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Evaluation of the antimicrobial activity of an antiseptic hydrogel prepared with silver nanoparticles (AgNPs) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*◊, ◊◊

Evaluación de la actividad antimicrobiana de un hidrogel antiséptico elaborado con nanopartículas de plata (AgNPs) contra *Staphylococcus aureus* y *Pseudomonas aeruginosa*

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ABSTRACT: In both developed and developing countries, food and consumption habits have led us to a dead end where most of the population suffers alterations in metabolism, which leads to diseases such as type 2 diabetes mellitus (DM), high blood pressure and chronic inflammation. DM can increase the risk of having some skin problems, such as bacterial and fungal infections that lead to ulcers that are very difficult to heal, and a large percentage of patients even lose their lower extremities. A proposal for the care of ulcer wounds, diabetic foot, burns, among others, is to implement patches and/or bandages with a silver nanoparticle gel used as an antiseptic, as an alternative administration system to antibiotics to control and inhibit the colonization of microorganisms such as super bacteria. Since silver nanoparticles have great potential in biomedical applications such as bactericidal, fungicidal, antiviral or agent healing, among others. Of all its uses, one of the most important is his employment as an agent antiseptic for hygienic and medical purposes. In this work we prepared a hydrogel with a concentration of 1000 µg/mL of Ag NPs, and stability studies were carried out: pH study of the final formulation and physical study to corroborate that the hydrogel maintains its appearance, color, odor and pH for 30 days at 25 °C and 40 °C. Its antimicrobial activity was evaluated and compared against a commercial cream containing silver sulfadiazine. From the Kirby-Bauer test, an average diameter of the inhibition zones for our hydrogel was obtained that was greater than that of the inhibition zones for the commercial cream. Ttherefore, the hydrogel prepared with AgNPs has good antimicrobial activity against *Staphylococcus aureus* and against *Pseudomonas aeruginosa*.

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KEYWORDS: metabolic diseases, antibiotic resistant microorganisms, silver nanoparticles (Ag-NPs), medical applications, chronic wounds.

RESUMEN: Tanto en los países desarrollados como en los países en desarrollo, los hábitos alimentarios y de consumo han llevado a un callejón sin salida donde la mayoría de la población sufre alteraciones metabólicas, lo cual conlleva a sufrir enfermedades como la diabetes mellitus tipo 2 (DM), la hipertensión arterial y la inflamación crónica. La DM puede aumentar el riesgo de padecer algunos problemas cutáneos, como infecciones bacterianas y fúngicas, provocando úlceras muy difíciles de cicatrizar, e incluso un gran porcentaje de pacientes pierde las extremidades inferiores. Una propuesta para la atención de heridas ulcerosas, pie diabético, quemaduras, entre otras, es implementar parches y/o vendajes con gel de nanopartículas de plata utilizado como antiséptico, como sistema de administración alternativo a los antibióticos para controlar e inhibir la colonización de microrganismos como las súper bacterias. Dado que las nanopartículas de plata tienen un gran potencial en aplicaciones biomédicas como bactericidas, fungicidas, antivirales o cicatrizantes, entre otras. De todos sus usos, uno de los más importantes es su empleo como desinfectante con fines higiénicos y médicos. En este trabajo, preparamos un hidrogel con una concentración de 1000 µg/mL de nanopartículas de plata (Ag NPs), y se realizaron estudios de estabilidad: estudio de pH de la formulación final y estudio físico para corroborar que el hidrogel mantiene su apariencia, color, olor y pH durante 30 días a 25 °C y 40 °C. Su actividad antimicrobiana también se evaluó y se comparó con una crema comercial que contiene sulfadiazina de plata. A partir de la prueba de Kirby-Bauer, se obtuvo un diámetro promedio de las zonas de inhibición para nuestro hidrogel mayor que el de las zonas de inhibición para la crema comercial; por lo tanto, el hidrogel elaborado con nanopartículas de plata presenta buena actividad antimicrobiana contra *Staphylococcus aureus* y contra *Pseudomonas aeruginosa*.
PALABRAS CLAVE: enfermedades metabólicas, microrganismos resistentes a antibióticos, nanopartículas de plata (AgNPs), aplicaciones médicas, heridas crónicas.

