

A NEW FORMULATION OF CINNAMON OIL AND CHITOSAN DEPOLYMERIZED AGAINST OPPORTUNISTIC MICROORGANISMS DURING WOUND HEALING

MARÍA LUISA DEL PRADO-AUDELO^{1,2}, ADRIANA ESTELA HERNÁNDEZ-TENORIO¹, MAYKEL GONZÁLEZ-TORRES³, JONATHAN J. MAGAÑA⁴, ROBERTO SÁNCHEZ-SÁNCHEZ⁵, MARÍA DEL PILAR GRANADA-MACÍAS⁶, SILVESTRE ORTEGA-PEÑA⁷, HERNÁN CORTES⁴, GERARDO LEYVA-GÓMEZ^{1*}

¹Department of Pharmacy, Faculty of Chemistry, National Autonomous University of Mexico, Ciudad Universitaria, Circuito Exterior S/N, Del. Coyoacán, Mexico City, Mexico

²Graduate Laboratory in Pharmaceutical Technology, FES-Cuautitlán, National Autonomous University of Mexico, Cuautitlán Izcalli, Mexico

³CONACyT-Biotechnology Laboratory, National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra, Mexico City, Mexico

⁴Genomic Medicine Laboratory, Department of Genetics, National Center for Research and Attention to Burns (CENIAQ), National Institute of Rehabilitation-Luis Guillermo Ibarra Ibarra (INR-LGII), Mexico City, Mexico

⁵Tissue Engineering Unit Cell Therapy and Regenerative Medicine, National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra (INR-LGII), Mexico City, Mexico

⁶Department of Biology, Faculty of Chemistry, National Autonomous University of Mexico, Ciudad Universitaria, Circuito Exterior S/N, Del. Coyoacán, Mexico City, Mexico

⁷Laboratory of Infectology, National Institute of Rehabilitation Luis Guillermo Ibarra Ibarra, Mexico City, Mexico

*corresponding author: gerardoleyva@hotmail.com

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Abstract

Infectious processes may delay wound healing. In this regard, there are diverse treatments available to prevent infections and promote wound healing. However, many of these present drawbacks, such as bacterial resistance, decrease their efficacy. In this respect, chitosan, a natural polymer with antibacterial and antifungal properties, may be useful in the process of skin repair. In this work, a cinnamon oil-based formulation with chitosan modified by gamma irradiation was developed. Moreover, disodium EDTA was added to create a combined antimicrobial effect against infections. The novel formulation showed percentages of inhibition of 43.64% for *E. coli*, 42.86% for *P. aeruginosa*, 80.27% for *C. albicans*, and 42.31% for *S. aureus*, as well as antibiofilm activity against *P. aeruginosa*. Our results suggest that the obtained formulation could be a new alternative treatment for chronic wounds.

Rezumat

Procesele infecțioase pot întârzia vindecarea rănilor. În acest sens, există diferite tratamente disponibile pentru prevenirea infecțiilor și vindecarea rănilor, dar rezistența bacteriană încă rămâne o problemă. În acest sens, chitosanul, un polimer natural cu proprietăți antibacteriene și antifungice, poate fi util în procesul de reparare a pielii. În această lucrare a fost elaborată o formulare pe bază de ulei de scorțișoară cu chitosan modificat prin iradiere gamma. Mai mult decât atât, EDTA disodic a fost adăugat pentru a crea un efect antimicrobian combinat împotriva infecțiilor. Noua formulare a înregistrat procente de inhibiție de 43,64% pentru *E. coli*, 42,86% pentru *P. aeruginosa*, 80,27% pentru *C. albicans* și 42,31% pentru *S. aureus*, precum și activitate antibiofilm împotriva *P. aeruginosa*. Rezultatele obținute sugerează că formularea ar putea fi un nou tratament alternativ pentru plăgile cronice.

