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Effect of chitosan membranes against gram-negative bacteria isolated from cutaneous ulcers

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Abstract

The objective of this study was to evaluate the *in vitro* effectiveness of membranes developed with pure chitosan and chitosan in a mixture with glycerol-honey against gram-negative bacteria isolated from skin ulcers. The membranes were prepared by the solvent evaporation technique. The identification and antibiotic sensitivity of microorganisms were determined in microplates, and *in vitro* tests were developed by the agar diffusion technique. The most frequently isolated microorganism was *Escherichia coli* with 43.75 % prevalence. All membranes showed antimicrobial effects by direct contact against *Proteus mirabilis*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Morganella morganii*. Antibiograms showed that most of these microorganisms are multi-resistant to antibiotics. All of this suggests that chitosan-based membranes are a safe alternative for the treatment of infected cutaneous ulcers compared to traditional antibiotics. The outcomes of this study confirm that membranes made of a biodegradable polymer, such as chitosan have activity against multidrug-resistant gram-negative bacteria that grow in infected skin ulcers.

Introduction

Chronic wounds cause severe financial problems due to the medical attention they require. These types of wounds are characterized by slow healing, or they may never heal, which generates physical and emotional stress to the patient. The degree of infection always depends on the species and concentration of the microorganism, as well as the response of the host (Akuzy *et al.* 2018). According to data reported by Davies *et al.* (2007), chronic wounds on the skin affect approximately 3 % of people over 60 years of age; additionally, these types of wounds maintain polymicrobial biota.

The microbial biota found in chronic wounds includes *Staphylococcus*, *Streptococcus*, *Pseudomonas* and coliforms. It is also possible to find anaerobic bacteria because conditions are generated for them to proliferate through the combination of necrotic tissue and low levels of oxygen. For Gentili *et al.* (2012), the presence of 10^5 or more cells per gram of tissue is a fundamental indicator to confirm that healing will be slow. Goswami *et al.* (2017) reported more frequent microorganisms to be *Staphylococcus aureus*, *Pseudomonas aeru-*

ginosa, *Staphylococcus epidermidis*, and *Candida albicans*. Specifically, *S. aureus* and *P. aeruginosa* are the cause of nosocomial infections with an incidence of 20 – 40 % and 5 – 15 %, respectively (Mama *et al.* 2014). Particularly, *P. aeruginosa* is an opportunistic gram-negative pathogen capable of causing severe infections in ulcers, in addition to being resistant to multiple antibiotics (Myhrman *et al.* 2013). Microorganisms of clinical origin develop various mechanisms of resistance to antibiotics due to prolonged therapies. Therefore, the selection of the type and dose of antibiotic is essential to prevent the spread of resistance between microorganisms and promote the healing of ulcers (Cunha *et al.* 2018).

Chitosan is a natural polymer obtained by deacetylation of chitin; it is composed of D-glucosamine and N-acetyl-D-glucosamine (Balti *et al.* 2017). Its biological applications are due to its biocompatibility, biodegradability, and its nontoxicity (Babushkina *et al.* 2015; El-Malek *et al.* 2017). The amine groups of chitosan provide the antimicrobial capacity (Ashrafi *et al.* 2018) and hemostatic properties. Due to its chemical properties, chitosan has the ability to form films, hydrogels, and fibers (Kiroshka *et al.* 2014; Khalil *et al.* 2015). Its potential application in the area of medicine is due to its affinity to aminoglycosides of human tissues (Babushkina *et al.* 2015). Additionally, it is characterized as being hypoallergenic (Koryagin *et al.* 2006). The mechanism of action of chitosan is that it alters the cell surface and its permeability, causing the leakage of intracellular substances. This is attributed to the fact that the positive charges of the amino group of chitosan interact with the negative charges of the surface of microorganisms (Demir *et al.* 2010; Uranga *et al.* 2019). According to Dragostin *et al.* (2016), when applying dressings based on chitosan for wound healing, chitosan is degraded into N-acetyl glucosamine, which is used to accelerate the re-epithelialization process.

