Original Article

Antibacterial Activity of Saliva Extraction of Leech *Hirudo medicinalis*

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Abstract

Background: Bacteria have developed techniques to withstand the majority of today's medicines. Leeches are considered as a potential treatment for diseases management for their capacity to prevent blood clotting while sucking, keep it in a liquid state for prolonged storage, and displace antibacterial activities. More study is needed to understand the biology of leeches and the chemical substrates of potential treatments. **Objectives:** Extract *Hirudo medicinalis* saliva, screening antibacterial activity of crude leech saliva extract (CLSE) on test isolates ATCC bacteria (*Escherichia coli*, *Psedumonas auroginosa*, *Streptococcus pyogenes*, and *Staphylococcus aureus*) by disc diffusion test. Screening antibacterial activity of silver nanoparticles (Ag-NPs) saliva on test isolates by agar well diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) test. **Materials and Methods:** Agar well diffusion method, antibacterial activities of CLSE on ATCC bacteria and (LSE-Ag NPs), micro broth dilution method and the agar diffusion test, (MIC) and (MBC) of LSE-Ag were determined. **Results:** Compared to AN_{30}, the CLSE had no effect whereas LSE-Ag stopped the growth of the test isolates at 15 µL, for MIC, MBC test (10 µL) AgNPs from high to low concentrations, accordingly, in comparison to positive and negative controls show significant differences with different concentrations of AgNPs for each bacterium, but there are no significant differences between the 600 µg/mL concentrations of AgNPs compared with positive control (antibiotics). **Conclusions:** *P. aeruginosa*, *S. pyogenes*, *E. coli*, and *S. aureus*-related infectious illnesses may be treated using LSE-Ag.

Keywords: Crud leech saliva extraction, minimum bactericidal concentration, minimum inhibitory concentration

Introduction

The lives of people and hospitalized patients have always been seriously threatened by pathogenic microorganisms, particularly bacteria that cause diseases.[1] It is one of the significant difficulties that doctors must deal with. Because of the toxicity of some chemically created medications and the pathogenic strains' demonstrated resistance to conventional antibiotics, early and correct identification and treatment of infectious diseases can be difficult. Pathogenic organisms employ a variety of strategies to resist conventional medications, involving, for example, the production of biofilms and/or capsules and the use of proton efflux pumps. This increases an organism's pathogenicity in turn.[2]

The segmented worm known as a leech (Hirudinea) belongs to Annelida.[3] The word "leech" comes from the Anglo-Saxon "loece," which meant "to heal." Leech usage is regularly depicted in Egyptian paintings from 1500 BC. Since ancient times, bloodletting has been utilized as a form of treatment for "local reduction."[4,5]

Leech species are divided into two groups based on how they feed. For example, ferocious leeches prey on a variety of invertebrates. The second kind of leeches are called sanguivorous leeches, and they are ectoparasites that eat vertebrate blood, including human blood. Leeches extract blood from their victim with the aid of their suckers and biting jaws, engorge to the utmost degree, and then spontaneously remove the desire to

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Quick Response Code:

Website: https://journals.lww.com/mjby

DOI: 10.4103/MJBL.MJBL_1063_23

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Submission: 23-Jul-2023 **Accepted:** 21-Aug-2023 **Published:** 30-Apr-2026

How to cite this article: Witwit LJ, Taj-Aldeen WR. Antibacterial activity of saliva extraction of leech *Hirudo medicinalis*. Med J Babylon 2026;23:304-11.

keep eating.[6] Both wet terrestrial environments and aquatic habitats (ponds, streams, lakes, and the ocean) are home to them.[7]

In June 2004,[8] the Food and Drug Administration gave its approval for the filing of medicinal leeches as a treatment option. Site biting, blood suction, and most significantly the injection of leech saliva, which contains a variety of bioactive chemicals, into the area are all associated to the therapeutic qualities of leeches.[9] Ingredients in leech saliva ensure the presence of anti-inflammatory, anticoagulant, platelet inhibitory, thrombin regulating, analgesic, extracellular matrix degradative, and antibacterial effects.[10]

