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ORIGINAL RESEARCH

Antimicrobial and Anti-Biofilm Properties of Ma'in Hot Springs Targeting Bacteria Isolated from Diabetic Foot Ulcers

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Introduction: Diabetic foot ulcers (DFUs) are a serious complication of diabetes, which is worsened by biofilm-forming bacterial infections that can contribute to antibiotic resistance and delayed wound healing. This study explores the antimicrobial and anti-biofilm properties of Ma'in Hot Springs Water (MHSW) against *Staphylococcus aureus* and *Pseudomonas aeruginosa* which are associated with DFU.

Methods: The chemical composition of MHSW was determined using LC-MS, UV-Vis spectroscopy, and heavy metal profiling. Antimicrobial efficacy was determined through minimum inhibitory concentration (MIC) determination, bacterial growth kinetics, and biofilm inhibition assays.

Results: The results demonstrated a dose-dependent antibacterial effect. Biofilm formation, exopolysaccharide production, and bacterial adhesion were reduced in treated samples. Moreover, MHSW disrupted virulence factors such as plasma coagulation and metallo-β-lactamase production. It was also found to be non-cytotoxic.

Discussion: These findings demonstrate the potential of MHSW as an alternative or adjunctive treatment for DFU infections. However, the presence of heavy metals exceeding safety limits requires further investigation to determine their optimal concentration for clinical usage.

Keywords: diabetic foot ulcers, biofilm, antimicrobial activity, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Ma'in hot springs

Introduction

Diabetic foot ulcers (DFUs) are a serious complication of diabetes mellitus. They contribute significantly to global morbidity and mortality. The lifetime risk of developing a foot ulcer in individuals with diabetes is in the range of 19% and 34%. The underlying causes of DFUs involve a combination of neuropathy, trauma, and, in many cases, peripheral arterial disease. Diabetic neuropathy results in foot deformities, callus formation, and reduced sensitivity to trauma or pressure.[1]

A major challenge in the management of DFUs is the presence of biofilms produced by bacteria.[2] Biofilm formation is an important factor in the progression and chronicity of DFUs. It contributes to the development of antibiotic resistance and creates barriers to wound healing.[3] Biofilms protect bacteria from the host's immune response and antimicrobial agents[4] by forming extracellular polymeric substances (EPS), which create a stable environment for bacterial survival, making it difficult to rid the wound of biofilm-producing bacteria. This protective layer allows bacteria to thrive in nutrient-rich conditions leading to chronic inflammatory responses that make the infection worse.[5] Effective treatment strategies that target these microbial communities can prevent disease progression and, in severe cases, prevent serious outcomes such as amputation or death.[2]

Diabetic foot ulcers are colonized or infected by a polymicrobial community consisting of both Gram-positive and Gram-negative bacteria. Meta-analysis of hundreds of clinical samples globally shows that *Staphylococcus aureus* is the most frequently isolated organism, followed by *Pseudomonas spp*. and *Escherichia coli* as well as *Proteus* and

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Enterococcus species.[6] Biofilms are estimated to be present in up to 60–80% of non-healing wounds.[7] Among biofilm producers, *S. aureus* and *P. aeruginosa* are prevalent due to their extracellular matrix production, toxin release, and enhanced drug resistance.[8]

Recent research demonstrates the potential of utilizing natural and renewable resources, especially in the discovery of antimicrobial compounds.[9] Hot springs and volcanic environments are excellent sources of novel natural products, such as alkaloids, polyketides, and peptides. These ecosystems harbor microorganisms that have evolved special mechanisms to survive in extreme conditions. This results in the biosynthesis of compounds with different chemical properties and biological activities.[10]

The Ma'in hot springs, which are located approximately 58 kilometers south of Amman, Jordan, are a valuable yet underexplored resource. It is situated 150 meters below sea level and the water in these springs ranges in temperature from 42–63°C. Studies have demonstrated that Ma'in hot springs water (MHSW) possesses antimicrobial activity against certain Gram-positive bacteria. This activity is due to its unique chemical composition, which includes high concentrations of inorganic compounds such as chloride and sulfide, as well as heavy metals like cadmium (Cd), chromium (Cr), nickel (Ni), and manganese (Mn).[11] Moreover, sediment sampling found culturable antibiotic-producing Actinobacteria, including taxa related to Nocardiopsis, Streptomyces, and Nocardioides. This shows Ma'in's value for bioprospecting and the need for further chemical characterization of bioactive compounds.[12]

Harnessing the antimicrobial and anti-biofilm properties of MHSW is an effective approach to managing bacterial infections in DFUs, especially those complicated by the formation of biofilms. Therefore, this study aims to explore the antimicrobial and anti-biofilm potential of MHSW, which remains largely under-investigated despite its unique chemical composition and biological activity. This research will investigate the efficacy of MHSW against bacterial biofilms associated with DFUs which might provide a novel and natural approach to treating these challenging infections.