The strategy of using pure chitosan and its topical combinations as an alternative to antibiotics has been proposed to health institutions. In reference to the above, the aim of this work was to evaluate the *in vitro* effectiveness of films developed with pure chitosan and in mixture with glycerol-honey against gram-negative bacteria isolated from cutaneous ulcers. Honey was incorporated into chitosan films as an adjuvant agent because it has been used to eliminate infections, is a healing agent in skin ulcers (Alam *et al.* 2014) and is an anti-inflammatory agent (Meo *et al.* 2017). According to Basualdo *et al.* (2007), honey promotes the formation of granulation tissue, the growth of the epithelium and healing. In addition, honey has antimicrobial activity against *S. aureus*, *P. aeruginosa* and *K. pneumoniae* (El-Malek *et al.* 2017).

Experimental

Physicochemical characteristics of chitosan

In this research we worked with chitosan obtained in our laboratory (Sánchez-Duarte *et al.* 2012). The humidity and ash content of the chitosan were 92.42 ± 0.07 % and 0.37 ± 0.02 %, respectively. Its molecular weight was 119.48 kDa and the degree of deacetylation was 84.59 ± 0.87 %. Additionally, the functional groups characteristic of chitosan were identified by Fourier transform infrared spectroscopy (FTIR**)**.

Preparation of chitosan membranes

The membranes were prepared using the solvent evaporation technique. Six different formulations were developed, three were of pure chitosan at 1, 2 and 3 % in acetic acid at 1 % (w/v), another of 2 % chitosan with 0.2325 ± 0.01 g of glycerin and the other two formulations were 2 % chitosan and honey (95 : 5 v/v), glycerin was added to one of them. The honey was diluted in water (80 : 20 v/v). All the mixtures were homogenized until all the components were fully incorporated. Specifically, 10 mL of each of the formulations were measured independently and a plastic mold was poured and subsequently dried at 40 °C for 24 h. Finally, the membranes were detached from the molds and stored in sterile plastic bags.

Sample collection

The study was descriptive and cross-sectional. The samples were taken from infected ulcers of patients hospitalized by a doctor specialized in epidemiology. Specifically, the sampling was done

in the ulcer center with sterile cotton tipped applicator and was introduced in a transport device Stuart (COPAN Transystem^{R}, Brescia, Italy) to keep the isolate in good condition. Once all the samples were collected, they were transported in a hermetically sealed container to a laboratory certified in microbiological analysis and were analyzed in a period no longer than one hour. All patients involved in the trial confirmed their participation with informed consent and the research protocol was approved by the Institutional Ethics Committee.

Isolation, identification and antibiotic sensitivity

To favor the isolation and identification of microorganisms, samples were seeded by cross-streaking on MacConkey agar (BD Bioxon, Cuautitlán Izcalli, Estado de México, México) for gram-negative bacteria and on Mannitol salt agar (BD Bioxon, Cuautitlán Izcalli, Estado de Mexico, Mexico) for gram-positive bacteria, and the plates were incubated at 37 °C for 24 h. For fungi, the samples were seeded on Biggy agar (BD Bioxon, Cuautitlán Izcalli, Estado de Mexico, Mexico) by sowing and sweeping, and the plates were incubated at 30 °C for 24 h. Subsequently, the identification of microorganisms was performed by the broth microdilution technique. The isolated colonies were taken with a Prompt^{TM} inoculation rod and placed in Prompt^{TM} inoculation bottles until reaching a concentration of 0.08 on the MacFarland standard. The microplates were inoculated with 100 µL in each well and incubated at 37 °C for 24 h. For the gram-negative bacteria, the SIEMENS type 44 microplates were used (B1017-305) and for gram-positive type 33 (B1017-211). Finally, the plates for the identification of the microorganisms were read using LabPro Command Center Software (MicroScan® LabPro$_{TM}$), which indicates the percentage of certainty corresponding to the microorganism identified. Antibiotic sensitivity was measured by the minimum inhibitory concentration (MIC) according to the CLSI (Clinical and Laboratory Standards Institute 2014) criteria. The antibiotics studied were ampicillin, amikacin, ampicillin/sulbactam, cefuroxime, cefotetan, ticarcillin/clavulanate, piperacillin/tazobactam, cefazolin, cefotaxime, ceftazidime, imipenem, ceftriaxone, moxifloxacin, cefepime, gentamicin, trimetropin/sulfa-methoxazole, levofloxacin, ciprofloxacin, cefota-xime/clavulanate, ceftazidime/clavulanate, tobra-mycin, meropenem and aztreonam. One combination for gram-negative (*Klebsiella pneumoniae)* is shown, of which the identification was confirmed by biochemical tests and the sensitivity and resistance to antibiotics were evaluated simultaneously.

