International Journal of Design & Nature and Ecodynamics
Vol. 21, No. 6, June, 2026, pp. 1801-1810
Journal homepage: http://iieta.org/journals/ijdne

Partially Purified Hirudin from *Hirudo medicinalis* as a Sustainable Natural Alternative for Controlling Biofilm Formation in *blaCTX-M*-Producing *Citrobacter freundii* from Burn and Wound Infections

Shahrazad Najem Abdu-Allah[1*], Batool Abd Al Ameer Baqer[1], Maysoon Kh. Abbas[2], Eman Jassim Mohammed[1]

[1] Microbiology Department, College of Science, Mustansiriyah University, Baghdad 10052, Iraq
[2] Biology Department, College of Science, Mustansiriyah University, Baghdad 10052, Iraq

Corresponding Author Email: shah3314@uomustansiriyah.edu.iq

https://doi.org/10.18280/ijdne.210625

Received: 13 March 2026
Revised: 20 May 2026
Accepted: 27 May 2026
Available online: 30 June 2026

Keywords:
Citrobacter freundii, Hirudo medicinalis biofilm, antimicrobial agents, Congo red agar method, tissue culture plate method

ABSTRACT

Citrobacter freundii is an opportunistic pathogen. Its ability to produce biofilms and its high antimicrobial resistance were observed, especially among isolates containing the *blaCTX-M gene*, which is linked to resistance to oxyimino-cephalosporins such as cefotaxime (CTX). To evaluate the biofilm-forming ability of *Citrobacter freundii* and antibiotic resistance patterns, the presence of the *blaCTX-M* gene in *C. freundii* isolates was assessed, and the effectiveness of partially purified hirudin from *Hirudo medicinalis* against biofilm was evaluated. This study was performed from December 2022 to February 2024. A total of 150 skin swabs were collected from wounds and burns. Biofilm production was quantitatively assessed using the tube method (TM), the Congo red agar method (CRAM), and the tissue culture plate method (TCPM), and molecular screening was performed to investigate and extract the *blaCTX-M* gene using polymerase chain reaction (PCR). Of the 150 samples, 17 isolates were confirmed as *C. freundii*. The results show that 11 (64.70%) isolates formed strong slime, but 6 (35.29%) of the isolates did not produce a slime layer. As for the molecular detection of the *blaCTX-M* gene, it showed that 10 isolates (58.8%) out of 17 isolates of *Citrobacter freundii,* which formed biofilm, carried this gene (544 base pairs), and these 10 isolates were also producers of broad-spectrum beta-lactamase enzymes according to phenotypic detection, while 7 isolates (41.2%) did not carry this gene. All isolates were resistant to at least one antibiotic (100%) and exhibited high MAR indices; the highest susceptibility was observed to imipenem (100%), followed by ciprofloxacin (41.17%) and gentamicin (23.52%). The results showed clear and statistically significant differences between the isolates compared to the control group before treatment with partially purified hirudin. A statistically significant difference was found between them, $p = 0.00002$ ($p < 0.001$). After treatment with partially purified hirudin, a statistically significant difference was also found between them, $p = 0.000001$ ($p < 0.001$). Most importantly, the results showed clear and significant differences between the isolates before and after treatment with partially purified hirudin, indicating the antimicrobial efficacy of the extract, with a *p-value* of 0.000178. *C. freundii* clinical isolates appear multidrug-resistant (MDR) and have the ability to produce biofilm. Purified hirudin exhibited a marked depressed effect on biofilm formation by multidrug-resistant *C. freundii* from burns and wounds.

1. INTRODUCTION

Citrobacter freundii is one of the most dangerous pathogenic microorganisms that has raised significant concerns about community health. It is a globally widespread and ubiquitously bacteria, considered a nosocomial infection, and is considered an opportunistic clinical disease affecting a wide spectrum of illnesses. It also exhibits resistance to antibiotics [1, 2].

Modern medicine has recently focused on complementary medicine methods and their potential mechanisms of action. Many researchers have studied leech therapy, an ancient practice, to investigate its potential effects on various diseases, such as inflammatory conditions, arthritis, and post-surgical recovery. Leeches are the most commonly used type of leech for therapeutic purposes, and many different types have been tested and researched all over the world. Leeches secrete more than 20 biologically active substances, including hirudin, antistatin, aeglin, guamarin, saratin, substituents, complement inhibitors, and carboxypeptidase inhibitors. These substances exhibit anti-inflammatory, analgesic, antiplatelet, anticoagulant, thrombin regulator, extracellular matrix, and

antimicrobial effects. Further research may reveal a wider range of these effects. This technique is inexpensive, effective, and easy to apply, and its mechanisms of action in treating certain diseases have been elucidated. It is clear that microbial phototherapy is a complementary or supplementary option to conventional treatment. It is part of many therapies with different specialties, and it secretes various types of bioactive substances that differ between species. Each species must be evaluated individually in terms of its therapeutic potential and the molecules it secretes. New substances may be discovered, and these could be the basis for future therapies [3].

Although it is commonly considered a portion of the human gut microbiota but progressively involved in healthcare-associated infections [1]. Some isolates of *C. freundii* have been correlated with nosocomial opportunistic infections affecting various tissues, including the biliary tract, liver, bloodstream, urinary tract, large and small intestines, peritoneum, bone, and wounds, particularly in immunocompromised individuals [4, 5].

Citrobacter freundii is increasingly identified as someone of importance causative agent of nosocomial infections and serves as a significant reservoir of antimicrobial resistance determinants [6].

Citrobacter freundii has become resistant to antibiotics, causing the widespread use of broad-spectrum antimicrobials [7]. Antibiotics, for example, fluoroquinolones, aminoglycosides, nitrofurantoin, carbapenems, and cephalosporins are standard antibiotics for treating infections caused by this bacterium [8]. This raises concerns about the increasing resistance of *Citrobacter freundii* to many antibiotics. Low-virulence *Citrobacter species* that remain in the host for a long time can contribute to the progression of pathogens through the accumulation of resistance genes to multiple antimicrobials [9-11].

The emergence of multidrug-resistant and extensively drug-resistant enteric bacteria, such as *C. freundii*, poses a significant threat to human health [12]. Antithetrapy for AmpC-*Citrobacter* may inhibit the production of beta-lactamases by bacteria. Antibiotics are not available to treat this bacterium because it produces and contains chromosomal beta-lactamase enzymes [13, 14].

The objective of this study is to evaluate the biofilm-forming ability of *Citrobacter freundii* and the antibiotic resistance pattern, and assess the effectiveness of medicinal leech extract against biofilm.

2. MATERIALS AND METHODS

2.1 Sample isolation and diagnosis

Seventeen isolates of *Citrobacter freundii* were obtained from burn and wound patients in various hospitals in Baghdad, Iraq. Most of the isolates were collected from 150 skin swabs taken from both sexes of different ages between December 2022 and February 2024. Morphological, biochemical, and IMViC tests, Triple Sugar Iron agar test, as well as the API 20E system, were performed on the isolates.