Methods and Materials

Sampling of MHSW

MHSW samples were collected from Ma'in hot springs located in Ma'in, Jordan (N 35–36-36, E 31–36-40) during April 2024. The in situ temperature of water was 59°C and the in situ pH was 7.8. Water was collected in sterile glass bottles, sealed with molten wax, and transported to the laboratory for further analysis. Water was collected using all sterile precautions and stored in a sterile glass bottle (250 mL). Immediately after collection samples were placed in an insulated cooler with ice packs (maintained at 4°C) and transported to the laboratory (time from collection to laboratory receipt <6 hours). Upon arrival samples were stored at 4°C and processed within 24 hours for chemical and microbiological analyses.

MHSW Preparation for Experiments

Water samples were sterilized using a 0.2 μm pore size filter under vacuum. Subsequently, 5 mL of MHSW was inoculated onto Mueller–Hinton agar (Hi-Media Labs, India) and incubated at 37°C for 24 hours to check for microbial contamination. The inoculum was carefully spread across the agar surface to ensure uniform distribution rather than absorption into the medium. Each assay was conducted in triplicate. To account for the hot spring origin of the water, additional plates were incubated at elevated temperatures (45°C and 55°C) to monitor the growth of thermotolerant microorganisms. Moreover, selected plates were incubated for extended periods (up to 72 hours) to detect any slow-growing microbial contaminants. The absence of bacterial growth under all tested conditions confirmed the sterility of the filtered water, making it suitable for subsequent antimicrobial and anti-biofilm assays.

Mineralogical and Chemical Analyses

Liquid Chromatography-Mass Spectrometry

LC-MS analysis was conducted based on the method provided by Zhang et al[13] with slight modifications. LC-MS analysis was performed using an Orbitrap Exploris 240 mass spectrometer (Thermo Fisher Scientific Inc) equipped with an atmospheric pressure chemical ionization (APCI) source in positive ion mode. Key settings included a capillary

temperature of 155°C, an APCI vaporizer temperature of 400°C, a corona discharge current of 8 μA, a sheath gas flow set to 40, and an auxiliary gas flow at 10 (arbitrary units). The data-dependent analysis involved multiple scan events: MS1 (full mass spectrum, m/z 100–1250), followed by MS2 analysis of the most intense ion from MS1, MS3 analysis of the most intense ion from MS2, and so on up to MS12. Detection was performed with an isolation width of m/z 5.0, a collisional dissociation energy of 35%, and an activation Q value of 0.15. Compound structures were identified by comparison with authentic standards.

UV-Visible Spectra

For UV-Vis measurements, we used the protocol devised by Giannakopoulos et al.[14] UV-Vis measurements were performed using a Hitachi U-1900 single-beam UV/Vis spectrophotometer, scanning wavelengths from 200 to 1000 nm at a speed of 800 nm/min. Samples were filtered through prewashed 0.45 μm Whatman fiber filters and adjusted to pH 7.0 to account for variations in light absorption caused by protonation states of organic acids.[15]

Heavy Metals Analysis

Metal concentrations in water samples were accurately measured using a multi-standard calibration method. Calibration standards included a 10 mg/mL multi-element solution with 29 metals (ICP-MS Standard, 5% HNO_3), a 10 mg/mL mercury standard (5% HNO_3), and a 10 mg/mL rare-metal standard (5% HNO_3), all from PerkinElmer.[16]

Inoculum Preparation

Two standard bacterial strains *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) along with 10 pre-characterised MDR and biofilm-positive strains from laboratory stock (5 *S. aureus* and 5 *P. aeruginosa*) were used in this study. These stock strains were originally isolated from diabetic foot ulcer patients at the University of Tabuk, Saudi Arabia and preserved for research purposes. Of the 5 *S. aureus* (SA) strains, all were methicillin-resistant (MRSA; oxacillin MIC > 4 mg/l) and all 5 *P. aeruginosa* (PA) were MBL positive. Each bacterial strain taken from the stock cultures was streaked on the Nutrient agar plate (incubated at 37°C for 24 hours). The bacterial suspension was prepared by suspending three bacterial colonies from each plate in sterile 0.9% sodium chloride to acquire 0.5 McFarland scale (10^8 CFU per mL) as inoculums to determine the MIC value.