In vitro assays of chitosan membranes against gram-negative bacteria

The *in vitro* tests were determined by the Kirby-Bauer agar diffusion technique. Specifically, for each isolate a dilution in broth was made in an inoculation bottle Prompt^{TM}, and with a sterile swab, the boxes were inoculated in Mueller Hinton agar in seeding by sweeping. Each membrane was divided into disks with a diameter of 16.4 ± 0.10 mm. Three discs were placed with a sterile clamp on the inoculated medium. In addition, two discs of Whatman No. 1 paper impregnated independently with 1 % acetic acid and 0.9 % sodium chloride were placed as blanks. The plates were incubated at 37 ° C for 24 h, and finally, the increase in area and the inhibition halo generated by each membrane was measured.

Results

Phenotypic identification of microorganisms in skin ulcers

In total, 23 ulcers of infected patients were sampled. Gram-negative and gram-positive bacteria were found with 51.61 % and 29.03 % prevalence, respectively. In addition, it was possible to isolate *Candida albicans* with a prevalence of 19.35 %. Previous studies related to gram-positive bacteria were published, particularly with regard to *S. aureus* (Escárcega-Galaz *et al.* 2017).
Specifically, this work focused on gram-negative bacteria. From all of the ulcers sampled, 16 gram-negative strains were isolated. Among them, *Proteus mirabilis, Escherichia coli, Enterobacter aerogenes, Pseudomona aeruginosa, Klebsiella pneumoniae* and *Morganella morganii* were

identified. Table 1 shows a comparison of the microorganisms isolated in this investigation with respect to that reported by other authors.

Table 1. Comparative analysis of incidences of gram-negative bacteria in skin ulcer.

Microorganisms	**This work**	**Mama *et al.* (2014)**	**Kateel *et al.* (2018)**	**Malik *et al.* 2013)**
Proteus mirabilis	12.50	-	-	1.5
Escherichia coli	43.75	20	34.69	27.8
Enterobacter aerogenes	6.25	-	-	-
Pseudomonas aeruginosa	12.50	8	30.61	15.6
Klebsiella pneumoniae	18.75	10	-	5.8
Morganella morganii	6.25	-	-	0.7
Klebsiella species	-	-	12.63	-
Proteus species	-	-	5.10	-
Klebsiella oxytoca	-	-	-	7
Proteus vulgaris	-	-	-	3.5

Antibiotic sensitivity of gram-negative bacteria

Bacterial resistance to the 23 antibiotics studied is presented in Table 2 (SI-2). Regardless of the phenotype, it was found that all isolated gram-negative bacteria were resistant to ampicillin (CMI >16 $\mu g.mL^{-1}$), ampicillin/sulbactam (CMI >16/8 $\mu g.mL^{-1}$cefuroxime (CMI >16 $\mu g.mL^{-1}$), cefazolin (CMI >16 $\mu g.mL^{-1}$), cefotaxime (CMI >32 $\mu g.mL^{-1}$), moxifloxacin (CMI >4 $\mu g.mL^{-1}$), cefepime (CMI >16 $\mu g.mL^{-1}$) and ciprofloxacin (CMI >2 $\mu g.mL^{-1}$). However, this group of bacteria showed sensitivity to cefotetan (MIC <16 $\mu g.mL^{-1}$), piperacillin/tazobactam (MIC <8 $\mu g.mL^{-1}$), imipenem (MIC <4 $\mu g.mL^{-1}$), cefotaxime/clavulanate (MIC <0.5/4 $\mu g.mL^{-1}$), ceftazidime/K clavulanate (MIC <0.25/4 $\mu g.mL^{-1}$) and meropenem (MIC <4 $\mu g.mL^{-1}$).