According to studies,[11-13] the production of harmful compounds is reduced as a result of the "green synthesis" of silver nanoparticles (Ag-NP). Important kinds of pathogenic bacteria are particularly poisonous to silver ions and silver-based compounds.[14]

According to numerous studies,[15-18] numerous industries, including catalysis, optoelectronics, imaging and diagnostics, antimicrobials, and therapeutics, use silver nanoparticles. Silver is an effective antibacterial agent with minimal toxicity to humans and a wide spectrum of *in vitro* and *in vivo* applications, according to significant study.[19] Therefore, the investigation of the antibacterial activity of raw and Ag-NPs mediated saliva of *Hirudo medicinalis* against various harmful bacteria is the main objective of the study.

Materials and Methods

The period of our present study is extended from September 2022 to May 2023 and this study is performed in Malaria and Vector Research Group (MVRG) Biotechnology Research Center (BRC) Pasteur Institute of Iran which included the following steps.

Leeches sampling and maintenance

The Bandar-e-Anzali rice farm in northern Iran's city provided the leeches, which were subsequently processed and housed in well-aerated plastic containers filled with non-chlorinated tap water in a separate room at room temperature. Every 2 days, the water was changed routinely.[20]

Leech feeding and saliva extraction

Twelve weeks were spent starving leeches. A method described in the literature [20,21] was slightly modified to get saliva.

Briefly, parafilm membrane (PARAFILM "M"; Bemis) was stretched over a funnel containing 0.001 M arginine (0.02 g) and 0.15 M (0.08 g) saline solution. 10 mL of distilled water and the solution were added to the newly created device. While the solution was kept at 37°C, leeches were permitted to draw the solution through the membrane. Leeches were permitted to suck till they were satisfied. Soon after they emerged from the membrane, leeches were stopped by putting them in a plastic bowl with ice all around them for 5–10 min. With the use of this technique, leeches can complete whatever they have eaten by throwing it up. To finish the saliva collection, leeches were softly embraced from the back near anterior (mouth) sucker. Bloody substances were not collected; rather, they were thrown out. Centrifuging the collected fluid took place for 10 min at 4°C and 9000 rpm. Crude leech saliva extract (CLSE) was the name given to the centrifuged supernatant. The entire gagged fluid was collected and kept in a refrigerator at 4°C.

Microbial strains

Gram-positive bacterial spp. (*Streptococcus pyogenes* ATCC104030 and *Staphylococcus aureus* ATCC25923) and Gram-negative bacterial spp. (*Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922) from the bacterial bank of Institute Pasteur of Iran were used as reference strains of human pathogens.

Antimicrobial activity

Disc diffusion testing, minimum inhibitory concentration testing, and minimum bactericidal inhibitory testing were used to determine CLSE's antimicrobial efficacy.[22]

Reagent required

Mueller-Hinton agar (MHA; Sigma Aldrich, UK), antibiotic discs ($Amikacin_{30}$µg, $Ceftriaxon_{30}$µg, and $Ampicillin_{10}$µg; PADTAN TEB), McFarland standard 0.5 (1×10^8 Colony Forming Unit [CFU]/mL), inoculum and paper filter discs (saturated tablets with LSE overnight and stored in refrigerator).