Antibacterial Susceptibility Assay

Kirby Bauer disk diffusion method was used for susceptibility test as per Clinical and Laboratory Standards Institute guideline on Mueller–Hinton agar.[17] The following antibiotics were used in this study: Amikacin (30 μg), Ceftazidime (30 μg), Cefepime (30 μg), Levofloxacin (5 μg), Tobramycin (10 μg), Piperacillin (100 μg), Imipenem (10 μg), Cefoperazone (75 μg), Cefoperazone/Sulbactam (75/10 μg), Cefotaxime (30 μg), Cefotaxime/Clavulanic Acid (30/10 μg), Piperacillin/Tazobactam (100/10 μg), Cefepime/Clavulanic Acid (30/10 μg), Sparfloxacin (5 μg), Erythromycin (15 μg), Gentamicin (10 μg), Oxacillin (1 μg), Ciprofloxacin (5 μg), Cefoxitin (30 μg), and Vancomycin (30 μg) [Hi-Media Labs, India]. Interpretation of result as suggested by the manufacturers recommendation (Hi-Media labs, Mumbai, India). To check its efficacy, we ran the MLB and Biofilm test again as described by Baniya et al.[18]

Colony Numbers of S. aureus and P. aeruginosa

The *S. aureus* cells were washed three times with sterile saline solution. This involved suspending the cells and then centrifuging them at 6000 × g for 10 minutes at 4°C to remove any acidic hot-spring water or hydrochloric acid. After washing, the bacteria were resuspended in 1 mL of sterile saline solution. The bacterial count was determined by plating on soybean-casein digest agar with lecithin and polysorbate 80, using the ten-fold dilution method.

Biofilm Formation

Formation was quantified following previously established protocols with slight modifications[19,20] using 96-well flat-bottom plates. An overnight culture was used to inoculate 10 mL trypticase soy broth (TSB) + 1% glucose and incubated for 24 h at 37°C, then standardized by measuring OD600 and adjusting each culture to an OD600 of 0.5, corresponding to 10^8 CFU/mL.

Standardized cultures were then diluted 1:100 in fresh medium. About 200 µL of the diluted culture were added to individual wells of a flat-bottom 96-well plate. Sterile broth served as blank and control strains were processed similarly. After a 24 h incubation at 37°C the wells were gently tapped and washed four times with PBS (pH 7.2) to remove planktonic cells. Adherent biofilm was fixed with 2% sodium acetate, stained with 0.1% crystal violet, rinsed, dried, and the bound dye was quantified by measuring OD at 590 nm. All experiments were done in duplicate and blank OD averages were subtracted from test values. Based on OD590 readings, biofilms were classified as weak (0.1 to ≤0.400), moderate (>0.400), or strong (>0.800).

Minimum Inhibitory Concentration (MIC) Activity of MHSW

MIC testing was performed by broth microdilution in sterile flat-bottom 96-well plates in accordance with Clinical and Laboratory Standards Institute guidelines.[21] Mueller–Hinton broth (MHB) was used to prepare the test series of hot-spring water (MHSW) at 80, 70, 60, 50, 40, 30, 20 and 10% (v/v). About 200 µL of each concentration of MHSW (20–25°C) was dispensed per well in technical duplicate. Bacterial inocula (5 *S. aureus* and 5 *P. aeruginosa* isolates from laboratory stock) were prepared from 18–24 h non-selective agar cultures, adjusted to 0.5 McFarland and diluted in MHB so that the addition of 20 µL inoculum to each well produced a final inoculum of approximately 5×10^5 CFU/mL (final volume 220 µL). Each plate contained a growth control (MHB + inoculum, no MHSW), a sterility control (MHB only) and quality-control strains (*P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923) processed identically. Baseline optical density at 620 nm (OD620) was recorded immediately after inoculation. Plates were incubated in ambient air at 35 ± 2°C and re-read at 20–24 h. Then, the optical density values of the wells taken initially were subtracted from the final values recorded after 24 hours. By finding out the lowest concentration of the hot spring water which had a lower optical density value than the optical density of the control, the MIC value was determined.

Effect of Sub-MIC of MHSW on the Growth Rate and CFU of Standard Strains

To determine the growth rate and CFU count of standard strains, we used the methodology of Mira et al[22] with slight modifications. Overnight cultures of *P. aeruginosa* and *S. aureus* were grown at 37°C in 10 mL tryptic soy broth (TSB) in sealed 15 mL tubes without aeration for 16–18 h. Cultures were pooled, centrifuged at 4,000 rpm for 15 min at 4°C, and resuspended in 4 mL M9 buffer to obtain a 4X concentrated bacterial stock, corresponding to 2.5×10^8-2.5×10^9 CFU/mL. This stock was diluted into fresh TSB containing MHSW at 0.25×, 0.5×, and 0.75× MIC, with a TSB-only culture as control, and incubated at 37°C for 24 h. After incubation, cultures were serially diluted in M9 buffer (tenfold dilutions, up to 10^{-8}), and appropriate dilutions were spread-plated on TSB agar to obtain countable colonies. Plates were incubated overnight at 37°C, and CFU/mL was calculated and expressed as $\log_{10}$ CFU/mL.