Medical samples were cultured on brain heart infusion broth, nutrient agar, blood agar, Salmonella-Shigella (SS) agar, MacConkey agar and Mueller-Hinton agar according to needs, and the colonies were diagnosed using microbiological techniques (morphology and biochemistry) [15].

2.2 Genetic detection of the *blaCTX-M* gene using polymerase chain reaction

A single-molecular polymerase chain reaction (PCR) assay was performed to amplify fragments of the *blaCTX-M* gene in all *Citrobacter freundii* isolate strains under study.

2.2.1 DNA extraction

DNA was extracted by suspending three colonies of each *Citrobacter freundii* isolate on MacConkey agar plates in 500 µL of nuclease-free water (Bromage, USA) and heating them for 10 minutes at 90 ℃ using a water bath. The samples were then centrifuged for 10 minutes at 10,000 rpm and used as a template for bacterial DNA analysis using PCR.

2.2.2 Primers and amplification reactions used

The primers and amplification reactions used in this study were as follows: the forward primer sequence of the *blaCTX-M* gene (5-TTTGCGATGTGCAGCACCA GTAA-3) and the reverse primer sequence of the *blaCTX-M* gene (5-CGATATCGTTGGTG GTGCCATA-3) (Alpha DNA, Canada) [16, 17].

Amplification reactions (25 µL per sample) were performed using Mastermix 2X mix (Kappa Corporation, India) (12.5 µL), forward initiator (1.5 µL), reverse initiator (1.5 µL), nuclease-free water (4.5 µL), and DNA sample (5 µL). The thermal cycling conditions included an initial denaturation process at 95 ℃ for 5 minutes, followed by 30 cycles at 94 ℃ for 30 seconds, then annealing at 55 ℃ for 40 seconds, and then elongation at 72 ℃ for 50 seconds. The cycle concluded with a final elongation at 72 ℃ for 10 minutes.

2.2.3 Agarose gel electrophoresis

Gel electrophoresis was used to detect the PCR products, which were imaged using ethidium bromide and a UV light documentation system [17].

2.3 Antibiotic susceptibility testing

Clinical bacterial isolates from burns and wounds were cultured on Mueller-Hinton agar using the standard Kirby-Bauer disc diffusion method, according to the 2020 CLSI [16]. The antibiotic discs used in this study included Amoxicillin (25 µg), Aztreonam (15 µg), Ciprofloxacin (10 µg), o-Trimoxazole (25 µg), Gentamicin (10 µg), Imipenem (10 µg), Nitrofurantoin (300 mcg), Ofloxacin (5 µg), Penicillin G (10 U), Piperacillin (100 mcg), Tobramycin (10 µg), Amikacin (10 µg), Ampicillin (10 µg), Ceftriaxone (30 µg), Cephalexin (30 µg), Piperacillin/Tazobactam (100/10 µg M-H agar plates were prepared and coated with a 0.5 M McFarland swab-modified bacterial suspension, followed by antibiotic tablets. The plates were incubated for 24 hours at 37 ℃. The diameter of the inhibition zone was measured and calculated to determine antibiotic resistance using CLSI criteria [16].

The result was that the bacteria were MDR, meaning they were resistant to one or more of three agents [12].

2.4 Examination of biofilm-producing organisms

Screening for biofilm production for all isolates was done using the tube method (TM), Congo red agar method (CRAM), and tissue culture plate method (TCPM).

2.4.1 Phenotypic features of biofilm production in Congo Red agar

The isolates were grown on CRA plates, previously prepared by adding 0.8 g/L of Congo red and 50 g/L of sucrose (from British Drug Houses/England) and 1 L of brain heart infusion agar (Himedia - India). The plates were then incubated for one day at 37 ℃ and 8 hours at room temperature. This allowed for the observation of black, rough colonies produced by the slime-producing strains, distinguishing them from non- biofilm producing isolates (red smooth colonies), and the results appeared as in Figure 1.

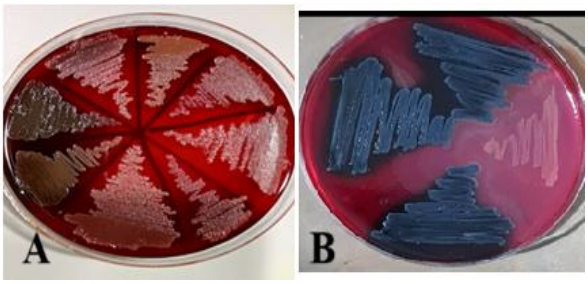

Figure 1. Congo red agar (CRA) assay for slime production. (A) Red, smooth colonies indicating non-slime-producing isolates, (B) black colonies indicating slime-producing (biofilm-producing) isolates

2.4.2 Phenotypic features of biofilm creation by the Tube Method test

The TM was used to establish biofilms. The vaccine was placed on 5 mL of heart and brain extract broth with a ring of bacterial culture prepared overnight in a glass tube and then incubated at 37 ℃ for 24 hours. The tubes were then washed with P-buffered saline and air-dried. The dry tubes were stained for 15 minutes with 0.1% C.V. dye, then washed with deionized H_2O to eliminate additional dye. The tubes were allowed to dry, and biofilm formation was investigated. Most tubes showed observable biofilm along the bottom and the wall, indicating positive biofilm formation. Some tubes also showed a colored disc at the air-liquid border, indicating negative biofilm formation [15, 18].

The optical absorption (ODc) values were calculated separately for each experimental group as the average of three empty control wells containing only sterile heart and brain broth. The isolates were ranked according to their ability to form biofilms (S = strong biofilm formation, M = moderate biofilm formation, W = weak biofilm formation, N = no biofilm formation) according to the equation shown in Table 1.

Table 1. The mean optical density (OD) value obtained for all isolates and the strength of biofilm formation [19, 20]

Averaged OD Value	Biofilm Production
ODc ≥ ODi	Non-adherent
2 × ODc ≥ ODi ≥ ODc	Weakly adherent
4 × ODc ≥ ODi ≥ 2× ODc	Moderately adherent
ODi > 4 × ODc	Strongly adherent

Note: ODc = Optical density for control, ODi = Optical density for isolates.

2.4.3 Phenotypic features of biofilm formation by microtiter plate method or tissue culture plate method

Tissue culture was performed on plates. Five milliliters of heart and brain extract broth was inoculated with a ring of freshly cultured isolates into 96 wells, and the plates were incubated under ideal growth conditions. The bacterial suspension was diluted 1:4. A sterile polystyrene plate containing wells, each holding 200 µL of the prepared bacterial suspension, was used. Fresh culture medium was placed in the wells. The plates were incubated at 37 ℃ for 24 h. The plates were gently shaken to eliminate the contents of the wells and washed repeatedly with 200 µL of phosphate-buffered saline solution. They were then incubated at 50 ℃ for one hour to fix the biofilms formed by the adhering bacteria. The stable biofilms were stained with C.V. (0.1%) for half an hour, and the stain was removed with deionized water. They were air-dried, and the results were read using ELISA (600 nm wavelength) to determine the optical density of the stained bacterial biofilms [15-18].