Keywords: cinnamon oil, chitosan, gamma radiation, infections

Introduction

A wound is defined as a disruption to normal anatomical structure and function [28]. According to the U.S. National Institute of Health, 80% of human infections involve mechanisms of antibacterial resistance, which delay wound healing [6]. These mechanisms are mediated by bacterial biofilms; in these systems, microbial colonies are immobilized within a matrix composed of polysaccharides, proteins, nucleic acids,

and lipid molecules, conferring them tolerance to antibiotics and immunological protection [30]. In recent years, interest in essential oils as treatments against biofilm formation has increased due to its potential to decrease antimicrobial resistance [13]. In this regard, Cinnamon essential oil (CO) is an important candidate being composed mainly of trans-cinnamaldehyde, o-methoxy-cinnamaldehyde, cinnamyl aldehyde, benzaldehyde, phenyl ethanol, borneol,

eugenol, coumarin, and cinnamic acid [29]. CO has demonstrated activity against biofilm cultures of *Streptococcus mutans* and *Lactobacillus plantarum* [23]; the effect appears to depend on the high hydrophobicity of CO, which would disrupt the lipid bilayer of the cell membrane, increasing proton permeability. To improve the antibiofilm-associated properties of CO and to balance its toxicity in high concentrations, this compound could be mixed with different excipients, such as EDTA or chitosan [11, 14]. The mechanism of action of EDTA is based on its ability to remove Mg^{2+} and Ca^{2+} ions from the external cell wall of Gram-negative bacteria, affecting the integrity of the bacterial membrane [27]. On the other hand, the natural polymer chitosan is widely used as wound dressing [1, 4] and as antibacterial agent. Interestingly, the depolymerization process of chitosan by means of gamma radiation increases its antimicrobial effects, according to previous studies [2, 15].

For these reasons, the aim of this work was to develop a new treatment based on CO, EDTA, and irradiated chitosan against persistent wound microorganisms.

Materials and Methods

Materials

Chitosan (low-molecular-weight), and glacial acetic acid were obtained from Sigma-Aldrich® (Merck KGaA01, 104 Darmstadt, Germany), whereas disodium EDTA, CO, and polysorbate 80 were purchased from Droguería Cosmopolita (Mexico City, Mexico). Argentafil® silver sulfadiazine (Grossman Laboratories, Mexico) was employed as reference for the antimicrobial and antibiofilm tests. The microorganisms utilized were *Escherichia coli* (ATCC® 25922™), *Staphylococcus aureus* (ATCC® 2913™), *Pseudomonas aeruginosa* (ATCC® 27853™) and *Candida albicans*.

Preparation of samples

A solution of chitosan in acetic acid (2% w/v) was irradiated under cobalt-60 gamma irradiation at a dose of 25 KGy. The irradiated chitosan was mixed with EDTA, maintaining constant stirring until EDTA was completely dissolved. CO and polysorbate 80 (Tween 80) were mixed and added drop-wise into the irradiated chitosan-EDTA solution. Stirring was conserved until homogeneity and the pH was controlled to maintain a value of 5.0. The final formulation was filtered using 0.20 µm membranes.

Table I
Concentrations of irradiated formulation with cinnamon oil pH 5.0

Excipient	Concentration (%)
Cinnamon oil	0.93% v/v
Disodium EDTA	1.86% w/v
Irradiated chitosan	1.85% w/v
Tween 80	4.63% v/v
Acetic acid 0.5%	90.73% v/v

Table 1 presents the concentrations employed for elaboration of the formulation. Regarding the physicochemical characterization, the formulation and excipients were lyophilized (-49°C, 0.05 mBar, and 24 h).

Physicochemical characterization

Infrared spectroscopy (FT-IR)

To determine the possible interactions of the formulation excipients, lyophilized samples were analysed by FT-IR using an FTIR Nicolet 6700 (Thermo Fisher Scientific®, USA). The scanning range was 4000 - 400 cm^{-1} .

Thermogravimetric analysis (TGA)

To evaluate the thermal stability of the excipients and the formulation, lyophilized samples were tested using a calorimeter (Q5000; TA Instruments, DE, USA) at a heating rate of 10°C/min, with a temperature range of 0 - 800°C, and under a nitrogen atmosphere.

Differential scanning calorimetry (DSC)

To evaluate the thermal properties of the excipients and the formulation, the lyophilized samples were tested using a calorimeter (Q2000; TA Instruments, DE, USA) at a heating rate of 10°C/min, with a temperature range of 20 - 350°C, under a nitrogen atmosphere.

