang MC, Chien CT, Lai WF. 2012. Acceleration of wound healing in diabetic rats by layered hydrogel dressing. *Carbhydr. Polym.* **88:** 809-819.
26. Lin T, Lin L, Zeng P. 2014. Development of a duplex real-time PCR method for the pharmaceutical rapid microbial detection of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J. Biosci. Med*. **2:** 12-19.
27. Lodise TP, Lomaestro Ben GJ, Drusano GL. 2008. Larger vancomycin doses (at least 4 grams per day) are associated with an increased incidence of nephrotoxicity. *Antimicrob. Agents Chemother*. **52:** 1330-1336.
28. Maguire GP, Arthur AD, Boustead PJ, Dwyer B, Currie BJ. 1998. Clinical experience and outcomes of community acquired and nosocomial methicillin-resistant *Staphylococcus aureus* in a northern Australian hospital. *J. Hosp. Infect*. **38:** 273-281.
29. Masson-Meyers DS, Enwemeka CS, Bumah VV, Andrade TAM, Cashin SE, Frade MAC. 2013. Antimicrobial effects of *Copaifera langsdorffii* oleoresin infected rat wounds. *Int. J. Appl. Microbiol. Sci*. **2:** 9-20.
30. Mayer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, Melaughhlin JL. 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med*. **45:** 31-34.
31. Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van Eldere J, Glupczynski Y, *et al*. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin. Microbiol. Infect*. **13:** 560-578.
32. Mizuno K, Yamamura K, Yano K, Osada T, Saeki S, Takimoto N, *et al*. 2002. Effect of chitosan film containing basic fibroblast growth factor on wound healing in genetically diabetic mice. *J. Biomed. Mater. Res*. **64:** 177-181.
33. Mock JJ, Barbic M, Smith DR, Schultz DA, Schultz S. 2002. Shape effects in plasmon resonance of individual colloidal silver nanoparticles. *J. Chem. Phys*. **116:** 6755-6760.
34. Mohammed FA. 2010. Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against gram-positive and gram-negative bacteria. *J. Nanomed*. **6:** 103-109.
35. Motoshima M, Yanagihara K, Fukushima K, Matsuda J, Sugahara K, Hirakata Y, *et al*. 2007. Rapid and accurate detection of *Pseudomonas aeruginosa* by real-time polymerase chain reaction with melting curve analysis targeting *gyrB* gene. *Diagn. Microbiol. Infect. Dis*. **58:** 53-58

36. Moura LIF, Dias AMA, Carvalho E, De Sousa HC. 2013. Recent advances on the development of wound dressings for diabetic foot ulcer treatment. *Acta Biomater*. **9:** 7093-7114.
37. Nagai G, Takano M. 2014. Entry of aminoglycosides into renal tubular epithelial cells via endocytosis-dependent and endocytosis-independent pathways. *Biochem. Pharmacol*. **90:** 331-337.
38. O'Loughlin A, O'Brien T. 2011. Topical stem and progenitor cell therapy for diabetic foot ulcers. *Stem Cells Clin. Res*. **978:** 579-604.
39. O'Meara S, Nelson EA, Golder S, Dalton JE, Craig D, Iglesias C. 2006. Systematic review of methods to diagnose infection in foot ulcers in diabetes. *Diabet. Med*. **23:** 341-347.
40. Parasuraman S, Raveendran R, Kesavan R. 2010. Blood sample collection in small laboratory animals. *J. Pharmacol. Pharmacother*. **1:** 87-93.
41. Pelgrift RY, Friedman A.J. 2013. Nanotechnology as a therapeutic tool to combat microbial resistance. *Adv. Drug Deliv. Rev*. **65:** 1803-1815.
42. Rathur HM, Bloulton AJM. 2005. Recent advances in the diagnosis and management of diabetic neuropathy. *J. Bone Joint Surg*. **87:** 1605-1610.
43. Regiel A, Irusta S, Kyzioł A, Arruebo M, Santamaria J. 2013. Preparation and characterization of chitosan–silver nanocomposite films and their antibacterial activity against *Staphylococcus aureus*. *Nanotechnology* **24:** 015101.