In vitro assays of chitosan membranes against gram-negative bacteria

To determine the potential antimicrobial activity in a clinical setting, chitosan membranes were tested against gram-negative bacteria isolated from ulcers of hospitalized patients. *In vitro* tests with chitosan-based membranes were adverse for all microorganisms. For all bacterial isolates, the only effect was found by direct contact without the formation of the inhibition halo. Membranes tend to modify their size in relation to the concentration of chitosan. Table 3 shows the percentage of increase in area observed for each bacterium. As the concentration of chitosan in the membrane increased, its percentage increasing in area also increased. Fig. 1 shows an inhibition assay with *Escherichia coli* in chitosan membranes formulated with glycerol and honey.

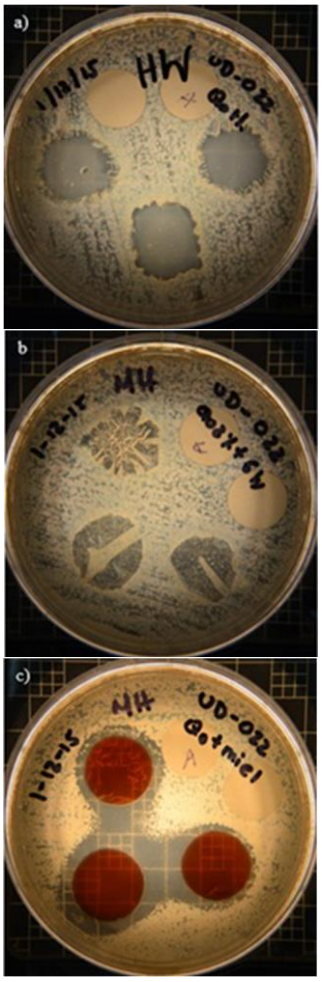

Fig. 1. *In vitro* microbiological assay with *E. coli* in the chitosan 1 % (**a**), chitosan 2 % + glycerol (**b**) and chitosan 2 % + honey (**c**) membranes.

Table 2. Antibiogram of the 16 isolated skin ulcers.

Antibiotic	UD-003 *P. mirabilis*	UD-004 *E. coli*	UD-005 *E. coli*	UD-006 *E. coli*	UD-008 *E. coli*	UD-009 *E. coli*	UD-010 *E. aerogenes*	UD-011 *P. aeruginosa*	UD-013 *P. mirabilis*	UD-016 *K. pneumoniae*	UD-017 *M. morganii*	UD-019 *K. pneumoniae*	UD-022 *E. coli*	UD-025 *K. pneumoniae*	UD-028 *P. aeruginosa*	UD-029 *E. coli*
Ampicillin	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16
Amikacin	R>32	R>32	R>32	R>32	R<4	R<4	R>32	R>32	R>32	R>32	R>32	R>32	R>32	S<4	S<4	R>32
Ampicillin/Sulbactam	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8	R>16/8
Cefuroxime	R>16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16
Cefotetan	R>32	S<16	R>32	R>32	S<16	S<16	R>32	R>32	R>32	R>32	S<16	S<16	R>32	S<16	R>32	R>32
Ticarcycline/K Clavulanate	R>64	R>64	R>64	R>64	R>64	R>64	S<16	R>64	R>64	R>64	R>64	R>64	R>64	S<16	R>64	R>64
Piperacillin/Tazobactam	R>64	R>64	R>64	R>64	S<8	S<8	S<8	R>64	R>64	S<8	R>64	S<8	R>64	S<8	S<8	R>64
Cefazolin	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16
Cefotaxime	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32
Ceftazidime	R >16	R >16	R >16	R >16	R >16	R >16	S<2	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16
Imipenem	R>8	S<4	S<4	S<8	S<4	S<4	S<4	R>8	R>8	R>8	S<4	S<4	S<4	S<4	R>8	S<4
Ceftriaxone	R>32	S<8	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32	R>32
Moxifloxacin	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4
Cefepime	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16
Gentamicin	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	S<1	S<1	S<1	R>8
Trimethoprim/Sulfamethoxazole	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	S<2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38	R>2/38
Levofloxacin	R>4	R>4	R>4	R>4	R>4	R>4	S<2	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4	R>4
Ciprofloxacin	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2	R>2
Cefotaxime/K clavulanate	R>4/4	R>4/4	R>4/4	R>4/4	S<0.5/4	S<0.5/4	S<0.5/4	R>4/4	R>4/4	R>4/4	S<0.5/4	S<0.5/4	R>4/4	S<0.5/4	R>4/4	R>4/4
Ceftazidime/K Clavulanate	R>2/2	R>2/2	R>2/4	R>2/4	S<0.25/4	S<0.25/4	S<0.25/4	R>2/4	R>2/4	R>2/4	S<0.25/4	S<0.25/4	R>2/4	S<0.25/4	R>2/4	R>2/4
Tobramycin	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	R>8	S<1	S<1	R>8	R>8
Meropenem	S<4	S<4	S<4	S<4	S<4	S<4	S<4	R>8	R>8	R>8	S<4	S<4	S<4	S<4	S<4	R>8
Aztreonam	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	R >16	S<8	R >16