Antimicrobial test

To evaluate the antimicrobial activity of the formulation, the disk diffusion test was employed [3]. Microbial strains of *Escherichia coli* (ATCC® 25922™), *Staphylococcus aureus* (ATCC® 2913™), *Pseudomonas aeruginosa* (ATCC® 27853™) and *Candida albicans* were inoculated onto Mueller-Hinton agar plates, at a concentration of 1.5×10^8 CFU/mL. Sterile filter-paper discs (5 mm in diameter) were impregnated with each of the samples and placed in the inoculated agar. Then, the plates were incubated at 37°C for 48 h. After the incubation time, the inhibition zones were measured. The experiment was performed three times in independent sessions.

Anti-biofilm test

To analyse the anti-biofilm activity of the formulation, the disk diffusion method in agar was applied. For strains of *Escherichia coli* and *Pseudomonas aeruginosa* the Mueller-Hinton agar was employed; whereas for *Staphylococcus aureus* and *Candida albicans*, the Mueller-Hinton agar was supplemented with sheep blood 5%. Argentafil® silver sulfadiazine 1% was used as positive control, whereas as negative control injectable water was selected. Each agar plate was inoculated with the microorganisms (1.5×10^8 CFU/mL) and incubated at 37°C for 24 h to form the biofilm. Subsequently, the disks impregnated with the solutions-of-interest (formulation, negative and positive controls) were placed on the plates and incubated for 12 h at 37°C, after which the zones of inhibition surrounding the antibiotic disks were measured and recorded. The experiment was performed three times in independent trials.

Results and Discussion

In this work, a new treatment for the inhibition of opportunistic microorganisms in wound healing based on CO, irradiated chitosan, and EDTA (pH 5.0) was elaborated, characterized, and evaluated *in vitro*.

Physicochemical characterization

FT-IR

In Figure 1, the FT-IR spectra of the excipients and formulation are presented. In the IR spectrum of disodium EDTA (line a), the stretch of the C=O bond was able to be identified, which was found to be $1,674\text{ cm}^{-1}$, whereas the stretch of the $-\text{CH}_2$ and $-\text{OH}$ bonds were observed at $1,476$ and $1,390\text{ cm}^{-1}$, respectively. Lines (b) and (c) reveal the FT-IR spectra corresponding to chitosan and irradiated chitosan, respectively. In agreement with the literature [18], on chitosan, a typical band was observed at 3450 cm^{-1} , corresponding to the $-\text{OH}$ group, while at 2883 cm^{-1} , the band related to the $-\text{CH}_2$ stretching vibration was found. In addition, the bands of $-\text{NH}_2$ and $-\text{CH}_3$ vibration were observed at 1595 and at 1374 cm^{-1} , respectively [12]. On the other hand, in the irradiated chitosan spectra, the bands of the main functional groups are also clearly present. However, interestingly, the intensity of the peaks within the range from $1650 - 800\text{ cm}^{-1}$ was higher than the peaks of un-irradiated chitosan. This behaviour could be explained by the depolymerization of the chitosan, which generates bonus C=O functional groups [31]. Finally, the formulation spectrum demonstrated the characteristic bands of the excipients (Figure 1, line d), proving the cohesion of the excipients.

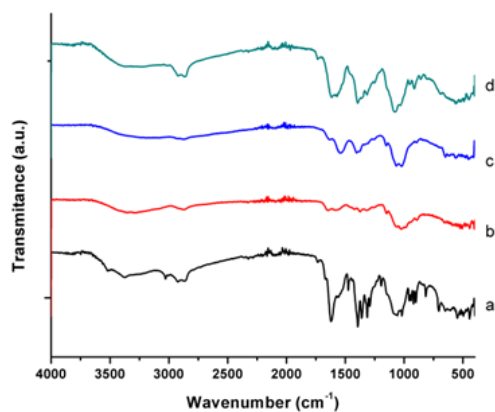


Figure 1.

FT-IR spectra of: EDTA (a), chitosan (b), irradiated chitosan (c) and the formulation (d)

Thermogravimetric analysis

The thermograms of the excipients and the formulation are presented in Figure 2. Line (a) depicts the thermogram of EDTA. For chitosan (line b), a slight loss of mass related to the humidity of the samples is observed between 40 and 150°C . Likewise, the second thermal event, observed between 270 and 310°C is related to chitosan decomposition, involving the depolymerization

phenomena and degradation of the glucopyranose units and their subsequent oxidation. It is noteworthy that, for the irradiated chitosan (line c), degradation reactions were found between 130°C and 360°C , suggesting that the molecular weight is lower than that of the un-irradiated chitosan, due to that it requires a lower temperature to achieve degradation [12]. This behaviour suggests that the thermal stability of the chitosan molecule decreases when it is modified, particularly, by gamma radiation [10]. Finally, the thermogram of the formulation (line d) presents thermal events at 98 , 140 and 220°C , approximately. The differences between the thermograms, (a, b, c and d), could be due to the interactions and cohesion between the excipients into the formulation. This result, along with those of the IR analysis, show that new bonds exist in the formulation.