2.5 Extraction and partial purification of Hirudin from *Hirudo medicinalis*

2.5.1 Preparation of *Hirudo medicinalis* crude extract

Fourteen 10 cm long *Hirudo medicinalis* leeches were prepared to destroy bacteria. The leeches were cut into pieces and then ground in a homogenizer with the addition of 5 mL of normal saline solution, and put in the shearer at 25 ℃ for 2 days, obtain a homogeneous suspension, centrifuge for 20 minutes at 3000 g, filter the liquid through a Millipore 0.45 mm filter, and keep the extract at 4 ℃ to use [21], as shown in Figure 2.

Figure 2. *Hirudo medicinalis*

2.5.2 Partial purification of Hirudin

The crude extracts obtained from *Hirudo medicinalis* leeches were centrifuged at a speed of 10,000× for 20 minutes at a temperature of 4 ℃ to remove undissolved material and cell remnants. The filtrate was then filtered through a 0.22 µm membrane filter. The filtrate was used in a DEAE-Sepharose ion-exchange chromatography column pre-neutralized with 20 mM Tris-HCl buffer (pH 8.0). The associated proteins were separated using a linear gradient of sodium chloride (0-1.0 M), and the fragments that showed antithrombin activity were collected. The active fractions were concentrated using ultrafiltration through a membrane with a molecular weight cutoff of 3 kDa.

Based on the sequential purification steps, the resulting fractions were considered hirudin-rich. Protein analysis was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which revealed a dominant protein band corresponding to the predicted molecular weight of hirudin, indicating partial purification. The biological activity of the enriched fractions was confirmed using an antithrombin activity assay. The partially purified protein was obtained as a 2 mg/mL basal solution. A 1 mg/mL dilute solution was prepared, and 100 µL of the dilute protein was mixed with an equal volume (100 µL) of bacterial culture in a

96-well microplate, resulting in a final protein concentration of 0.5 mg/mL in contact with the bacterial cells [22-24].

2.5.3 In vitro inhibitory effect of partially purified Hirudin on isolates producing biofilm

To study the inhibitory effect of partially purified hirudin from *Hirudo medicinalis* on biofilm of isolates, the highest biofilm-producing isolates of *Citrobacter freundii* were selected to be tested.

A modified spectrophotometric method was used to detect biofilm inhibition [25]. Biofilm inhibition was performed using 96-well microplates. In vitro, bacteria were cultured in heart and brain extract (BHI) broth and incubated at 37 ℃ for 18 h. The bacterial culture was diluted in BHI broth, then its concentration was adjusted using a 0.5 McFarland tube. The partially purified protein was obtained as a stock solution with a concentration of 2 mg/mL. A diluted solution was prepared at a concentration of 1 mg/mL, and 100 microliters of the diluted protein were mixed with a similar volume (100 microliters) of bacterial culture in a 96-well microplate, resulting in a final protein concentration of 0.5 mg/mL in contact with the bacterial cells. The plates were incubated for 24 h at 37 ℃. The wells were then washed in sterile physiological saline to remove any adhering bacteria and stained using the same procedure as above to detect biofilm formation.

2.6 Ethical considerations

Approval was obtained from the Ethics Committee at the College of Science, Mustansiriyah University (Approval No. BSCMU/12022/00092M, November 1, 2022). The procedures followed the ethical guidelines outlined in the Declaration of Helsinki, and written informed consent was obtained from all individual participants included in the study.

3. RESULTS

3.1 Collection and identification of bacterial isolates

A whole of 17 isolates of *Citrobacter freundii* were obtained from burns and wounds from various hospitals in Baghdad. Most of the isolates were collected from 150 skin swabs.

3.1.1 Phenotyping of isolates

Citrobacter spp. isolates underwent microscopic examination, and all isolates appeared to be Gram-negative. Minor bars that occurred as a sole cell or in pairs [1, 2].

On MacConkey agar, all isolated were appear as large, dry, smooth, elevated colonies and pink color because they are slow lactose fermenters (Figure 3).

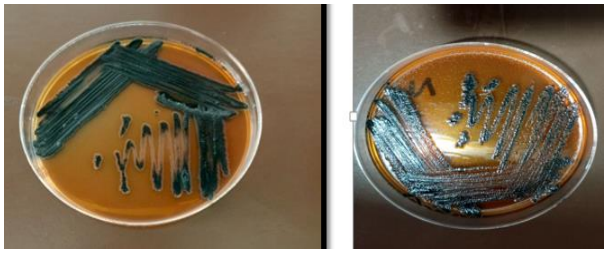

Figure 3. *Citrobacter freundii* on Salmonella-Shigella (SS) agar

Citrobacter species on SS agar appear as small to medium-sized colonies, typically colorless to pale yellow, often with a distinct black center. Due to their ability to produce H_2S and ferment lactose (slowly or partially), they can resemble Salmonella colonies, though *Citrobacter* is generally distinguished by its ability to ferment lactose (pink on MacConkey). Because *Citrobacter* can mimic pathogen appearances on SS agar, additional testing (like lactose fermentation on MacConkey or biochemical tests) is necessary for confirmation [1, 2].

3.1.2 Biochemical tests

Isolates were obtained from MacConkey agar and brain heart infusion (BHI) broth and subjected to biochemical tests. All isolates gave a (-) result for oxidase and a (+) result for catalase tests. Catalase examination is used to recognize the catalase enzyme in bacteria, which is a very important enzyme responsible for the degradation of harmful hydrogen peroxide to harmless oxygen and water [2, 3].

IMViC test was tested using a sequence of 4 different biochemical experiments to classify and organize bacteria, particularly members of the *Enterobacteriaceae*. This was done to identify the specific type of bacteria, particularly Gram-negative bacteria, as it identifies and classifies members of the *Enterobacteriaceae* family.

3.2 Genetic detection of the *blaCTX-M* gene in *C. freundii* strains using polymerase chain reaction

All seventeen *C. freundii* isolates were subjected to PCR testing for the presence of the *blaCTX-M* gene. The results, shown in Figure 4, indicate the presence of the *blaCTX-M* gene (544 base pairs) in 10 (58.8%) of the 17 *C. freundii* isolates, while 7 (41.2%) did not carry the *blaCTX-M* gene. The seventeen *C. Freundii* isolates carrying the *blaCTX-M* gene were also phenotypically detectable and produced Extended-Spectrum β-Lactamase (ESBL) enzymes. Isolates were phenotypically positive for ESBL production but did not carry the *blaCTX-M* gene and were perhaps resistant to cephalosporins without the presence of the CTX-M enzyme in Figure 4.