Introduction

Metabolic diseases, long considered of minor importance, are now taking place as the main threats to human health. DM is the most common non-communicable disease worldwide and the fourth to fifth cause of death in developed countries, and also now in developing countries. The global number of people with DM will increase from 220 million to 300 million people by 2025 (Tripathi *et al*., 2006).

Chronic complications of diabetes mellitus affect many organ systems and are responsible for most morbidity and mortality. Chronic complications can be divided in vascular and non-vascular, a serious example of these complications is the important pressure zones for DM, since an untreated injury in these areas can lead to diabetic foot, others like burns or varicose ulcers (figure 1) are also relevant because chronic wounds promote a high colonization of microorganisms (Baczako *et al*., 2019; Armstrong *et al*., 2023).

Regarding bacterial infections and inflammation, it poses another severe threat to human health and the social economy in developed countries. In addition, the existence of super-bacteria and the increasingly severe phenomenon of antibiotic resistance highlight the development of new antibacterial agents (Fang *et al*., 2023).

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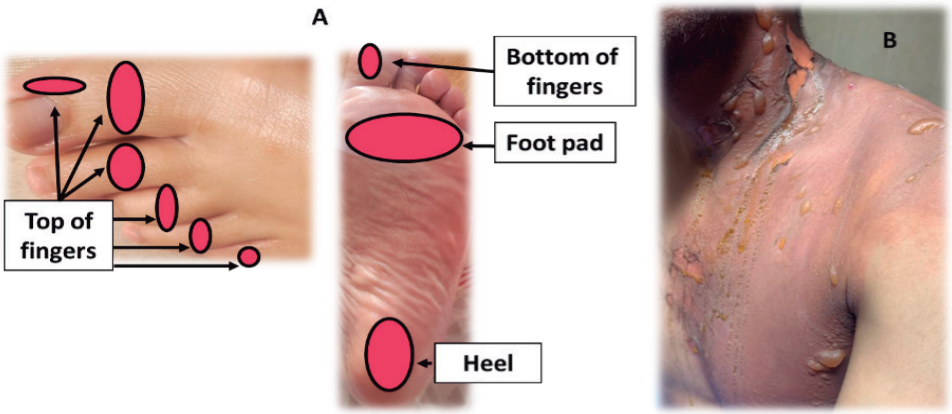
FIGURE 1. A) Important pressure zones for DM. B) Burns.



Source: Author's elaboration.

Staphylococcus aureus is the most relevant pathogenic species within its genus, since it is mainly responsible for causing various infections such as skin infections, pneumonia, endocarditis and osteomyelitis. *S. aureus* has a high level of antibiotic resistance and is a common cause of infections in hospitals and the community (Ahmad-Mansour *et al*., 2021); It is also one of the most common bacteria found in chronic wounds.

Instead, *Pseudomonas aeruginosa* is a common, Gram-negative environmental organism due to its natural resistance to antibiotics and the ability to form biofilms. Infection with this pathogen can cause severe therapeutic problems like respiratory infections, urinary tract infections, wound infections, blood infections, eye infections, among others (Mielko *et al*., 2019), principally in chronic wounds and especially in pressure ulcers and burns.

Although the use of topical antibiotics has been the frequently used treatment to control the colonization and proliferation of these pathogens in wounds, treatment with silver has revolutionized infection care due to its beneficial effects. However, it has been shown that treatments containing only the silver ion can alter healing, causing cosmetic abnormalities in the skin. Virulence factors and biofilms constitute attractive targets for the prevention of infections caused by multidrug-resistant bacteria (Kim *et al*., 2019; Reese *et al*., 2022).