Table 3. Area increase [%] of chitosan membranes.

Membrane	*P. mirabilis*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*E. aerogenes*	*M. morganii*
Ch 1 %	31.9 ± 20.6^{a}	28.0 ± 21.1^{a}	30.86 ± 19.3^{a}	26.1 ± 14.1^{a}	27.6 ± 5.9^{a}	32.8 ± 8.8^{b}
Ch 2 %	36.6 ± 24.9^{ab}	45.4 ± 55.5^{bc}	36.00 ± 18.4^{a}	30.7 ± 20.7^{ab}	46.8 ± 56.2^{ab}	39.2 ± 4.5^{d}
Ch 3 %	64.6 ± 63.5^{c}	54.4 ± 27.4^{c}	39.07 ± 35.7^{a}	44.8 ± 36.9^{ab}	58.0 ± 40.6^{ab}	54.7 ± 19.8^{bc}
Ch 2 % + g	23.2 ± 5.2^{a}	34.0 ± 17.8^{ab}	26.10 ± 14.3^{a}	29.2 ± 14.7^{ab}	23.6 ± 14.8^{a}	30.7 ± 5.0^{a}
Ch 2 % + h	32.4 ± 12.1^{ab}	35.2 ± 12.4^{ab}	34.52 ± 2.5^{a}	54.9 ± 52.5^{c}	30.8 ± 40.9^{ab}	46.9 ± 0.0^{cd}
Ch 2 % + h + g	51.4 ± 6.3^{bc}	46.0 ± 14.9^{bc}	37.99 ± 4.1^{a}	35.7 ± 38.9^{ab}	64.9 ± 18.4^{c}	76.0 ± 18.1^{e}

Initial area: 213.82 mm^2 (n = 6).

Discussion

Mama *et al.* (2014) reported that 87.4 % of 150 samples were positive for pathogens, of which 53 % were gram-negative bacteria and 47 % gram-positive. Other authors report that the prevalence for *P. aeruginosa* is in the range of 20 – 30 % in ulcers (Schmidtchen *et al.* 2003).

Today, a large number of microorganisms have developed resistance to antibiotics, which is why antibacterial drugs are required as new alternatives (Babushkin *et al.* 2015; El-Malek *et al.* 2017). Mama *et al.* (2014) reported that most isolates are resistant to ampicillin and cephalexin with 96 % and 92.4 %, respectively. On the other hand, Malik *et al.* (2013) reported that resistance to antibiotics among microorganisms is very variable and illustrated it for *P. aeruginosa* (63.7 %), *P. mirabilis* (57.5 %), *M. morganii* (57.5 %), *E. faecalis* (55.2 %), *Acinetobacter sp.* (51.9 %), *P. vulgaris* (50.3 %), *E. coli* (45.9 %), *K. pneumoniae* (44.8 %), *S. aureus* (44.3 %), *K. oxytoca* (42.9 %), and *Coryneform sp.* (37.1 %). The antibiotics that they evaluated included penicillins, cephalosporins, monobactams, carbapenems, aminoglycosides, chloramphenicols, quinolones, and fluoroquinolones, β-lactam inhibitors, macrolides, lincosamides, and glycol-peptide. Finally, Aspiroz *et al.* (2017) sampled two patients with ulcers in the leg and mentioned that *Pseudomonas aeruginosa* tends to present a greater recurrence of multi-resistance to antibiotics.