In parallel, 15 two-fold serial dilutions of each treated culture were prepared in M9 and dispensed into 96-well plates (4 replicates, 200 µL/well) and 384-well plates (6 replicates, 80 µL/well). Blank wells (M9 only) were included as negative controls. OD_{600} was then measured every 5 min for 30 min using a Biotek Epoch 2 plate reader, and values were corrected by subtracting the media background.

Plasma Coagulation by S. aureus

For plasma coagulation assay, protocol devised by Katz[23] and Rakotovao-Ravahatra et al[24] was used with slight modifications. Two *S. aureus* strains (SA-1 and SA-2) were cultured in 5 mL of brain heart infusion broth at 37°C for 24 hours with agitation. After incubation, the cultured supernatants were harvested by centrifugation at 500 × g for 30 minutes at room temperature. Cell suspensions of the *S. aureus* strains containing $2.3–8.6 \times 10^7$ CFU and the cultured supernatants were then inoculated into 0.45 mL of rabbit plasma supplemented with sub-MIC of MHSW and 0.01 M phosphate-buffered saline in microcentrifuge tubes. Plasma coagulation was evaluated at 4 hours and reconfirmed at 24 hours of incubation at 37°C.

Observation of Metallo-Beta-Lactamase Detection by P. aeruginosa

The reconfirmation of Metallo-β-lactamase (MBL) was conducted using the Imipenem E-strip impregnated with/without EDTA. Initially, the test organism was inoculated onto Mueller–Hinton agar plates, with the opacity adjusted to match 0.5 McFarland standards (1.5×10^8 CFU/mL).

Effect of MHSW MIC on Biofilms Formation

Effect of MHSW on Mono Species Biofilm

For this test, isolates used were *Pseudomonas aeruginosa* (PA3, PA5) and *Staphylococcus aureus* (SA1, SA2). Bacteria in microtiter plates were treated with MSHW at concentrations of 0.25x, 0.5x, and 0.75x of the MIC. The mixtures were incubated at 37°C for 24 h. Biofilm inhibition was quantified. Positive controls lacked additives, while negative controls contained only TSB. After incubation, plates were rinsed with PBS (pH 7.4), stained with 0.1% crystal violet for 30 min, and excess dye was removed. The stain was solubilized in 95% ethanol, and biofilm OD was measured at 590 nm.[25]

Effect of MHSW on Mixed Species Biofilm

Mixed assays used two defined dual-species combinations: PA3 + SA1 and PA5 + SA2. This inhibition assay followed the same initial steps as the mono-species method. Mixed biofilm production was assessed as described by Zubair et al[25] and Zhang et al.[26] Overnight cultures of mixed strains (PA3 + SA1, PA5 + SA2) were diluted to 1×10^6 CFU/mL and combined equally (1:1). A 100 µL mixed suspension and 100 µL of TSB with MHSW (0.25x, 0.5x, and 0.75x) were added to 96-well plates. After 24 h incubation at 37°C, plates were rinsed with PBS, stained with 0.1% crystal violet for 30 min, washed, and solubilized in 95% ethanol. Biofilm OD was measured at 590 nm.

MIC Effect on Exopolysaccharides (EPS)

EPS was extracted from cell-free supernatants of test strains treated with MHSW (0.25×, 0.5×, and 0.75× MIC) and untreated controls by adding three volumes of chilled ethanol and incubating at 4°C for 16–18 h. For *P. aeruginosa*, EPS was quantified using the Dubois phenol-sulfuric acid method with a glucose standard curve (0–100 µg/mL). Swarming motility was assessed by point-inoculating strains at the center of LB agar plates (with or without MHSW at MIC) using sterile toothpicks. Plates without MHSW served as controls. After incubation at 37°C for 24 h, the swarm zone diameter was measured.

For *S. aureus*, EPS quantification followed the phenol-sulfuric acid method with minor modifications.[27] Overnight cultures were centrifuged (8000 rpm, 30 min, 20°C), and the supernatant was mixed with three volumes of chilled ethanol and incubated overnight at −20°C. EPS pellets were recovered (7000 rpm, 30 min, 5°C), resuspended in MilliQ water, and reacted with 80% phenol and concentrated H_2SO_4. After incubation at 25°C for 10–20 min, absorbance was measured at 490 nm, and EPS concentration was calculated from a glucose standard curve (Y = 0.1546X - 0.0067), expressed in µg/mL.