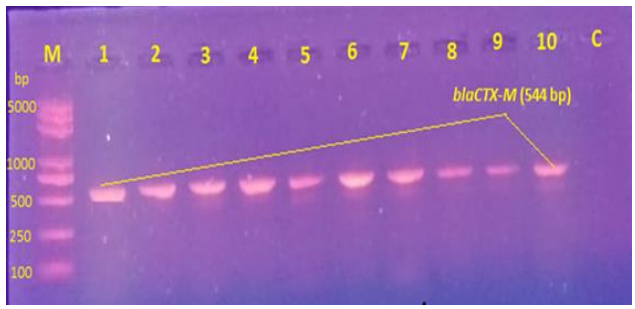

Figure 4. Electrophoresis of the polymerase chain reaction (PCR) product of *blaCTX-M* (544 bp) in *C. freundii* isolates
Note: Lane M: 100 pb DNA ladder (Kapa, India); lanes 1-10: *C. freundii* isolates; lane C: Negative control (had all PCR mixture including water instead of DNA template). Detection was done on agarose gel (1%) at 5 V/cm for one hour, stained with ethidium bromide, and visualized on a UV transilluminator documentation system

3.3 Antimicrobial susceptibility of *C. freundii* isolates

3.3.1 Standard disc diffusion technique

In vitro antibiotic sensitivity testing was performed on all *C. freundii isolates* by a CD diffusion test towards 16 different

antimicrobial agents. The isolates exhibited diverse levels of resistance to the Aminoglycoside class, including Tobramycin (100% R) and Amikacin (100% R), Gentamicin (76.47%), a βeta-Lactam class including piperacillin/tazobactam, amoxicillin, aztreonam, penicillin G, and ampicillin were resistance (100% R), cephalosporins class including ceftriaxone (100% R) and cephalexin (100% R). While isolates showed a high level of sensitivity to the Carbapenems class, including imipenem (100% S), the fluoroquinolones class, including ciprofloxacin, was resistant (58.82%), while showing a high level of resistance toward ofloxacin (100% R); all isolates showed a high level of resistance toward nitrofurantoin and co-trimoxazole (100% R). The overall susceptibility patterns of isolates are displayed in Figure 5 and Tables 2 and 3.

Figure 5. *C. freundii* on Mueller-Hinton agar shows an inhibition zone surrounding the antibiotic disc

Table 2. Antibiotic susceptibility testing of *Citrobacter freundii* isolates determined by the Kirby–Bauer disk diffusion method, showing inhibition zone diameters (mm)

Antibiotic Disks	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
Amoxicillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aztreonam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ciprofloxacin	10	25	15	10	25	10	30	25	27	10	10	25	15	25	12	12	15
Co-Trimoxazole	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gentamicin	0	25	0	10	25	0	0	30	25	0	0	0	0	0	0	0	0
Imipenem	30	30	35	30	25	30	30	30	25	25	25	30	25	35	25	25	30
Nitrofurantoin	0	0	0	0	0	0	0	10	0	0	0	10	0	0	0	0	0
Ofloxacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillin G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piperacillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tobramycin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amikacin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ampicillin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ceftriaxone	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cephalexin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Piperacillin/Tazobactam	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Note: C = Citrobacter isolates, 0 = A value of 0 mm indicates the absence of an inhibition zone around the antibiotic disc.

Table 3. Percentages of *C. freundii* susceptibility to 16 antibiotics

Antibiotics Disc	Term	Number and Percentage of Resistant	Number and Percentage of Sensitive
Amoxicillin	AX	17(100%)	0%
Aztreonam	AZ	17(100%)	0
Ciprofloxacin	CIP	10(58.82%)	7 (41.17%)
Co-Trimoxazole	CO	17(100%)	0%
Gentamicin	GEN	13 (76.47%)	4 (23.52%)
Imipenem	IMP	0%	17(100%)
Nitrofurantoin	NIT	17(100%)	0%
Ofloxacin	OF	17(100%)	0%
Penicillin G	P	17(100%)	0%
Piperacillin	PIP	17(100%)	0%
Tobramycin	TOB	17 (100%)	0%
Amikacin	AK	17 (100%)	0%
Ampicillin	AP	17(100%)	0%
Ceftriaxone	CEA	17(100%)	0%
Cephalexin	CN	17(100%)	0%
Piperacillin/Tazobactam	PIP	17(100%)	0%

3.4 In vitro inhibitory effect of Partial Purified Hirudin on *Citrobacter freundii* biofilm

The biofilm producer's isolates exhibited three different categories: 10 of isolates 58.82% were produced strong-biofilm, while other 3 of isolates 17.64% (3/17) were none-biofilm producer, 1 isolate 5.88% was produced moderately biofilm and 3 isolates 17.64% was produced weakly biofilm as shown Table 4, while results demonstrated from the CRAM that out of 11 *C. freundii* 64.70% (11/17) of isolates were biofilm formation, while, 35.29% (6/17) isolates were identified as non-biofilm formation, The biofilm producer's isolates exhibited three different categories: 11 of isolates 64.70% were produced strong-biofilm, while other 6 of isolates 35.29% were none- biofilm producer and non isolates produced weakly or moderately biofilm, While results shown from the TM that out of 13 *C. freundii* 76.47% (13/17) of isolates were biofilm formation, while 23.52% (4/17) isolates were identified as non-biofilm formation. The biofilm producer isolates exhibited three different categories: 10 of the isolates (58.82%) produced strong biofilm, while the other 4 isolates (23.52%) were non-biofilm producers; 1 isolate (5.88%) produced moderate biofilm, and 2 isolates (11.76%) produced weak biofilm, as shown in Table 4.

In this study, results proved that of the 17 bacterial isolates included in this study, fourteen isolates were able to form biofilms, representing 82.35% of the total isolates. Because the aim of this experiment was to study the effect of partially purified hirudin on biofilm-forming bacteria, only these 14 isolates were included in the subsequent analyses. The remaining three isolates (17.64%), which did not exhibit biofilm-forming ability, were excluded from this part of the study. The results in Table 5 presented that the isolates *C. freundii*-1 (0.522 S), *C. freundii*-13 (0.346 S), *C. freundii*-3

(0.560 S), *C. freundii*-4 (0.529 S), *C. freundii*-10 (0.365), *C. freundii*-11 (0.616), *C. freundi*-15 (0.544), *C. freundi*-16 (0.453) and *C. freundi*-17 (0.333) formed strong biofilm, while the isolates *C. freundi*-7 (0.299) formed moderate biofilm, and the isolates *C. freundi*-2 (0.211), *C. freundi*-12 (0.205), and *C. freundi*-14 (0.207) formed weak biofilm. Therefore, Tables 6-9 present the results obtained from the 14 biofilm-forming isolates only. A statistically significant difference was observed between the control group and the biofilm isolates before treatment with partially purified hirudin (1 mg/ml) extracted from the medicinal leech (*Hirudo medicinalis*). The *p*-value was 0.00002 ($p < 0.001$), as shown in Table 5. The result showed that after the treatment with (1 mg/mL) partially purified hirudin, all the isolates of *C. freundii* formed weak and non-biofilm, the control group and biofilm isolates treated with partially purified hirudin from the medicinal leech (*Hirudo medicinalis*) showed a significant difference; the probability value was statistically significant, $p = 0.000001$ ($p < 0.001$), as shown in Table 6. The tissue culture method on plates showed a light purple color (weak biofilm). After treatment with partially purified hirudin from *Hirudo medicinalis*. These creatures, through which they secrete saliva containing biologically active substances, possess therapeutic properties.