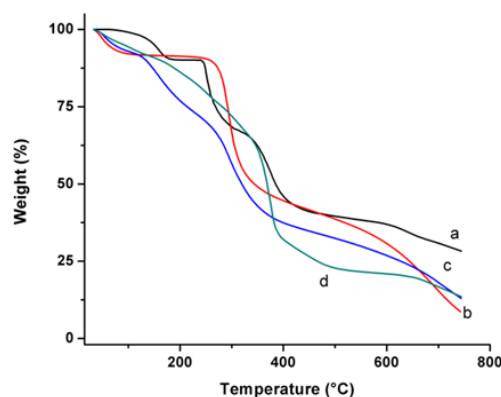


Figure 2.

Thermograms of EDTA (a), chitosan (b), irradiated chitosan (c) and the formulation (d)

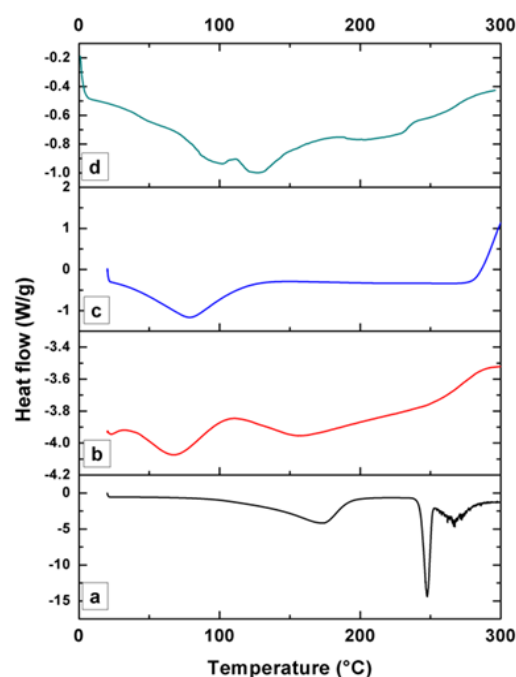


Figure 3.

DSC profiles of EDTA (a), chitosan (b), irradiated chitosan (c) and the formulation (d)

Differential scanning calorimetry

Figure 3 presents the first thermal event for chitosan and irradiated chitosan (lines (b) and (c), respectively) between 20°C and 130°C, which corresponds to an endothermic peak associated with the evaporation of water. Likewise, the thermal event for chitosan (line b) that begins at 300°C could indicate degradation of the molecule [8, 9, 24, 25]. As can be observed, there are slight changes between the DSC profile of chitosan (line b) and that of the irradiated chitosan (line c). This difference could be related to the depolymerization of the chitosan chain. Finally, line (d) shows the thermal profile of the formulation; similarly to the TGA profile, the thermal events of the excipients are mixed due to the interaction of these in the process of obtaining the formulation.