MIC Effect on Alginate

Bacterial strains were added to nutrient broth amended with or without sub-MICs of MHSW and incubated overnight at 37°C. The amount of alginate produced was measured by the addition of boric acid-sulfuric solution (4:1) to test bacteria placed in an ice bath. To this mixture, 0.2% carbazole solution prepared in ethanol was added and incubated at 55°C after vortexing for 10 s. Quantification was done at 530 nm.[28]

MIC Effect on Cell Surface Hydrophobicity (CSH)

The microbial adhesion to hydrocarbons assay assessed the cell surface hydrophobicity of the test strains, both treated and untreated. Briefly, overnight cultures of the bacterial strains (1 mL) were added to microcentrifuge tubes containing 100 µL xylene and MHSW sub-inhibitory concentrations. Untreated bacterial cultures served as control groups. The samples underwent vigorous vortexing for 2 minutes, followed by a room temperature incubation for 10-minute to facilitate phase separation. The absorbance of the aqueous phase was then measured at 530 nm, and the percentage of hydrophobicity was calculated using the formula: %Hydrophobicity = (1 − OD after vortexing/OD before vortexing) × 100. This quantitative analysis provided insights into the changes in microbial surface properties under the experimental conditions.[19]

MIC Effect on Disruption of Preformed Biofilm

Biofilms were first allowed to form by incubating cultures in microtiter plates for 24 h at 37°C. Following incubation, non-adherent cells were removed through a washing step. Fresh growth medium, either containing or lacking 0.5 times

the minimum inhibitory concentration of MHSW, was then added to each well and incubated for an additional 24 hours at 37°C. After this period, any remaining non-adherent cells were washed away, and the adherent cells, representing the biofilm, were stained with crystal violet. Subsequent quantification of the biofilm biomass was performed by measuring the absorbance at 585 nm, as per the previously established protocol.[19]

Effect of MHSW on Immature Biofilms

Two *Staphylococcus aureus* isolates (SA-1 and SA-2) were cultivated, with inocula adjusted to 3.4×10^8 CFU/mL and 5.1×10^8 CFU/mL, respectively. Cell suspensions were inoculated into 2.5 mL of rabbit plasma (BioRad, cat no. 56352), which was placed on 1.77 cm^2 coverslips within tissue culture dishes. After 24 h of incubation at 37°C, early biofilm structures (immature biofilms) had formed on the coverslips. The coverslips were gently washed five times with 1 mL of sterile saline, then placed in fresh rabbit plasma either alone (control) or supplemented with MHSW (60%, pH 5.4) or 1 N HCl. After another 24 h incubation at 37°C, attached cells were quantified. Coverslips were washed with sterile saline, and adherent cells were detached by sonication (60% power, 45 s, 4°C). The suspensions were serially diluted, plated on tryptic soy agar, incubated at 37°C for 24 h, and colonies enumerated manually. Results were expressed as CFU/mL. Plasma coagulation was observed concurrently.

Inhibitory Effect of Sub-MIC on Attachment

Cell suspensions of test strains containing 0.5 McFarland suspension were inoculated into 2.5 mL of rabbit plasma either alone (control) or supplemented with Sub-MIC on coverslips in tissue culture dishes (n = 5). After incubation for 24 h at 37°C, the number of cells attached to each coverslip was determined.[29]

Cytotoxicity Tests

Cell Viability Assay

The biocompatibility of MHSW was evaluated using an MTT assay on L929 murine fibroblasts (Sigma Aldrich) and human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors by Ficoll gradient centrifugation. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 100 IU/mL penicillin streptomycin at 37°C in a humidified 5% CO_2 incubator and used in the exponential growth phase. For the assay, 1.0×10^4 cells per well were seeded in 100 µL complete medium in 96 well plates and incubated 24 h to allow attachment. MHSW was prepared in complete medium at concentrations corresponding to 2× MIC, 1× MIC, 0.75× MIC, 0.5× MIC and 0.25× MIC, and 100 µL of each concentration was added to wells while negative control wells received medium alone; plates were incubated 24 h at 37°C, 5% CO_2. After treatment, wells were gently washed once with PBS (for non-adherent PBMCs, wash carefully to avoid cell loss), and MTT reagent was prepared and added by combining 10 µL of 0.25 mg/mL MTT with 90 µL fresh medium per well; plates were incubated 3–4 h at 37°C, protected from light, until formazan crystals formed. The medium was then removed and 100 µL isopropanol was added to each well to dissolve the formazan with gentle shaking, and absorbance was measured at 570 nm with a reference at 690 nm. Viability for each treatment was calculated as [(Abs_treatment − Abs_blank)/(Abs_negative_control − Abs_blank)] × 100, setting the negative control as 100% viability; all conditions were performed in triplicate, and results are reported in percentage.[30]