Metallic nanoparticles of silver (AgNps), gold (AuNPs), zinc oxide (ZnO NPs), copper and iron oxides, among others, have great potential in the pharmaceutical industry since these materials can be synthesized and modified in a relatively simple way (Chávez-Sandoval *et al*., 2021; Medina-Solano *et al*., 2024).

AgNPs have many advantages, such as the quantum confinement of electronic movement in semiconductor nanoparticles, surface plasmon reso-

nance in some metallic particles or super magnetism in magnetic nanomaterials. Although several theories have been formulated about the antimicrobial mechanism of silver nanoparticles, their bacteriostatic effect is related to various biological processes. These processes include the generation of reactive oxygen species (ROS) and the induction of oxidative stress (OS) through enzyme inactivation. One of the key mechanisms of action is the denaturation of disulfide bonds present in bacterial proteins, which ultimately leads to cell death. These properties confer great benefits to the use of nanoparticles compared to current commercial drugs.

In this work we prepared a hydrogel with a concentration of 1000 μg/mL of AgNPs, its antimicrobial activity was evaluated and compared against a commercial cream containing silver sulfadiazine in *S. Aureus* and *P. aeruginosa,* our results suggest that the combination of gels and AgNPs displays the great potential to manage superbug-infected trauma.

Materials and methods

All materials used in this work were reagent grade and all tests were done in triplicate.

Synthesis and characterization of AgNPs

The method described by Chávez-Sandoval *et al*. (2021) was used, whit some modifications as described below.

Briefly: Sodium citrate was used as the reducing agent. JT Baker brand silver nitrate was used as the precursor.

Once the nanoparticles were obtained, they were stored at 4 ºC for characterization and subsequent use.

- Sodium citrate (Na3C6H5O7) 0.5 g
- Silver nitrate (AgNO3) 0.2 g
- Distilled water 50 mL

UV-visible spectrophotometry

A Perkin Elmer spectrophotometer, model Lambda XLS, was used.

Transmission electron microscopy (TEM)

The equipment used was a scanning electron microscope in transmission mode of the JEOL brand model JEM-ARM200F (Japan) of the Electron Microscopy Laboratory of the Center for Nanoscience and Micro and Nanotechnologies (CNMN) of the IPN in Mexico City.

Microorganisms

The *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains were obtained from the UPIBI-IPN strain collection.

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Basic tests for the study of antimicrobial activity

Minimum inhibitory concentration (MIC)

This test measures antimicrobial susceptibility by determining the smallest amount of an agent, which is generally an antibiotic, capable of completely inhibiting the growth of a given bacterial strain.

It is ideal for non-demanding growth bacteria such as *S. aureus* and *P. aeruginosa*. In addition, it is a method that allows determining the inhibitory activity and the bactericidal activity (Andrews *et al*., 2001).

Formulation and preparation of the hydrogel

The AgNPs have antimicrobial activity by themselves, however, they have a low binding affinity, so it is recommended to use hydrogels as an effective vehicle to improve this antimicrobial activity, since in combination with the appropriate polymers, their antimicrobial activity can be enhanced. The gel produced belongs to the classification of "hydrogel" which is a three-dimensional network composed of flexible polymer chains that absorb a large amount of water, although insoluble, they swell in the presence of it, significantly increasing its volume while maintaining its shape until reaching the physicochemical balance (Gao *et al*., 2016).

In this work the hydrogel was prepared according to the methodology of Algin *et al*. (2020) and adding silver nanoparticles, according to the AgNPs concentrations obtained from the MIC test, for each microorganism.

Once the hydrogel was obtained, it was compared against a commercial cream using the Kirbi-Bauer test.

Kirby-Bauer test

The Kirby-Bauer test was performed to compare the antimicrobial activity of the hydrogel, made from silver nanoparticles, against the antimicrobial activity of a commercial silver sulfadiazine cream, widely used to prevent and treat infections in wounds and burns (Reese *et al*., 2022). The mechanism of action of sulfadiazine cream is based on the combination of sulfadiazine, which acts as an inhibitor of folic acid synthesis, and silver ions, which interfere with the function of bacterial enzymes and proteins (De Francesco *et al*., 2022).