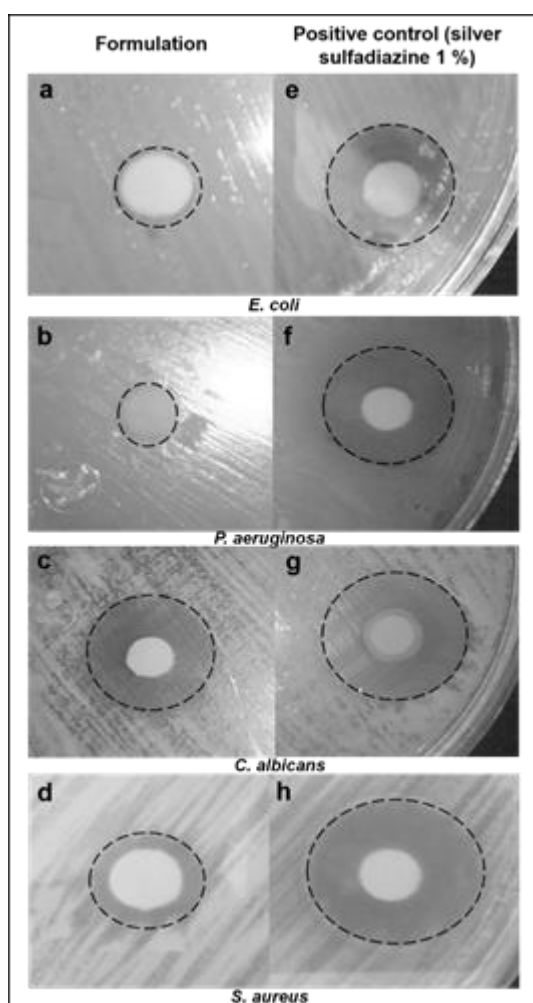


Figure 4.

Inhibition halo of formulation (a, b, c and d) and positive control, silver sulfadiazine 1% (e, f, g and h)

Antimicrobial test

In order to determine the antimicrobial capacity of the new formulation, inhibition tests were performed on four of the characteristic strains considered as opportunistic in wound healing. The zone of inhibition

was measured after 48 h of incubation; these results are shown in Figure 4. The percentage of inhibition of the formulation with respect to the positive control was 43.64% for *E. coli* ($p < 0.05$), 42.86% for *P. aeruginosa* ($p < 0.05$), 80.27% for *C. albicans* (ns), and 42.31% for *S. aureus* ($p < 0.05$). Although the positive control exhibited a higher microbial inhibition compared to the formulation, it should be considered that the formulation is not an antibiotic itself. Silver sulfadiazine is a treatment-of-first-choice in hospitals in several countries worldwide, but also there are reports of slight resistance to silver [19].

In this regard, our formulation combines the antimicrobial mechanisms of disodium EDTA, irradiated chitosan in acid medium, CO, and avoids the use of silver. All of these components show antimicrobial activity; thus, their combination renders the formulation a potent natural option. Interestingly, the formulation revealed the highest percentage of inhibition against the *C. albicans* strain (Figure 5).

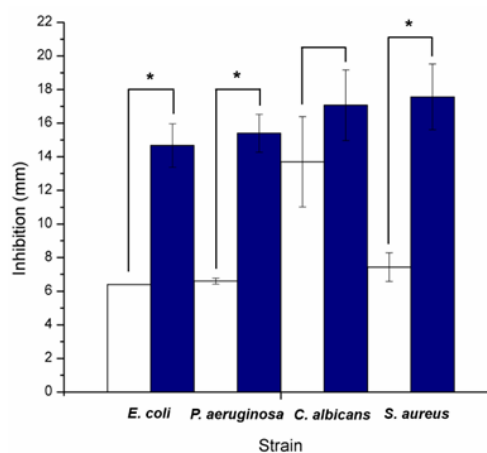


Figure 5.

Bacterial inhibition of formulation (white bars) vs. positive control, silver sulfadiazine 1% (blue bars)
* Indicates $p < 0.05$ as statistically significant

This polymorphic fungus is a member of the normal human microbiome. Although it usually resides as a life-long harmless commensal, under certain circumstances it can cause infections that range from superficial infections of the skin to life-threatening systemic infections [16, 22]. The inhibitory action of the formulation vs. *C. albicans* could be related to the presence of EDTA, because this agent possesses an antifungal potential. This effect could be explained by the chelating property of EDTA, which plays a critical role in morphogenesis and pathogenesis of *C. albicans* [22]. Likewise, the inhibition of different strains by CO has already been investigated, demonstrating interesting results. Prabuseenivasan *et al.* [20] investigated the microbial inhibition produced by different types of natural oils; for CO, inhibition vs. *S. aureus*, *P. aeruginosa*, and *E. coli* was 13.7, 21 and 21 mm, respectively. These values are higher than those of