Haemolyis Assay

Haemo-compatibility was assessed following ISO 10993-4 guidelines[31] using the protocol devised by Sæbø et al[32] with slight modifications. Fresh human erythrocytes (270 µL) were incubated in 96-well plates with MHSW at 2× MIC, 1× MIC, 0.75× MIC, 0.5× MIC and 0.25× MIC in RPMI (final volume 300 µL). Wells without MHSW were used as controls. After incubation for 1 h at 37°C and 5% CO_2, plates were centrifuged at 3,000 rpm for 5 min. The supernatants were transferred to new 96-well plates, and the absorbance at 541 nm was measured using a UV-visible spectrophotometer (Cary 60 UV-Vis spectrophotometer). All conditions were performed in triplicate and results reported in percentage.

Statistical Analysis

All experiments were performed in triplicates and the data obtained from experiments were presented as mean values. Differences between control and treatment groups were analyzed using one-way ANOVA followed by Dunnett's multiple-comparisons test to compare each treatment to the control.

Results

Mineralogical and Chemical Analyses

The mass spectrometric profile (Supplementary Figure 1a–m) revealed a stable recurrent fragment at m/z 114.0928 (with related ions at m/z 246.2471 and 136.1138), which might represent the organic ligand core. In contrast, the higher-m/z signals (m/z 354.3251, 453.3511, 679.5243, and 758.5837) are consistent with metal-ligand adducts or multi-ligand complexes. These assignments are supported by the UV-Vis spectrum (Figure 1), which displayed strong absorptions in the UV range (200–400 nm) and weaker features in the visible region. The intense UV bands are compatible with ligand-to-metal charge transfer involving the most abundant metals detected in the water (Cr and Fe), while the weaker visible absorptions may reflect d-d transitions of Cu or Ni complexes. By contrast, Zn and Cd are expected to influence primarily the ligand-centred UV transitions. Taken together, these findings suggest that the MS peaks represent a combination of organic ligand fragments and their coordination complexes with transition metals present in the sample.

The analysis of Ma'in hot spring water using ICP-MS reveals the presence of various heavy metals in different concentrations (PPM ± SE) (Table 1). Chromium (Cr) shows the highest concentration at 0.734 ± 0.047 ppm, followed by Iron (Fe) at 0.201 ± 0.043 ppm, and Manganese (Mn) at 0.197 ± 0.014 ppm. Zinc (Zn) and Copper (Cu) are present at 0.097 ± 0.017 ppm and 0.089 ± 0.019 ppm, respectively. Nickel (Ni) is detected at 0.067 ± 0.019 ppm, whereas Cadmium (Cd) and Lead (Pb) are present in much lower concentrations of 0.031 ± 0.001 ppm and 0.001 ± 0.0001 ppm, respectively.

Table 2 compares the concentrations of heavy metals in MSHW from our study with international standards and Ma'in water as reported elsewhere.[11] Cadmium (0.031 ppm) exceeds the limits determined by WHO, Europe, Canada, Australia, New Zealand, Japan, and the USA. Chromium (0.734 ppm) is significantly higher than the standard limit of 0.05 ppm in all guidelines set by WHO and countries. Copper (0.089 ppm) is below all standard levels, whereas Iron (0.201 ppm) is within or close to most limits except for New Zealand's stricter guidelines (0.01 ppm). Manganese (0.197 ppm) is below WHO's limit of 0.5 ppm but exceeds Europe and Canada's 0.05 ppm limit. Nickel (0.067 ppm) is above the 0.02 ppm limit in most guidelines. Lead (0.001 ppm) is below all standards of WHO and other countries, however, Zinc (0.097 ppm) is below its permissible levels, which range from 1 to 5 ppm depending on the country.