Crude leech salivary extract screening for antibacterial activity

Methodology of the Clinical and Laboratory Standards Institute,[23] in this experiment, gram positive and gram negative bacteria were used. *Escherichia coli* (25922) and *P. auroginosa* (27853), *S. aureus* (25923), and *S. pyogenes* (104030). The positive control was ($Amikacin_{30}$µg). The inoculum was made in a tube with 5 mL of phosphate buffer solution. The suspension's turbidity was fixed to 0.5 (1×10^8 CFU/mL), the McFarland level. The McFarland standard and inoculum tubes were put in front of a white sheet of paper with black dotted lines to accomplish this correction. Within 18–24 h after its manufacture, inoculum was used. A 50 µL bacterial inoculum was added to a MHA plate. In order for the agar medium to absorb the additional water favor, the plate was left at room temperature. The dish was placed in an incubator whereas the lid was slightly ajar to achieve this 4 mm is the optimal MHA thickness. After the medium has solidified, filter paper disc has been saturated with CLSE solution. The plate was then kept

at 37°C for a further 18-24 h. The zone of inhibition that developed around each well was measured using a caliper. A bacterium's susceptibility or resistance to an antibiotic is determined by the halo of growth inhibition that forms around the drug disc. Each antibiotic has a distinct range of bacterial growth suppression. To estimate the sensitivity or resistance, it is sufficient to measure the diameter of the lack of development using a ruler and then make a determination by checking the table.[23]

Synthesis materials and procedures of leech salivary extract-silver nanoparticle conjugate

-Silver nitrate ($AgNO_3$), crude extracted leech saliva.

A 50 mL plastic tube, an aluminum foil sheet, a 1000 µL pipette, a 200 µL pipette, a digital scale (Black Magic—Tackle) and a thermometer.

Ag-NPs synthesis

Using a chemical reduction method, synthesis was accomplished. The manufacture of silver nanoparticles from CLSE followed the procedure.[24]

Testing silver nanoparticles mediated by leech salivary extract for antibacterial activity

Using the agar well diffusion method developed by the Clinical and Laboratory Standard Institute,[22] and previously published for the antibacterial activity of CLSE,[25] the LSE-Ag at (10 µL) were tested for antibacterial activities on the test isolates.

Determining the leech salivary extract's minimal inhibitory concentration

We should prepare a standard count of the desired bacteria in the first step. For this purpose, we can prepare 5×10^4 CFU/well from each chosen bacteria for the minimum inhibitory concentration (MIC) micro dilution test and we should prepare 5×10^5 CFU/well for the MBC test, both in accordance with the requirement.[26]

Determining the lowest bactericidal concentration of silver nanoparticles mediated by leech salivary extract

Applying the technique outlined by,[27] determine the MBC of the LSE-Ag. Each not-growing well's whole contents were transferred to MHA plates and incubated at 35 ± 2°C at the following intervals. It took 24 h for the quickly developing Gram-negative rods *P. aeruginosa* and *E. coli*, and 48 h for *S. aureus* and *Streptococcus pyogenes*. Counting growing colonies and determining MBC. When there was no growth, it was considered bactericidal, and when there was growth, it was considered bacteriostatic.

Ethical approval

The study protocol was reviewed and approved by University of Babylon ethical committee according to the document number M221204 on December 6, 2022.

Results

Leeches feeding and saliva collection

Our current work demonstrates that when leeches were brought in close proximity to the parafilm membrane after 12 weeks of hunger, they began drinking the phagostimulatory fluid. Leeches were quite tolerant of the solution. Leeches' saliva was extracted right away once they descended from the membrane by first placing them in an ice container for 15 min. The extraction was finished by gently squeezing the leeches approaches the anterior (mouth) sucker from the back. The saliva of (70) leeches was gathered. The volume of the liquids that these leeches ingest ranges from 3 to 8 mL, and their body masses range from 0.08 to 3.07 g.

The antibacterial properties of crude leech salivary extract on test isolates Figure 1

The test isolates that the undiluted CLSE proved antibacterial against were listed in Table 1. The raw leech salivary extract could not stop the growth of any of the test isolates. The test isolates' growth was inhibited in zones of inhibition against *S. aureus*, *S. pyogenes, E. coli*, and *P. aeruginosa* when standard antibiotics ($Amikacin_{30}$ µg) were employed as the positive control.