It is important to mention that the commercial cream is formulated with a higher proportion of silver ions, according to its secondary packaging, it contains 280 mg of silver sulfadiazine for each g of the final formulation. Thus a 1:100 dilution was made accordingly that its silver concentration was comparable to the hydrogel prepared.

This test consists of insertion paper discs impregnated with antimicrobial agents on the surface of agar previously inoculated with the bacteria in our case *S. aureus* and *P. aeruginosa*.

The antimicrobial agent (AgNPs or commercial cream) contained in the disks diffuses through the agar, and as it moves away from the disk, its concentration decreases logarithmically, thus generating a gradient of the antimicrobial agent in the agar surrounding the disk (Yin *et al*., 2023).

Although there is still no standardized data regarding the inhibition diameter zones using silver nanoparticles, the sensitivity determination of the AgNPs was carried out by comparing their diameter with other antibiotics that are commonly used against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (tables 1 and 2).

Table 1. Determination of antibiotic sensitivity and resistance by the disk method for *Staphylococcus aureus*.

Staphylococcus aureus				
Antimicrobial agent	**Disk content µg**	**Inhibition halo (mm)**		
		Sensitive	**Intermediate**	**Resistant**
Cefazolin	30	≥18	15-17	≤14
Clindamycin	2	≥21	15-20	≤14
Erythromicyn	15	≥23	14-22	≤13
Gentamicin	10	≥15	13-14	≤12
Oxacilin	1	≥13	11-12	≤10
Penicilin G	10	≥29	–	≤28
Vancomycin	30	≥15	–	–

Source: Prepared by the authors based on data from American Society for Microbiology (2009).

Table 2. Determination of antibiotic sensitivity and resistance by the disk method for *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa				
Antimicrobial agent	**Disk content µg**	**Inhibition halo (mm)**		
		Sensitive	**Intermediate**	**Resistant**
Cefazolin	30	≥17	15-16	≤14
Clindamycin	75	≥21	16-20	≤154
Erythromicyn	30	≥23	15-22	≤14
Gentamicin	10	≥15	13-14	≤12
Oxacilin	30	≥19	15-18	≤14
Penicilin G	100	≥18	–	≤17
Vancomycin	10	≥15	13-14	≤12

Source: Prepared by the authors based on data from American Society for Microbiology (2009).

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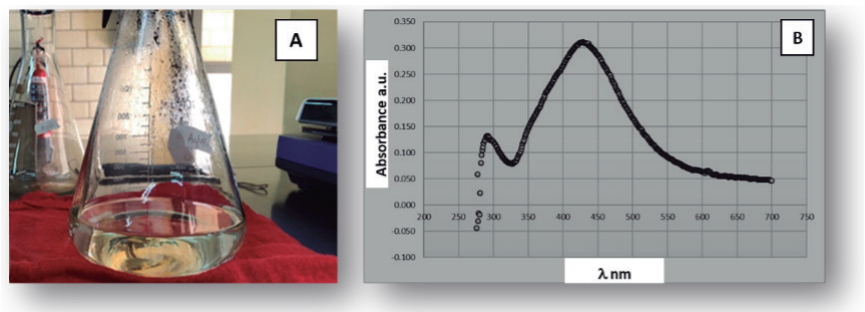
Results and discussion

Synthesis and characterization of AgNPs

We obtained a yellow colloid characteristic of the silver nanoparticles (Medina-Solano *et al*., 2024), and they were characterized by using a Perkin Elmer spectrophotometer, model Lambda XLS, from a wave sweeping in a range of 276 to 700 nm, obtaining a maximum lambda of 429 nm with an absorbance of 0.311 a.u. (figure 2). The appearance of absorption bands in the spectrum around wavelengths from 400 nm to 450 nm indicates the presence of AgNPs, the maximum lambda at these wavelengths is due to resonance absorption of surface plasmons.