Antibacterial Activity

The antibiotic sensitivity of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 demonstrated extreme variability. Resistance for Aztreonam was detected in the *P. aeruginosa* isolates PA2: 13 mm and PA3: 10 mm, while resistance was found with Imipenem for PA5: 10 mm, and wide variability in Piperacillin ranging from resistance (PA1: 8 mm) to strong sensitivity (PA4: 32 mm). Amikacin demonstrated consistent sensitivity across *P. aeruginosa* isolates, with inhibition

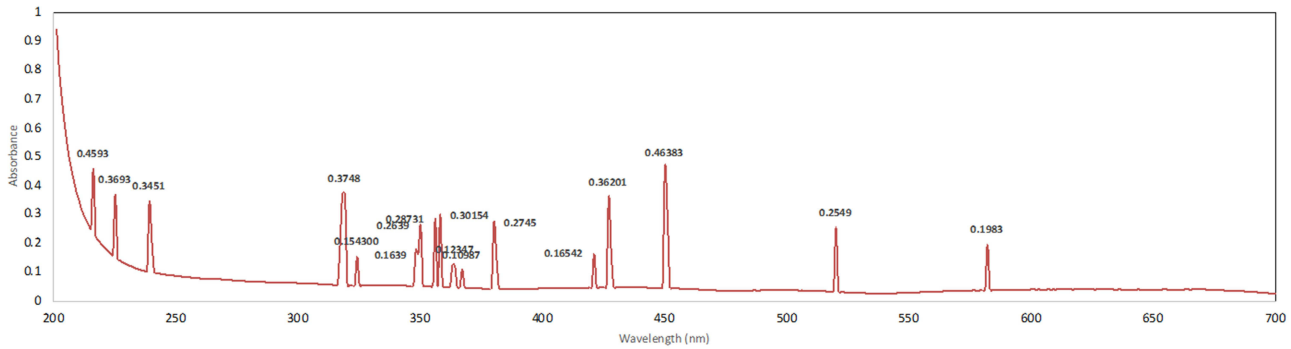

Figure 1 UV-visible spectra of MHSW.

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Table 1 Concentrations of Heavy Metals in Ma'in Hot Spring Water

Heavy Metals	Concentration (ppm) ± SE
Cadmium (Cd)	0.031 ± 0.001
Chromium (Cr)	0.734 ± 0.047
Copper (Cu)	0.089 ± 0.019
Iron (Fe)	0.201 ± 0.043
Manganese (Mn)	0.197 ± 0.014
Nickel (Ni)	0.067 ± 0.019
Lead (Pb)	0.001 ± 0.0001
Zink (Zn)	0.097 ± 0.017

Table 2 Comparison of Concentrations of Heavy Metals in MSHW from Our Study with International Standards as Reported Elsewhere

Heavy Metals	This Study	WHO	Europe	Canada	Australia	New Zealand	Japan	USA	Ma'in Water
Cadmium (Cd)	0.031	0.003	0.005	0.005	0.002	0.003	0.01	0.005	0.023
Chromium (Cr)	0.734	0.05	0.05	0.05		0.05	0.05	0.05	0.571
Copper(Cu)	0.089	2	2	2	1	2	1	1.3	0.07
Iron(Fe)	0.201		0.2	0.2	0.3	0.01	0.3	0.3	0.124
Manganese (Mn)	0.197	0.5	0.05	0.05	0.5	0.5	0.05	0.05	0.169
Nickel(Ni)	0.067	0.02	0.02	0.02	0.02	0.02			0.058
Lead(Pb)	0.001	0.01	0.01	0.01		0.01	0.01	0	0
Zink(Zn)	0.097	3			3		1	5	0.095

Notes: Taken from: Shakhatreh et al.[11]

zones of 21–31 mm, making it a reliable treatment option. For *S. aureus*, azithromycin, and erythromycin exhibited variable efficacy, with inhibition zones ranging from 19 mm (SA1) to 33 mm (SA2) for azithromycin, and from 7 mm (SA1) to 22 mm (SA5) for erythromycin. Ampicillin showed the largest inhibition zones (32–35 mm) across all *S. aureus* isolates. Ciprofloxacin and levofloxacin also proved effective with inhibition zones between 10–38 mm. Amikacin and ampicillin seem to be effective drugs against *P. aeruginosa* and *S. aureus*, respectively. However, variability in the susceptibility to other drugs emphasizes the necessity of conducting isolate-specific susceptibility testing (Table 3).

Minimum Inhibitory Concentration (MIC) Activity of MHSW

Table 4 shows the minimum inhibitory concentration (MIC) activity of MHSW against various strains of *P. aeruginosa* (PA1-PA5) and *S. aureus* (SA1-SA5). At lower concentrations (10–20%), all strains showed growth (G). At 30%, PA2 showed no growth (NG), however, all the other strains continued to grow. At 40%, growth inhibition was observed in PA2, PA4, SA3, and SA4. Complete inhibition of all strains occurred at 50% concentration and above ie 50–80%. Based on these results, an MIC of 50% MHSW was determined for both *P. aeruginosa* and *S. aureus* and was used in the following experiments.