Table 4. General results of isolates for biofilm in the tube method (TM), Congo red agar method (CRAM), and tissue culture plate method (TCPM)

Isolates No.	Biofilm Formation	TM (%)	CRAM (%)	TCPM (%)
Citrobacter freundii Isolate number 17	S	10 (58.82%)	11 (64.70%)	10 (58.82%)
	M	1 (5.88%)	0 (0%)	1 (5.88%)
	W	2 (11.76%))	0 (0%)	3 (17.64%)
	N	4 (23.52%)	6 (35.29%)	3 (17.64%)

Note: S = Strongly biofilm, N = Non-biofilm, W = Weakly biofilm, M = Moderately biofilm.

Table 5. Optical density for control (ODc) = 600 nm and for isolates (ODi) before treatment with partially purified hirudin by tissue culture plate assay

Isolate No.	Optical Density for Control (ODc) = 600 nm	Optical Density for Isolates (ODi) = 600 nm Before Treatment with Partially Purified Hirudin
C. freundii-1	0.071	0.522 S
C. freundii-13	0.088	0.346 S
C. freundii-3	0.071	0.560 S
C. freundii-4	0.071	0.529 S
C. freundii-17	0.088	0.333 S
C. freundii-6	0.071	0.466 S
C. freundii-11	0.088	0.616 S
C. freundii-15	0.088	0.544 S
C. freundii-16	0.088	0.453 S
C. freundii-10	0.071	0.365 S
C. freundii-7	0.071	0.299 M
C. freundii-12	0.088	0.205 W
C. freundii-2	0.071	0.211 W
C. freundii-14	0.088	0.207 W
C. freundii-8	0.071	0.038 N
C. freundii-9	0.071	0.042 N
C. freundii-5	0.071	0.047 N
Total	17	17
Mean	0.078	.3401
SD	0.008624	.191129
	p-value = 0.0002 ($p < 0.001$)	
	t. value = - 5.650	

Note: S = Strongly biofilm, M = Moderately biofilm, W = Weakly biofilm, N = non-biofilm. The ODc values were calculated separately for each experimental set as the average of three blank control wells containing sterile Brain Heart Broth only. The isolates are arranged according to their biofilm-forming ability rather than their numerical order.

3.5 Phenotypic detection of biofilm formation

Results demonstrated from the tissue culture flat plate method that out of the 17 bacterial isolates included in this study, 14 isolates (82.35%) were confirmed as biofilm producers and were selected for further evaluation of the antibiofilm activity of partially purified hirudin. The remaining 3 isolates (17.64%) did not exhibit biofilm-forming ability and were therefore excluded from this part of the analysis. Accordingly, Figure 6 presents the OD values of the 14 biofilm-forming isolates before and after treatment with hirudin (1 mg/mL), and Tables 7-9 summarize the statistical comparison between both groups using one-way Analysis of Variance (ANOVA).

The results, calculated using optical density at 600 nm for isolates that formed a biofilm before and after treatment with a partially purified hirudin extract from (*Hirudo medicinalis*), are shown in Table 6 (*p*-value = .000001).

The results before treatment with the partially purified hirudin, compared with after treatment, show a clear, significant difference indicating the extract's effectiveness, *p*-value = 0.000178, as an antimicrobial agent, as shown in Tables 7-9 and Figure 6.

Table 6. Optical density for control (ODc) = 600 nm and for isolates (ODi) after treatment with 1 g/mL of partially purified hirudin by tissue culture plate assay

Isolates No.	Optical Density for Control (ODc) = 600 nm	Optical Density for Isolates (ODi) = 600 nm After Treatment with Partially Purified Hirudin at a Concentration of 1 mg/mL
Ci.freundii-1	0.098	0.082 N
C. freundii-3	0.098	0.073 N
C. freundii-4	0.098	0.209 W
C. freundii-6	0.098	0.088 N
C. freundii-10	0.098	0.201 W
C. freundii-11	0.098	0.079 N
C. freundii-13	0.098	0.077 N
C. freundii-15	0.098	0.211 M
C. freundii-16	0.098	0.234 M
C. freundii-17	0.098	0.048 N
C. freundii-2	0.068	0.048 N
C. freundii-7	0.068	0.055 N
C. freundii-12	0.068	0.060 N
C. freundii-14	0.068	0.049 N
Total	14	14
Mean	0.0894	.10814
SD	0.14064	.07083
	p-value = 0.000001 ($p < 0.001$)	
	t. value = -0.9695	

Note: S = Strongly biofilm, M = Moderately, W = Weakly, N = None-biofilm. The ODc values were calculated separately for each experimental set as the mean of three blank control wells containing sterile Brain Heart Broth only.