Silver nanoparticles with leech salivary extract have antibacterial effects on test isolates

The antibacterial activity of silver nanoparticles against the two isolates, gram-positive *S. aureus* and *gram-negative E. coli*, is shown in Figure 2 at a concentration of 15 µL. Based on the molecular weight of $AgNO_3$, calculations were done to determine the right numbers. As a result, the following final concentrations of nanoparticles were produced.

1—0.1 g $AgNO_3$, 2—0.02 g, 3—0.001 g, 4—0.0006 g.

CRO_{30}µg used as positive control for *E. coli* and AM_{10} µg as positive control for *S. aureus.*

According to the last antibiogram results, we found that the suitable concentration of nanoparticle is 0.0006 g $AgNO_3$. Therefore, in order to determine the MIC of bacteria, serial dilutions were prepared according to Table 2.

Silver nanoparticles mediated by leech salivary extract: minimum bactericidal concentrations on test isolates

For *S. aureus, S. pyogenes, E. coli*, and *P. aeruginosa*, Table 3 showed the size of the inhibitory zone of (10 µL) of AgNPs from high to low concentrations, accordingly, in comparison to positive and negative control. The results

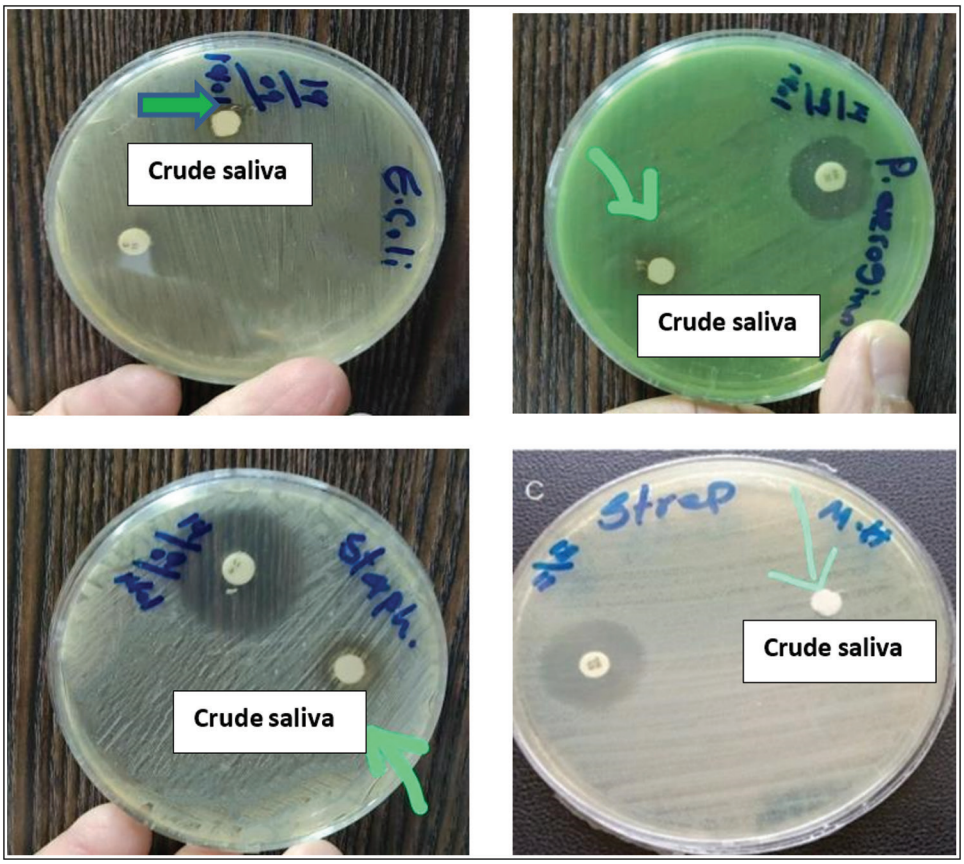

Figure 1: Antibacterial activity of crude *Hirudo medicinalis* saliva on test isolates (ATCC) bacteria compared with positive AN_{30}