FIGURE 2. A) Yellow colloid characteristic of AgNPs. B) UV-Vis characterization, the maximum lambda was 429 nm.



Source: Author's elaboration.

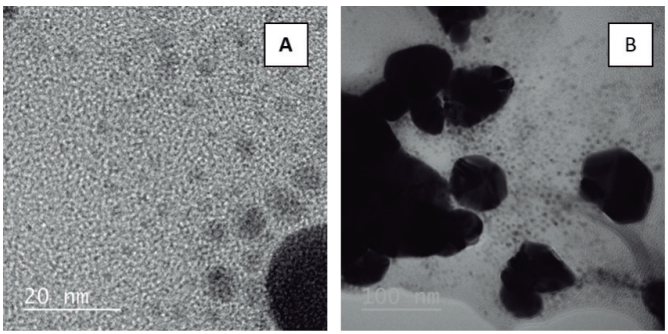
The color of the AgNPs can vary mainly due to the method used, as well as the concentration of the reducing agent, in this work we use sodium citrate as a reducing agent. Sodium citrate also acts as a stabilizer and pH mediator. The AgNPs thus obtained were kept refrigerated at a temperature of between 2-7 ºC until their characterization.

Transmission electron microscopy (TEM) characterization

The TEM analysis allowed us to observe that the AgNPs synthesized in this work are spherical with a variable size from 5 to 100 nm (figure 3). Spherical, polymorphic AgNPs of different sizes were observed. The variation in the size of the AgNPs can be attributed to the temperature, since, in general, the size of the nanoparticles increases with the reaction time.

We observed some agglomeration, so it is advisable to use other stabilizing agents as well as surfactants, which can protect the nanoparticles and prevent their agglomeration.

FIGURE 3. Silver nanoparticles obtained with sizes ranging from approximately 5 nm, image A, to 100 nm, image B.



Source: Author's elaboration.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration test was carried out to know the lowest concentration at which silver nanoparticles inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, to determine the concentration of AgNPs in the hydrogel formulation.

The concentration of the AgNPs in each tube is described in table 3.

A total of 11 concentrations were used.

Table 3. Concentration of AgNPs used for the MIC test.

Tube	AgNPs Concentration (µg / mL)
1	1000.00
2	500.00
3	250.00
4	125.00
5	62.50
6	31.25
7	16.62
8	7.81
9	3.90
10	1.95
11	0.97

Source: Author's elaboration.

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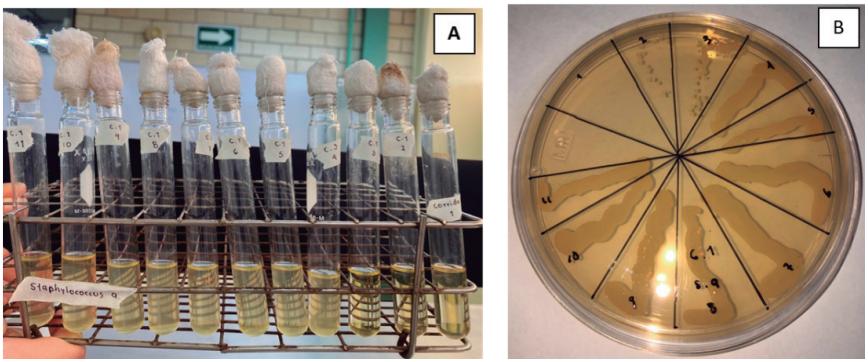
Staphylococcus aureus

As seen in figure 4, the minimum concentration at which there is inhibition of *Staphylococcus aureus* corresponds to 1000 µg/mL section number 1, and tube 1 in table 3.

FIGURE 4. A) Tube 1 contains the concentration of 1000 mg/mL and corresponds to the concentration where growth of *S. aureus* is no longer observed in panel B.