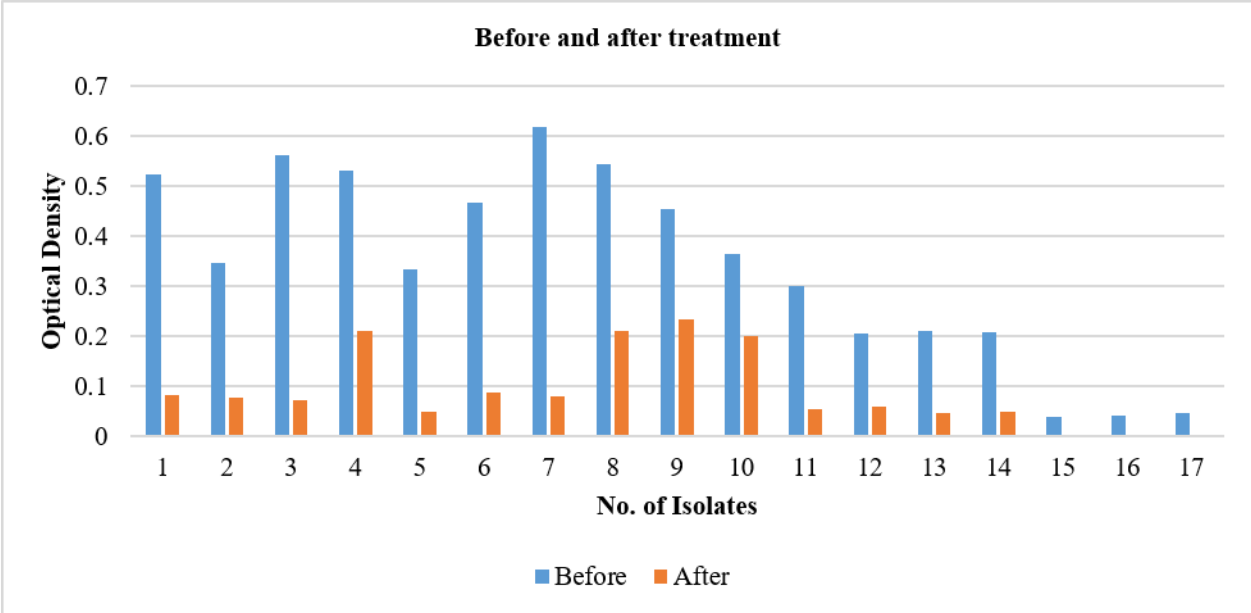

Figure 6. The optical density at 600 nm for biofilm-forming isolates before and after treatment with partially purified hirudin from *Hirudo medicinalis*

Table 7. Biofilm biomass (OD_{600}) of 14 biofilm-forming isolates before and after treatment with partially purified hirudin (ANOVA)

ANOVA: Single Factor					
Groups	**n**	**OD_{600} (mean ± SD)**	**Variance**	**F**	***p***
Before	14	0.340 ± 0.191	0.03653	18.47	0.000178
After	14	0.108 ± 0.071	0.00504		

Note: F: F-statistic.

Table 8. Descriptive statistics

Groups	n	Sum	Mean
Before	14	5.783	0.3402
After	14	1.512	0.1080

Table 9. One-way Analysis of Variance (ANOVA) summary

Source	SS	df	MS
Between groups	0.413858	1	0.413858
Within groups	0.64996	26	0.022412
Total	1.063819	27	

Note: SS: Sum of squares, df: Degrees of freedom, MS: Mean square.

4. DISCUSSION

These results can be explained by the mechanism proposed by research [16]. In general, Imipenem, Ciprofloxacin, and Gentamicin proved most effective against *C. freundii* isolates. However, isolates were highly resistant to Piperacillin/Tazobactam, Nitrofurantoin, Amikacin, Amoxicillin, Aztreonam, Ampicillin, Ceftriaxone, Ofloxacin, Penicillin G, Co-trimoxazole, Cephalexin, and Tobramycin.

Some researchers have shown that the medicinal leech (*Hirudo medicinalis*) typically carries a pure culture of *Aeromonas veronii* bv. *sobria* bacteria in its digestive tract. This is unusual in digestive tracts that are usually home to multiple microbes. Other types of bacteria may proliferate or survive in the medicinal leech's digestive tract. Using a colonization assay, researchers were able to compare the ability of clinical isolates and a symbiotic strain to colonize medicinal leeches experimentally. The symbiotic strain *A. veronii* bv. *sobria* proliferated well and persisted for at least seven days within the digestive tract. It was able to inhibit the proliferation of *Pseudomonas aeruginosa* and *Staphylococcus aureus* within the animal compared to their growth in the laboratory control sample, resulting in altered ingested blood within the digestive tract. Although both strains were able to persist in the digestive tract for a week, the number of viable *Escherichia coli* cells decreased approximately a thousandfold within 42 hours. By inhibiting the activation of the membrane-attack complex in the complement system present in the blood, it is possible to prevent this decrease in the number of viable *E. coli.* This indicates that the membrane-attack complex remains active within *H. medicinalis* and prevents the proliferation of susceptible bacteria. Thus, the antimicrobial properties of the blood of ingested vertebrates contribute to the specificity of the symbiosis between *A. veronii* and *H. medicinalis*, and to the changes that occur in the blood within the digestive tract of *H. medicinalis* [26].

The study [27] did not agree with our results, as it showed that 41.17% of bacterial isolates were sensitive to ciprofloxacin since it inhibits bacterial DNA gyrase, preventing supercoiling of DNA, which is required for chromosome compaction into the bacterial cell.

Cephalexin, one of the first-generation cephalosporins, was investigated in the current findings, in which 100% of the isolates were cephalexin-resistant. Ceftriaxone, one of the third-generation cephalosporins, was investigated in the current findings, in which 100% of the isolates were ceftriaxone-resistant. Resistance mechanisms, particularly the synthesis of large-scale beta-lactamase and other enzymes, may play a role in resistance to first-generation cephalosporins [28].

Citrobacter freundii isolates were tested as resistant to the class aminoglycoside Tobramycin (100%) and Amikacin (100%). Gentamicin's mode of action has long been assumed to involve the irreversible binding of the aminoglycoside to the 30S component of the bacterial ribosome. Inhibiting bacterial protein production [29].

Antibiotic resistance is a global problem. The *C. freundii* isolates in this study demonstrated resistance to multiple antibiotics. *C. freundii* is a major bacterial pathogen contributing significantly to hospital-acquired infections, a serious problem requiring urgent attention [14]. The significant prevalence of multidrug-resistant biofilm-forming organisms in our healthcare facility indicates a potential threat in our region. Therefore, regular monitoring of biofilms and antibiotic resistance among bacteria in clinical laboratories is recommended, along with the strict application of infection control and prevention measures.

This result is consistent with research [30], which stated that leech saliva extract had an inhibitory influence on some of the laboratory bacteria in its study. This result contradicts the report [31], and this may be attributed to the low concentration of bioactive molecules in the extract.

Leech saliva extracts demonstrated antibacterial activity against the microorganisms used in our study. Two studies [32, 33] explained that, like antimicrobial proteins and peptides, 4-bromobutyric acid, 6,17-octadine-1-ol acetate, and octadro-1,4,9,9-tetramethyl contribute to the efficacy of the leech saliva extract. The bioactive substances in the silver-treated leech saliva extract matrix may inhibit microbial growth by interacting with the thiol group in certain enzymes or respiratory enzymes, causing cell death or removal of reactive oxygen species that attack or accumulate within microbial cells. Medicinal leeches are reported to secrete saliva comprising approximately 60 diverse proteins [33, 34]. Leech secretions serve a variety of purposes beneficial to the leech during feeding, including increasing blood flow to the affected region. Many of these concealed proteins act as anticoagulants (such as hirudin), platelet accumulation inhibitors (commonly aperase, collagenase, and calin), vasodilators, and protease inhibitors [35]. In classical medicine, it was employed to treat many diseases, including middle ear infections, arthritis, inflammation of burns and wounds, and diabetic foot injuries. From the above results, it can be concluded that *Citrobacter freundii* clinical isolates was appearance MDR-phenotype and premium ability to produce biofilm, and clinical *Citrobacter freundii* isolates exhibited high resistance to commonly used antimicrobials. The TCPM is more sensitive than the CRA and TM in diagnosing *Citrobacter freundii* biofilm-producing strains. The CRAM is preferred as a complementary test due to its higher specificity compared to TCP. All three methods have proven effective in detecting biofilm-producing *Citrobacter freundii* isolates, and the three traditional tests confidently detected biofilm-producing isolates due to their appropriate sensitivity and specificity. This study showed a high prevalence of *C. freundii* isolates producing broad-spectrum beta-lactamase enzymes, as 10 (58.8%) of 17 *C. freundii* isolates were positive for producing broad-spectrum beta-lactamase enzymes by phenotypic detection, and the molecular investigation resulted in the presence of the *blaCTX-M* gene in 10 (58.8%) of 17 *C. freundii* strains. Also, *Hirudo medicinalis* extract shows high activity in preventing the formation of biofilm by *Citrobacter freundii* isolates.