Table 1: Antibacterial effects of crude leech salivary extract on a test isolate

Test isolates	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Escherichia coli*	*Pseudomonas aeruginosa*
Inhibition zones of antibiotic AN_{30} on test isolates (mm)	25.00 ± 4.100	25 ± 5.800	23.00 ± 4.400	22.00 ± 4.700
Inhibition zones of crude leech saliva on test isolates (mm)	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000

The means and standard deviation of values that differ significantly at $P > 0.05$

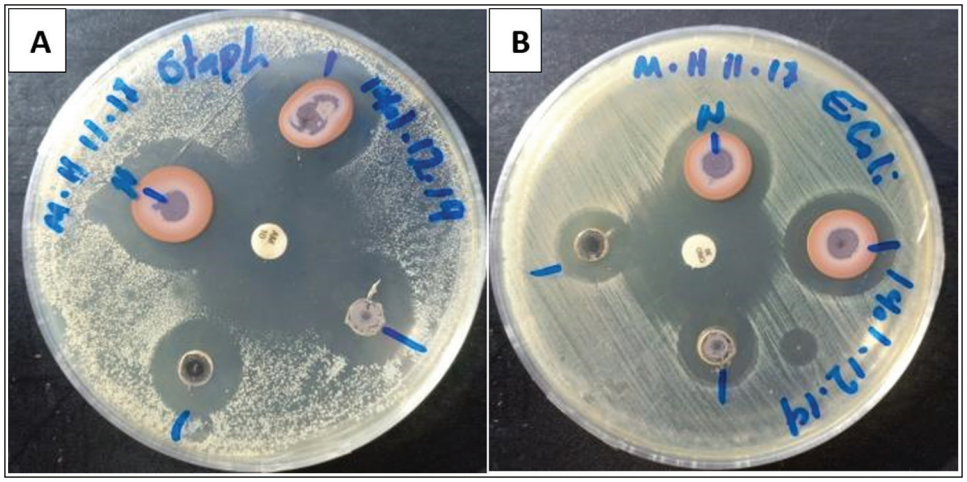

Figure 2: $AgNo_3$-leech saliva nanoparticle antibiogram. (A) *Staphylococcus aureus* and (B) *Escherichia coli.* Nanoparticle concentration in each well is (1) 0.1 g, (2) 0.02 g, (3) 0.001 g, and (4) 0.0006 g. Positive control in *E. coli* is CRO_{30} µg, in *S. aureus* is AM_{10} µg

Table 2: Serial dilutions were prepared in order to determine the minimum inhibitory concentration)MIC) of bacteria

No	1	2	3	4	5	6	7	8
Dilution factor	0 and Positive control	1/2	¼	1/8	1/16	1/32	Crude saliva	Negative control
$AgNO_3$ concentration (g)	0.0006	0.0003	0.00015	0.000075	0.0000375	0.00001875	0	0
PBS buffer (µL)	0	10	10	10	10	10	0	10
$AgNO_3$ Nano (µL)	10	10	10	10	10	10	0	0
Crude leech saliva	0	0	0	0	0	0	10	0

Dilution factor (PBS), positive control ($AgNO_3$), negative control (PBS)

Table 3: Size of inhibition zone of $AgNO_3$ from high to low concentration respectively in mm for *Staphylococcus aureus*, *Streptococcus pyogenes, Escherichia coli*, and *Pseudomonas aeruginosa* compare with positive control antibiotics