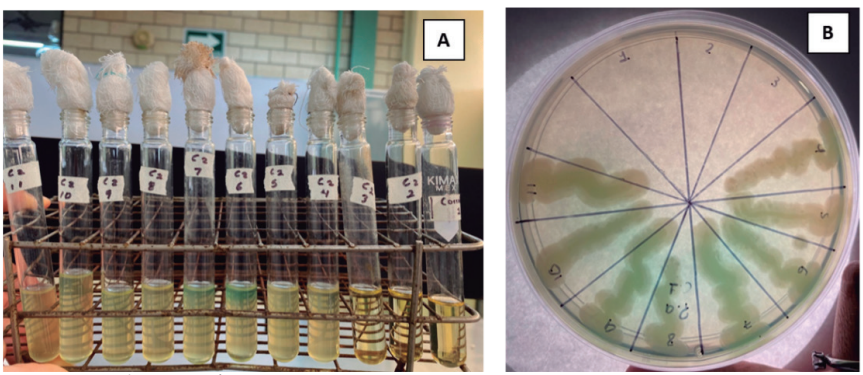


Source: Figure prepared by the authors.

Pseudomonas aeruginosa

In the case of *P. aeruginosa*, in figure 5, the minimum inhibitory concentration was 250 µg/mL, section 3, corresponding to tube 3 in table 3.

FIGURE 5. A) Battery tubes with different concentrations of AgNPs. B) Replating each concentration of AgNPs used.



Source: Author's elaboration.

Thus, it seems that the AgNPs synthesized in this work are more effective against *P. aeruginosa* than against *S. aureus*.

It is important to mention that the MIC test depends on the antibacterial activity of the AgNPs and this in turn is related to the synthesis method used, including reducing and stabilizing agents, since for cell disruption to be effective the concentration of the AgNPs, the charge in their surface, its morphology and especially its size, it has been reported that the smaller the AgNPs are, from 5 nm to 10 nm, the greater their antimicrobial effect (Nie *et al*., 2023; Medina-Solano *et al*., 2024).

Furthermore, there is no standardized data for the MIC test using silver nanoparticles and given that other protocols evaluate AgNPs with different characteristics, synthesized by various methods, a comparison cannot be made between the values obtained and those reported by previous research. Likewise, there is also no standardized MIC test data for antibiotics, since the articles reviewed works with different conditions and concluded that antibiotic therapy does not completely eradicate the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which could be because of the prescription of antibiotics in developing countries where the use of antibiotics is not yet well controlled (Sulis *et al*., 2022). This has led to the evolution of bacteria to resist antibiotics even at high concentrations, which is why AgNPs hydrogel are an excellent therapeutic alternative.

Formulation and preparation of the hydrogel

In accordance with the Official Mexican Standard NOM-073-SSA1-2015, stability of drugs and medications, as well as herbal remedies, in section 7.5.1, the conditions are stipulated according to the type of stability study, whether accelerated or intermediate condition, or long-term with the objective of demonstrating the useful life of the new drug (table 4).

Table 4. General study conditions for new medication.

Type of study	Storage conditions	Minimum period	Analysis frecuency
Accelerated stability	40 °C ± 2 °C / 75% ± 5% RH	3 months	1-3 months
Stability at intermediate condition	30 °C ± 2 °C / 65% ± 5% RH	6 months	3-6 months
Long term stability	25 °C ± 2 °C / 60% ± 5% RH	3 months (option 1)	3-6 months
	30 °C ± 2 °C / 65% ± 5% RH	12 months (option 2)	0, 3, 6, 9, and 12 months

Source: Author's elaboration.

These tests serve as quality control to ensure the effectiveness and safety of the medications. According to the results obtained, it is observed that our

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hydrogel maintains its pH, appearance, color and smell for 30 days. The hydrogel must have a pH of 7.0 and this pH must be maintained throughout its useful life since a lower or higher value can cause undesirable side effects such as irritation, pain, burning or itching since the purpose of the hydrogel is to provide a mechanical barrier, that is, it remains on the application surface, avoiding colonization of the wound by opportunistic microorganisms.