This study is able to manage the treatment failure of antimicrobial drugs and avoid persistent and chronic infections by using *Hirudo medicinalis* extract as an alternative treatment.

5. CONCLUSIONS

1. *Citrobacter freundii* clinical isolates appear to have an MDR phenotype and a high ability to produce biofilm.
2. Clinical *Citrobacter freundii* isolates show high resistance to often-used antimicrobials
3. The TCPM is more sensitive than the CRA and the T method in diagnosing *Citrobacter freundii* biofilm-producing strains. The CRA method is preferred as a complementary test due to its higher specificity compared to TCP. All three methods have proven effective in detecting biofilm-producing *Citrobacter freundii* isolates, and the three traditional tests confidently detect biofilm-producing strains due to their appropriate sensitivity and specificity.
4. Partially purified hirudin from *Hirudo medicinalis* shows high activity to prevent the formation of biofilm by *Citrobacter freundii* isolates.
5. This study is able to manage the treatment failure of antimicrobial drugs and avoid persistent and chronic infections by using *Hirudo medicinalis* extract as an alternative treatment.
6. Partially purified hirudin showed a clear ability to inhibit biofilm formation in MDR *Citrobacter freundii* isolates isolated from burn and wound infections. The observed activity indicates that hirudin may have value in the control of biofilm-associated infections caused by antibiotic-resistant bacteria. However, additional studies involving animal models and toxicity testing are needed before its clinical use can be considered.

ACKNOWLEDGMENT

The authors would like to express their sincere thanks and appreciation to the Microbiology Department, College of Science, Mustansiriyah University, for their continuous support, cooperation, and valuable assistance throughout this study.

REFERENCES

[1] Mboowa, G., Kidenya, B.R., Sserwadda, I., Kanyerezi, S., Akaro, I.L., Mkinze, B., Seni, J. (2025). Genomic characterization of Citrobacter freundii clinical isolates from Tanzania. Microbiology Resource Announcements, 14(8): e00363-25. https://doi.org/10.1128/mra.00363-25

[2] Al-Eqabi, S.R.S., Al-Abedi, G.J.K. (2021). Pathological, immunological, and hematological parameters associated with experimental infection of Citrobacter freundii in rabbits. Archives of Razi Institute, 76(6): 1607. https://doi.org/10.22092/ari.2021.356801.1911

[3] Sig, A.K., Guney, M., Guclu, A.U., Ozmen, E. (2017). Medicinal leech therapy—An overall perspective. Integrative Medicine Research, 6(4): 337-343. https://doi.org/10.1016/j.imr.2017.08.001

[4] Abbas, M.K., Abdu-Allah, S.N., Al Ameer Baqer, B.A. (2023). Study of the pathological antibacterial effects of tea extract and its role in reducing hypertension in pregnant women. Malaysian Journal of Microbiology, 19(1): 22. https://doi.org/10.21161/mjm.220008

[5] Duduveche, A.E. (2026). The *Citrobacter freundii* complex as an emerging pathogen: Genomic plasticity, virulence, and antimicrobial resistance. International Journal of Molecular Sciences, 27(5): 2378. https://doi.org/10.3390/ijms27052378

[6] Liu, L., Chen, D., Liu, L., et al. (2018). Genetic diversity, multidrug resistance, and virulence of *Citrobacter freundii* from diarrheal patients and healthy individuals. Frontiers in Cellular and Infection Microbiology, 8: 233. https://doi.org/10.3389/fcimb.2018.00233

[7] Ramos-Vivas, J., Chapartegui-González, I., Fernández-Martínez, M., et al. (2020). Adherence to human colon cells by multidrug-resistant Enterobacterales strains isolated from solid organ transplant recipients with a focus on *Citrobacter freundii*. Frontiers in Cellular and Infection Microbiology, 10: 447. https://doi.org/10.3389/fcimb.2020.00447

[8] Liu, L.H., Wang, N.Y., Wu, A.Y.J., Lin, C.C., Lee, C.M., Liu, C.P. (2018). Citrobacter freundii bacteremia: Risk factors of mortality and prevalence of resistance genes. Journal of Microbiology, Immunology and Infection, 51(4): 565-572. https://doi.org/10.1016/j.jmii.2017.08.016

[9] Pepperell, C., Kus, J.V., Gardam, M.A., Humar, A., Burrows, L.L. (2002). Low-virulence Citrobacter species encode resistance to multiple antimicrobials. Antimicrobial Agents and Chemotherapy, 46(11): 3555-3560. https://doi.org/10.1128/AAC.46.11.3555-3560.2002

[10] Sommer, M.O.A., Dantas, G. (2011). Antibiotics and the resistant microbiome. Current Opinion in Microbiology, 14(5): 556-563. https://doi.org/10.1016/j.mib.2011.07.005

[11] Ahmed, T., Islam, M.S., Haider, N., et al. (2023). Phenotypic and genotypic characteristics of antimicrobial resistance in Citrobacter freundii isolated from domestic ducks (Anas platyrhynchos domesticus) in Bangladesh. Antibiotics, 12(4): 769. https://doi.org/10.3390/antibiotics12040769

[12] Magiorakos, A.P., Srinivasan, A., Carey, R.B., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. Clinical Microbiology and Infection, 18(3): 268-281. https://doi.org/10.1111/j.1469-0691.2011.03570.x

[13] Choi, S.H., Lee, J.E., Park, S.J., et al. (2007). Prevalence, microbiology, and clinical characteristics of extended-spectrum β-lactamase-producing Enterobacter spp., Serratia marcescens, Citrobacter freundii, and Morganella morganii in Korea. European Journal of Clinical Microbiology & Infectious Diseases, 26(8): 557-561. https://doi.org/10.1007/s10096-007-0308-2

[14] Qiao, J., Chen, Y., Ge, H., et al. (2023). Coexistence of blaIMP-4, blaNDM-1 and blaOXA-1 in blaKPC-2-producing Citrobacter freundii of clinical origin in China. Frontiers in Microbiology, 14: 1074612. https://doi.org/10.3389/fmicb.2023.1074612

[15] Abdu-Allah, S.N., Mzhr, N.N., Alubadi, A.E.M., Shanyoor, G.J. (2019). Effect of cell-free supernatants of

Lactobacillus acidophilus and Bacillus subtilis against Staphylococcus aureus isolated from acne vulgaris and biofilm formation. Biochemistry & Cell Archives, 20(1): 1703-1709. https://doi.org/10.35124/bca.2020.20.1.1703

[16] Clinical and Laboratory Standards Institute. (2020). Performance standards for antimicrobial susceptibility testing. CLSI. https://www.nih.org.pk/wp-content/uploads/2021/02/CLSI-2020.pdf.