AgNPs con. (µg/mL) Bacteria	Inhibition zone in mm							$LSD_{(0.05)}$
	600	300	150	75	37.5	Positive control	Negative control	
	Mean ± SD							
S. aureus	26 ± 1.9^{d}	26 ± 1.9^{d}	21 ± 0.9^{c}	16 ± 2.2^{b}	0 ± 0^{a}	AM_{10} 35 ± 3.9^{e}	0 ± 0^{a}	1.247
S. pyogenes	27 ± 2.5^{d}	27 ± 2.5^{d}	27 ± 2.5^{d}	20 ± 1.3^{c}	15 ± 1.4^{b}	AN_{30} 25 ± 2.1^{d}	0 ± 0^{a}	2.163
E. coli	22 ± 3.1^{c}	22 ± 3.1^{c}	15 ± 1.4^{b}	15 ± 1.4^{b}	0 ± 0^{a}	CRO_{30} 30 ± 4.3^{d}	0 ± 0^{a}	1.573
P. auroginosa	23 ± 1.4^{d}	21 ± 0.9^{cd}	16 ± 2.2^{b}	16 ± 2.2^{b}	0 ± 0^{a}	CRO_{30} 20 ± 1.3^{c}	0 ± 0^{a}	2.350

Different letters refer to significant value at $P \leq 0.05$.

AM_{10}: Ampicillin (10 µg), AN_{30}: Amikacin (30 µg), CRO_{30}: Ceftriaxon (30 µg), LSD: least significant differences

Table 4: Test isolate exposed to the lowest inhibitory and bactericidal quantity of silver nanoparticles from leech salivary extract

Test isolates	Concentrations of LSE-Ag-NPs (µg/mL)		Interpretation
	MIC (µg/mL)	MBC (µg/mL)	
Escherichia coli	300	600	Sensitive
Staphylococcus aureus	300	600	Sensitive
Streptococcus pyogenes	75	150	Sensitive
Pseudomonas aeruginosa	300	600	Sensitive

reveal that there are significant differences with different concentrations of AgNPs for each bacterium, but there are no significant differences between the 600 µg/mL concentrations of AgNPs compared with positive control (antibiotics).

The minimal inhibitory and bactericidal concentration of silver nanoparticles mediated by leech salivary extract on test isolates was shown in Table 4. The LSE-Ag's MIC against *E. coli*, *S. aureus* and *P. aeruginosa* were (300 µg/mL) whereas the MBC of these bacteria were (600 µg/mL) and the MIC of the LSE-Ag against *S. pyogenes* was (75 µg/mL) whereas the MBC was 150 µg/mL [Figures 3 and 4].

Discussion

Due to the production of chemicals in its saliva as a result of its innate immune defense system, which is a result of its feeding process and surviving techniques in its habitat, *H. medicinalis* is one of the species that is most commonly used as a model in medicine. More than 100 substances are secreted by this organism, although only a small number of them have potent anti-inflammatory, antibacterial, analgesic, and anticoagulant activities.[28]

There are numerous ways to examine the saliva of various members of the leech family. Using the method outlined,[20] leech saliva extraction is accomplished in the current investigation. The animal's movement is stopped by the freezing temperature, which aids in maximizing the amount of saliva squeezed out of it. The freezing cold also forces the animal to regurgitate (vomit) the required sucked solution. We found that feeding the hungry leeches phagostimulatory fluid and then submerging them in ice in a plastic test tube made it much easier to collect the saliva. Last but not least, the animal is not killed by the ice, and it is sufficient to re-immerse him in water for him to resume all of his activities and continue to exist testing involved leaving routinely for illimitable amounts of time. The best outcome is found with phagostimulatory solutions containing 0.001 M arginine and 0.15 M sodium chloride.[29]

The results of the current investigation show that test isolates are not inhibited by CLSE. Low levels of bioactive compounds in the extract may be to blame for this.[30] The