The use of antibiotics is only recommended when the patient presents symptoms of infection. Some broad-spectrum medications used for ulcer infection are ampicillin/sulbactam, ticarcillin/clavulanate, coamoxiclav, clindamycin, quinolone, and cephalosporin. The antimicrobials that have been used to combat the infections are silver sulfadiazine, fusidic acid, metronidazole, sodium chloride, chlorhexidine, and iodine povidone (Howell-Jones *et al.* 2005).

Likewise, when the membranes of chitosan-glycerol are in contact with moisture, they decrease in size due to the membranes being plasticized. Chitosan-honey-bee membranes greatly increase in size by absorbing moisture from the culture medium because honey has the characteristic of being hygroscopic (Sasikala *et al.* 2013). Through statistical analysis, it was found that there was a significant difference between treatments for each of the microorganisms isolated. While for *P. aeruginosa*, there was no significant difference between the membranes.

Akyzu *et al.* (2018) reported zones of inhibition of 14.56 ± 0.81 mm for *P. microbilis* and 14.56 ± 0.92 mm for *P. aeruginosa*, the zone of inhibition for each microorganism increased by approximately 45 %. On the other hand, Michalska-Sionkowska *et al.* (2018) performed antimicrobial assays with 1 % chitosan membranes combined with collagen-gentamicin against *E. coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*; the zones of inhibition were approximately 25 – 30 mm.

As reported by Balti *et al.* (2017), chitosan does not have the ability to diffuse through agar due to its molecular weight, which is why it does not form halos of inhibition. For chitosan to possess the antimicrobial activity it must be solubilized at a low pH in organic acids (Simunek *et al.* 2010). In gram-positive bacteria, chitosan has a strong bactericidal

effect however, in gram-negative bacteria inhibition is considered slower and depends mainly on the molecular weight, degree of deacetylation, concentration and pH of chitosan (Mrázek *et al.* 2010).

The activity of chitosan against gram-negative bacteria is because the molecule tends to become polycationic below its pKa (pH 6.3) (Campana *et al.* 2017). In addition, the amino group of C2 can interact with the anionic components of negative surfaces, such as lipopolysaccharides and proteins, modifying their cellular permeability. Chitosan at concentrations of 0.1 $mg.mL^{-1}$ can bind to the negatively charged bacterial surface causing alteration of the cell membrane and leakage of intracellular components. When this polymer is found at high concentrations (2 and 5 $mg.mL^{-1}$), it tends to coat the bacterial surface, limiting the release of intracellular components and mass transfer (Khalil *et al.* 2015).

Bee honey has the potential to fight bacterial infections that have generated resistance to antibiotics and cannot be fought with traditional antibiotics (Kwakman *et al.* 2011). When the honey is at a pH of 3.1 – 4.5, the glucose is found as gluconolactone, which provides an environment that favors the activity of the fibroblasts and the healing of wounds. Together, the presence of hydrogen peroxide stimulates the generation of fibroblasts and angiogenesis (Alam *et al.* 2014).

In conclusion, the incidence of infected skin ulcers requires safe therapies that promote their healing. One answer to this problem is the use of chitosan membranes. These membranes have confirmed their activity by contact against gram-negative bacteria isolated from ulcers and that are resistant to antibiotics. Therefore, chitosan membranes are a viable alternative as antimicrobial agents for the treatment of infections without the risks involved with antibiotics.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical disclosures

Protection of human and animal subjects: the research protocol was approved by the Institutional Ethics Committee. All patients involved in the trial confirmed their participation with informed consent.

Confidentiality of data: the authors declare that no patient data appears in this article.

Right to privacy and informed consent: the authors declare that no patient data appears in this article.

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