[17] Warjri, I., Dutta, T.K., Lalzampuia, H., Chandra, R. (2015). Detection and characterization of extended-spectrum β-lactamases (blaCTX-M-1 and blaSHV) producing *Escherichia coli*, *Salmonella* spp. and *Klebsiella pneumoniae* isolated from humans in Mizoram. Veterinary World, 8(5): 599-604. https://doi.org/10.14202/vetworld.2015.599-604

[18] Mathur, T., Singhal, S., Khan, S., Upadhyay, D.J., Fatma, T., Rattan, A. (2006). Detection of biofilm formation among the clinical isolates of staphylococci: An evaluation of three different screening methods. Indian Journal of Medical Microbiology, 24(1): 25-29. https://doi.org/10.4103/0255-0857.19890

[19] Stepanović, S., Vuković, D., Hola, V., Bonaventura, G.D., Djukić, S., Ćirković, I., Ruzicka, F. (2007). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. Acta Pathologica, Microbiologica et Immunologica Scandinavica, 115(8): 891-899. https://doi.org/10.1111/j.1600-0463.2007.apm_630.x

[20] Klopper, K.B., Bester, E., van Schalkwyk, M., Wolfaardt, G.M. (2024). Highlighting the limitations of static microplate biofilm assays for antimicrobial testing. Environmental Microbiology Reports, 16(1): e13214. https://doi.org/10.1111/1758-2229.13214

[21] Al-Jumilly, E.F., Al-Safar, M.A., Alubade, E.S. (2017). Determination of minimal inhibition concentration and minimal bactericidal concentration of Hirudo medicinalis oil on pathogenic bacteria. Current Research in Microbiology and Biotechnology, 5(2): 1273-1277.

[22] Ewies, K.S., Rohde, J., Abdel-Maksoud, A.I., El Baz, A.F. (2025). Improving the chromatographic purification protocol for recombinant hirudin produced by Hansenula polymorpha. Egyptian Journal of Botany, 65(1): 1-11. https://doi.org/10.21608/EJBO.2024.288599.2842

[23] Bao, Z., Qi, X., Zhu, S., et al. (2025). Recent progress on formulations of hirudin. Pharmaceutical Sciences Advances, 3: 100078. https://doi.org/10.1016/j.pscia.2025.100078

[24] Abdulrazaq, R.A., Daham, R.I., Abdu-Allah, S.N. (2026). Characterization of a novel klebocin from Klebsiella pneumoniae: Broad-spectrum antimicrobial and anti-biofilm activities with high stability and selective toxicity. International Journal of Design & Nature and Ecodynamics, 21(4): 1037-1044. https://doi.org/10.18280/ijdne.210411

[25] Kheiri, F., Kermanshahi, R.K., Feizabadi, M.M. (2020). The inhibitory effects of Lactobacillus supernatants and their metabolites on the growth and biofilm formation of Klebsiella pneumoniae. Current Microbiology, 20(6): 902-912. https://doi.org/10.2174/1871526520666200106122632

[26] Indergand, S., Graf, J. (2000). Ingested blood contributes to the specificity of the symbiosis of Aeromonas veronii biovar sobria and Hirudo medicinalis, the medicinal leech. Applied and Environmental Microbiology, 66(11): 4735-4741. https://doi.org/10.1128/AEM.66.11.4735-4741.2000

[27] Rehman, A., Patrick, W.M., Lamont, I.L. (2019). Mechanisms of ciprofloxacin resistance in Pseudomonas aeruginosa: New approaches to an old problem. Journal of Medical Microbiology, 68(1): 1-10. https://doi.org/10.1099/jmm.0.000873

[28] Owaif, H.A.A., Aldulaimy, M.K., Abdulateef, S.A. (2023). The antibiotic resistance genes *blaSHV*, *blaOXA-48*, *blaTEM* and *blaIMP* in *Pseudomonas aeruginosa* isolated from respiratory tract infections in Baghdad, Iraq. International Journal of Biomedicine, 13(4): 341-344. https://doi.org/10.21103/Article13(4)_OA18

[29] Abbas, M.K., Abdul, F.R., Rasool, K.H. (2022). Immunological and enzymatic study of Staphylococcus aureus bacteria and fungi isolated from oral cavity. Research Journal of Pharmaceutical Technology, 15(7): 3119-3124. https://doi.org/10.52711/0974-360X.2022.00522

[30] Malik, B., Astuti, D.A., Arief, D.J.F., Rahminiwati, M. (2019). A study on antioxidative and antimicrobial activities of saliva extract of Indonesian local leeches. IOP Conference Series: Earth and Environmental Science, Tangerang, Indonesia, 251(1): 012061. https://doi.org/10.1088/1755-1315/251/1/012061

[31] Mostafa, A.A., Al-Askar, A.A., Almaary, K.S., Dawoud, T.M., Sholkamy, E.N., Bakri, M.M. (2018). Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi Journal of Biological Sciences, 25(2): 361-366. https://doi.org/10.1016/j.sjbs.2016.06.004

[32] Ojo, P.O., Babayi, H., Olayemi, I.K., Oladosun, O.P., Fadipe, L.A., Baba, E., Izebe, K. (2018). Anti-tubercular activities and molecular characterization of salivary extract of Hirudo medicinalis against Mycobacterium tuberculosis. Journal of Tuberculosis Research, 6(1): 1-9. https://doi.org/10.4236/jtr.2018.61001

[33] Rigbi, M., Levy, H., Eldor, A., et al. (1987). The saliva of the medicinal leech Hirudo medicinalis—II. Inhibition of platelet aggregation and leukocyte activity and examination of reputed anaesthetic effects. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 88(1): 95-98. https://doi.org/10.1016/0742-8413(87)90052-1

[34] El-Shimy, N. (1986). Distribution of the medicinal leech, Hirudo medicinalis, in the Middle East. Zoology in the Middle East, 1(1): 154-155. https://doi.org/10.1080/09397140.1986.10637544

[35] Abdul, F.R., Abbas, M.K., Rasool, K.H., Raheem, I.A. (2022). The contribution of interleukin (17) in Iraqi patients with type I diabetes and positive agglutination sera with Morganella morganii antigens. International Journal of Design and Nature and Ecodynamics, 17(5): 767-772. https://doi.org/10.18280/ijdne.170514