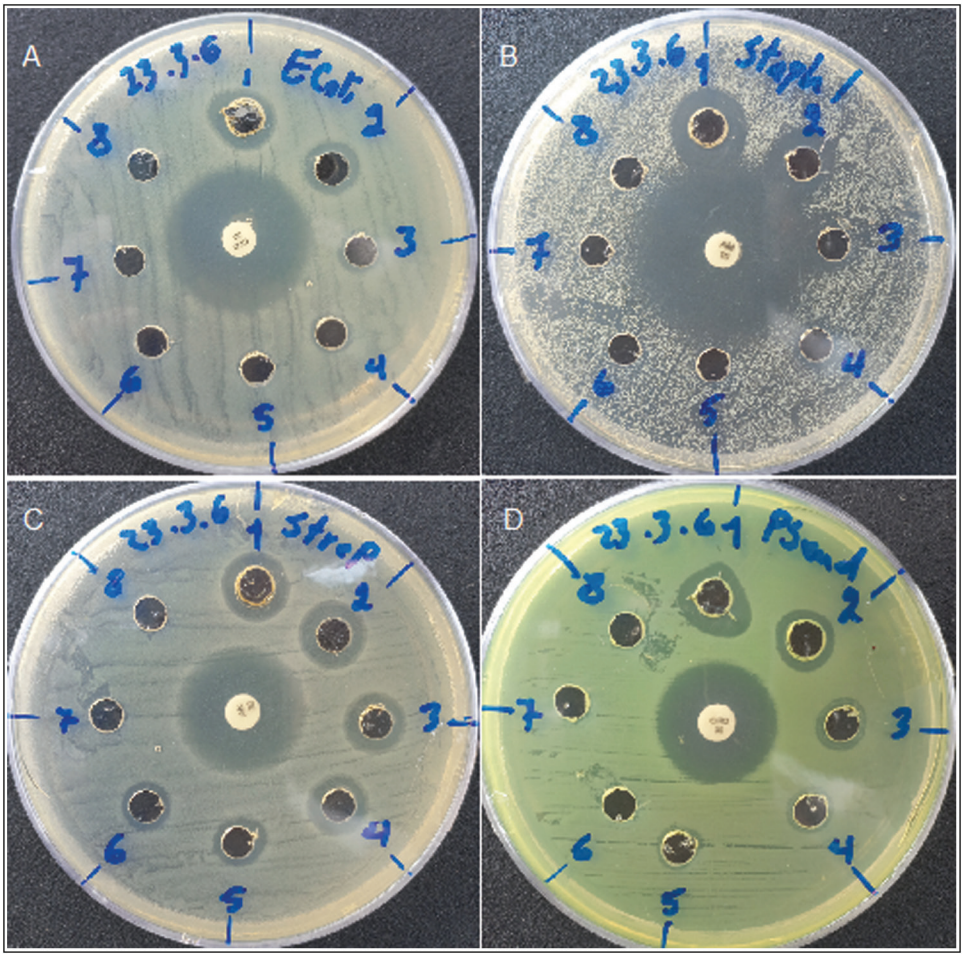

Figure 3: Disk diffusion susceptibility test. (A) *Escherichia coli*, (B) *Staphylococcus aureus*, (C) *Streptococcus pyogenes*, and (D) *Pseudomonas aeruginosa*. Serial dilutions in all plate are equal and include: Well 1; 600 μg/mL as positive control, well 2; 300 μg, well 3; 150 μg/mL, well 4; 75 μg/mL, well 5; 37.5 μg/mL, well 6; 18.75 μg/mL, well 7; crude saliva, well 8; negative control. Proportional standard sensitive antibiotic for each bacterium is, *E. coli*; Ceftriaxone ($CRO_{30}\mu g$), Staph; Ampicillin ($AM_{10}\mu g$), Strep; Amikacin ($AN_{30}\mu g$), Pseudomonas ($CRO_{30}\mu g$)