the tested formulation (7.3, 6.6 and 6 mm, respectively). However, the concentration of CO employed by Prabuseenivasan *et al.* [20] was 1:20, whereas that for our experiment was 1:100, indicating that the level of inhibition is dose-related. In a similar approach, Carvalhinho *et al.* [7] explored the inhibition of CO (15 μ L), obtaining a halo of 35 mm. These results indicate that a high concentration of CO is necessary to obtain a remarkable inhibition; however, it should be noted that high concentrations of this natural essential oil may produce cytotoxic effects [23]. On the other hand, antifungal activity against *C. albicans* is greater when the molecular weight of chitosan is low and the degree of deacetylation is high. Chitosan interacts with the cell wall due to the presence of sialic acid on surface, which causes an alteration in permeability, consequently, the filtration of intracellular components can inhibit the synthesis of DNA and RNA [26]. The inhibitory effect of the formulation on *C. albicans* is especially important for the treatment of chronic wounds, such as diabetic foot ulcers, where *Candida spp.* is predominant and resistant to the action of antifungal and antimicrobial agents such as amphotericin B, chlorhexidine, nystatin and fluconazole [17].

Antibiofilm test

Biofilms are complex structures involving different species of microorganisms, embedded in a polysaccharide matrix. Due to their structure, biofilms are resistant to disinfectants or detergents and lead to complications in many fields, such as in the food industry and in medical surgery. The antibiofilm activity of the formulation and the positive control against *P. aeruginosa* can be observed in Figure 6. Interestingly, the formulation presented an average of 13 mm of inhibition halo, while the positive control, which is widely used for the treatment of chronic wounds, did not exhibit activity *vs.* biofilm. Diverse mechanisms could explain the antibiofilm activity demonstrated by the formulation. For example, it has been reported that acetic acid could eradicate the mature biofilms of *P. aeruginosa*, even at low concentrations such as 0.5% [5]. Furthermore, the authors reported that in acetic acid at different values of pH (4.33 - 6), the acid was completely bactericide, killing all tested bacteria. On the other hand, chelating agents such as EDTA are capable of destabilizing biofilm sequestering iron, zinc, magnesium and calcium [21], in addition to the mechanisms of destabilization by the electrostatic charges of chitosan. Therefore, the combined antimicrobial effect of the excipients of the formulation obtained represents an alternative against biofilms produced by *P. aeruginosa*, compared with Argentafil[®] silver sulfadiazine, as treatment for wounds. An antibiofilm effect against the other bacteria was not observed in the evaluated concentration nor with the positive control.

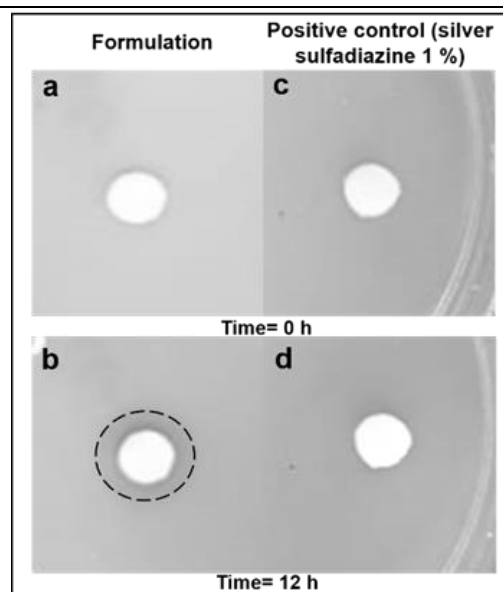


Figure 6.

Inhibition halo in *P. aeruginosa* biofilm of the formulation at 0 h (a) and at 12 h (b); positive control, silver sulfadiazine 1% at 0 (c) and at 12 h (d)

Conclusions

The mechanisms of bacterial resistance to antibiotics remain a serious health problem worldwide, and molecular alternatives are still limited. Particularly, during the healing progresses of severe wounds, the health state of patients becomes critical due to infectious processes. In this work, we developed a new antiseptic solution consisting of CO, chitosan modified by gamma irradiation, and EDTA. In addition, this study addressed a strategy of diluted CO to reduce the toxic effects on damaged tissue and took advantage of combined antimicrobial effects with EDTA and chitosan. The results indicated a percentage of effectiveness of between 50% and 80% against opportunistic bacteria and the biofilm of *P. aeruginosa*. Although the formulation is not an antibiotic form, it could be an excellent option for application as treatment in chronic wounds, considering the phenomenon of bacterial resistance. Therefore, this new proposal represents a biocompatible alternative of clinical interest to eradicate biofilms.

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Conflict of interest

The authors declare no conflict of interest.

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