44. Rey-Juradoa E, Tudób G, Soyc D, González-Martína J. 2013. Activity and interactions of levofloxacin, linezolid, ethambutol and amikacin in three-drug combinations against *Mycobacterium tuberculosis* isolates in a human macrophage model. *Int. J. Antimicrob. Agents* **42:** 524-530.
45. Saha N, Saarai A, Roy N, Kitano T, Saha P. 2011. Polymeric biomaterial based hydrogels for biomedical applications. *J. Biomater. Nanobiotechnol*. **2:** 85-90.
46. Sasikala L, Durai B. 2015. Development and evaluation of chitosan honey hydrogel sheets as wound dressing. *Int. J. Pharma Bio. Sci*. **6:** 26-37.
47. Savov E, Chankova D, Vatcheva R, Dinev N. 2002. In vitro investigation of the susceptibility of *Acinetobacter baumannii* strains isolated from clinical specimens to ampicillin/ sulbactam alone and in combination with amikacin. *Int. J. Antimicrob. Agents* **20:** 390-392
48. Schwartz JA, Lantis JC, Gendics C, Fuller AM, Payne W, Ochs DA. 2013. Prospective, non-comparative, multicenter study to investigate the effect of cadexomer iodine on bioburden load and other wound characteristics in diabetic foot ulcers. *Int. Wound J*. **10:** 193-199.
49. Shaheen SM, Yamaura K. 2002. Preparation of theophylline hydrogels of tactic poly (vinyl alcohol)/NaCl/H_2O system for drug delivery system. *J. Control. Release* **81:** 367-377.
50. Shankar EM, Mohan V, Premalatha G, Srinivasan RS, Usha AR. 2005. Bacterial etiology of diabetic foot infections in South India. *Eur. J. Intern. Med*. **16:** 567-570.
51. Smith CR, Maxwell RR, Edwards CQ, Rogers JF, Lietman PS, Hopkins J. 1978. Nephrotoxicity induced by gentamicin and amikacin. *Med. J*. **142:** 85-90**.**
52. Sondi I, Salopek-Sondi B. 2007. Silver nanoparticles as antimicrobial agent: case study on *E. coli* as a model for gram-negative bacteria. *J. Colloid Interface Sci*. **275:** 177-182.
53. Spilker T, Coenye T, Vandamme P, LiPuma JJ. 2004. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other *Pseudomonas* species recovered from cystic fibrosis patients. *J. Clin. Microbiol.* **42:** 2074-2079.
54. Stashak TS, Farstvedt E, Othic A. 2004. Update on wound dressings: indications and best use. *Clinic. Tech. Equine Pract*. **3:** 148-163.
55. Sugandhi P, Prasanth D. 2014. Microbiological profile of bacterial pathogens from diabetic foot infections in tertiary care hospitals, salem diabetes & metabolic syndrome. *Diabetes Metab. Syndr*. **8:** 129-132.
56. Tally FP, Gorbach LS. 1977. Amikacin therapy of gram-negative bacteremia. *Am. J. Med.* **62:** 936-939.
57. Tao Y, Qian LH, Xie J. 2011. Effect of chitosan on membrane permeability and cell morphology of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Carbohydr. Polym.* **86:** 969-974.
58. Ternent L, Dyson RJ, Krachler AM, Jabbari S. 2015. Bacterial fitness shapes the population dynamics of antibiotic-resistant and -susceptible bacteria in a model of combined antibiotic and anti-virulence treatment. *J. Theor. Biol*. **372:** 1-11.
59. Wei D, Sun W, Qian W, Ye Y, Ma X. 2009. The synthesis of chitosan-based silver nanoparticles and their antibacterial activity. *Carbohydr. Res*. **344:** 2375-2382.
60. Wild S, Roglic G, Green A, Sicree R, King H. 2004. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care* **27:** 1047-1053.
61. Zuo GY, Han ZQ, Hao XY, Han J, Li ZS, Wang GC. 2014. Synergy of aminoglycoside antibiotics by 3-benzylchroman derivatives from the Chinese drug *Caesalpinia sappan* against clinical methicillin-resistant *Staphylococcus aureus* (MRSA). *Phytomedicine* **21:** 936-941.