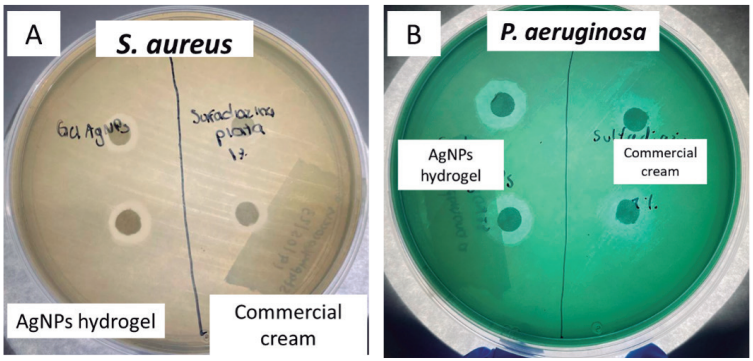
However, it is necessary to measure its viscosity, extensibility and extrusion for at least 6 months to evaluate its adhesion and extensibility on the skin, as well as determine its stability and expiration date. However, these tests go beyond the scope of this work.

Kirby-Bauer test

With Kirby-Bauer test we observe that after 48 hours there is antimicrobial effectiveness of both preparations against *P. aeruginosa*, however, a greater inhibition is observed by the AgNPs hydrogel that obtained an inhibition zone of up to 20 mm, with an average of 15 mm, against the sulfadiazine cream, whose resulting halo was 10 mm (table 2), demonstrating that the antibacterial action of the AgNPs hydrogel is greater than that of the commercial cream (figure 6).

During the analysis of the antimicrobial effectiveness test of the hydrogel and the commercial cream against *Staphylococcus aureus*, we observe that both products showed relatively low inhibition halos, however, it is notable that the hydrogel presented a slightly larger halo (13 mm) compared to cream (10 mm) (figure 6 and table 5).

FIGURE 6. Antibiogram result of the Kirby-Bauer test for microorganisms studied, A) *S. aureus*, and, B) *P. aeruginosa* both treatment with AgNPs hydrogel and commercial cream.



Source: Author's elaboration.

Although we mention that our results are not comparable with results reported with antibiotics, the largest inhibition zone observed for the hy-

Table 5. The average diameter of the haloes generated for *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Inoculum	Average diameter (mm)	
	AgNPs hydrogel	Commercial cream
Staphylococcus aureus	13.00	10.00
Pseudomonas aeruginosa	15.00	10.00

Source: Author's elaboration.

drogel against *Pseudomonas aeruginosa* was 15 mm, which could be considered like the values reported as sensitive for antibiotics such as gentamicin and tobramycin (≥15 mm).

On the other hand, the inhibition of *Staphylococcus aureus* was considerably lower, similar only with antibiotics such as oxacillin (tables 1-2).

The improved results of the AgNP hydrogel compared to the commercial cream may be because the silver nanoparticles integrate more efficiently into cells, inhibit microorganisms, and facilitate healing.

Conclusions

Silver nanoparticles were synthesized that showed a maximum lambda of 429 nm by UV-Vis spectrometry.

Transmission electron microscopy (TEM) revealed that the synthesized silver nanoparticles have a spherical morphology, with different sizes and some agglomeration.

A hydrogel was formulated with the silver nanoparticles obtained, which complies with the quality control of pH and stability after 30 days, according to Mexican regulations.

The hydrogel demonstrated higher antimicrobial effectiveness compared to commercial silver sulfadiazine cream at the same concentration, against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Conflict of interest

The authors declare that there is no conflict of interest of any kind.

Author contributions

Blanca Estela Chávez Sandoval: Conception and design of the article. Methodological development, writing of the original draft and review, and final editing of the text.
Alejandra Saraí Espinosa Vega and *Grecia Nuñez Tahuilán*: Data mining, analysis and interpretation.

María de Lourdes Moreno Ribera and *María Isabel García-Ventura*: Methodological development, data mining, analysis and interpretation.
José Abraham Balderas: Review and final editing of the text.

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