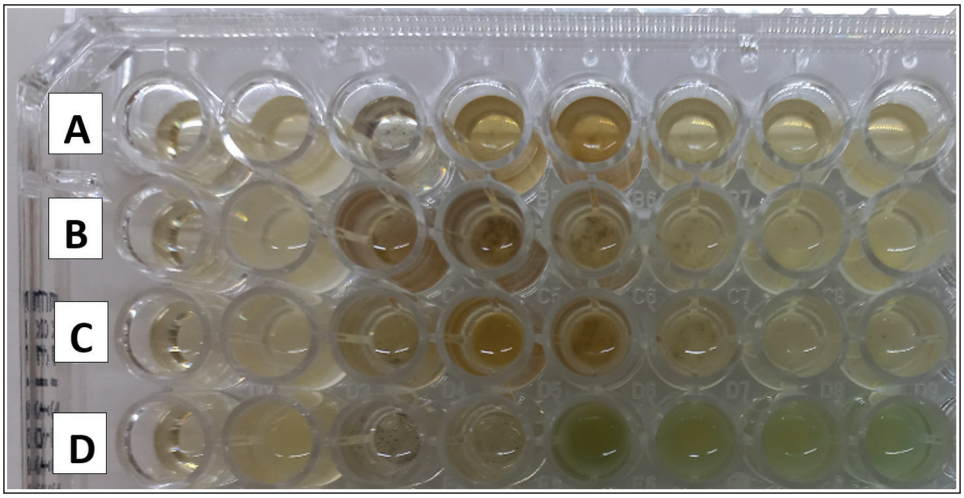

Figure 4: (A) *Escherichia coli*, (B) *Staphylococcus aureus*, (C) *Streptococcus pyogenes*, and (D) *Pseudomonas aeruginosa*. Columns 1: Negative control 100 μL Mueller Hinton broth only, 2: Positive (growth) control, 3-8: serial dilution including 3 = 600 μg, 4 = 300 μg, 5 = 150 μg, 6 = 75 μg, 7 = 37.5 μg, and 8 = 18.75 μg

author[25] concurs this finding conflicts with the findings of,[31] who claimed that several of the test isolates utilized in this study were inhibited by leech salivary extract.

A vital component that shields the body from germs, silver may readily bind to DNA, peptides, antibodies, and enzymes.[32,33]

It has been shown that silver nanoparticles)LSE-Ag) mediated by leech salivary extract can inhibit the growth of *P. aeruginosa*, *S. pyogenes*, *S. aureus*, and *E. coli*.[21] claim that the bioactive components that cause the activity of LSE include proteins and antimicrobial peptides (AMP). The submicron (minute) size of silver nanoparticles, which allows them to act as a drug carrier by absorbing bioactive molecules (hydrophilic and hydrophobic) into their matrix, may be the cause of the observed activity in the LSE-Ag as contrasted to the pure LSE counterpart. By interacting with the thiol group of specific enzymes or respiratory enzymes, the bioactive compounds in the matrix of the LSE-Ag may inhibit bacterial growth, leading to their eradication, or the formation of reactive oxygen species, which damages cells and accumulates in pits on the cell wall, or both. Microorganisms experience denaturation and cell death as a result of these occurrences because holes develop in their membranes.[31,34] According to reports, silver nanoparticles infiltrated the bacteria and injured them by interacting with their DNA and other phosphorus- and sulfur-containing compounds. The findings of this work may have an impact on the development of innovative Ag-NP-based antibacterial systems for use in medicine.[35]

Conclusion

From the present study it is concluded that the CLSE showed no inhibitory effect on the test isolates. The present investigation revealed the potential of silver nanoparticles obtained from leech saliva as a rich source of antibacterial agent. The leech salivary extract-mediated silver nanoparticles (LSE-Ag) were observed to inhibit the growth of (ATCC) isolates *P. aeruginosa*, *E. coli*, *S. aureus*, and *S. pyogenes*. According to the antibiogram results, we found that the suitable concentration of nanoparticle is 0.0006 g $AgNO_3$.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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