Table 3 Antibiotic Sensitivity of *S. aureus* and *P. aeruginosa* Strains

	S, I, R (mm)	PA1	PA2	PA3	PA4	PA5	S, I, R (mm)	SA1	SA2	SA3	SA4	SA5
Aztreonam: AT	22, 16–21, 15	28	**13**	**10**	25	19	ND	ND	ND	ND	ND	ND
Imipenem: IMP	19, 16–18, 15	21	26	**12**	18	**10**	ND	ND	ND	ND	ND	ND
Piperacillin: PI	22, 18–21, 17	**8**	17	21	32	26	ND	ND	ND	ND	ND	ND
Amikacin: AK	18, 15–17, 14	21	26	31	25	28	17, 15–16, 14	21	**13**	20	28	18
Ceftriaxone: CTR	21, 14–20, 13	17	**9**	21	27	28	21, 14–20, 13	21	18	**12**	**10**	20
Ciprofloxacin: CIP	25, 19–24, 18	22	27	19	**14**	18	21, 16–20, 15	**10**	28	35	19	22
Levofloxacin: LE	22, 15–21, 14	17	23	**10**	16	22	19, 16–18, 15	21	**10**	38	35	29
Norfloxacin: NX	17, 13–16, 12	24	21	**11**	19	**8**	17, 13–16, 12	28	34	16	**12**	27
Ofloxacin: OF	16, 13–15, 12	17	25	33	28	**10**	18, 15–17, 14	**8**	18	32	37	27
Ampicillin: AMP	ND	ND	ND	ND	ND	ND	29, –, 28)	32	**25**	35	34	30
Azithromycin: AZM	ND	ND	ND	ND	ND	ND	18, 14–17, 13	19	33	27	32	**12**
Erythromycin: E	ND	ND	ND	ND	ND	ND	23, 14–22, 13	**7**	16	18	21	22

Notes: (Green color indicates resistance value). S,I,R: zone size of standard as per manufacturers guideline and CLSI.
Abbreviations: PA, *P. aeruginosa*; SA, *S. aureus*; AK, Amikacin 30mcg; AT, Aztreonam 30mcg; IMP, Imipenem 10mcg; PI, Piperacillin 100 mcg; CTR, Ceftriaxone 30mcg; CIP, Ciprofloxacin 5mcg; LE, Levofloxacin 5mcg; NX, Norfloxacin 10mcg; AMP, Ampicillin 10mcg; AZM, Azithromycin 15mcg; OF, Ofloxacin 5mcg; E, Erythromycin 15mcg; S, sensitive (mm); I, Intermediate (mm); R, Resistant (mm); ND, Not Done.

Table 4 Minimum Inhibitory Concentration (MIC) Activity of MHSW

MHSW Conc.	PA1	PA2	PA3	PA4	PA5	SA1	SA2	SA3	SA4	SA5
10%	G	G	G	G	G	G	G	G	G	G
20%	G	G	G	G	G	G	G	G	G	G
30%	G	NG	G	G	G	G	G	G	G	G
40%	G	NG	G	NG	G	G	G	NG	NG	G
50%	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
60%	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
70%	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
80%	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

Abbreviations: G, Growth; NG, No growth.

Biofilm Formation Assay

Table 5 shows the biofilm formation abilities of *P. aeruginosa* and *S. aureus* strains. Among *P. aeruginosa*, PA3 and PA5 were identified as strong biofilm producers, PA1 showed intermediate biofilm formation, PA2 was weak, and PA4 was negative for biofilm production. For *S. aureus*, SA1 and SA2 were strong biofilm producers, SA5 was weak, and SA3 and SA4 were negative for biofilm production. Based on these results, the strong biofilm producers (PA3, PA5, SA1, and SA2) were selected for further experiments.

Table 5 Biofilm Formation Assay

Biofilm	*P. aeruginosa*	*S. aureus*
Positive	PA3, PA5	SA1, SA2
Intermediate	PA1	–
Weak	PA2	SA5
Negative	PA4	SA3, SA4

Abbreviations: *PA, P. aeruginosa; SA, S. aureus.*

MIC of MHSW for Standard Strains

Figure 2 shows the growth patterns of *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) in the presence of Ma'in Hot Spring Water (MHSW) at various MIC fractions (0.25×, 0.5×, and 0.75×). In the control group (no MHSW), both bacteria continued to grow, with increasing OD values over time. For *S. aureus*, growth was slightly reduced at 0.25× MIC, more inhibited at 0.5× MIC, and nearly completely suppressed at 0.75× MIC (Figure 2a). Similarly, *P. aeruginosa* showed dose-dependent inhibition and almost complete suppression at 0.5× MIC and 0.75× MIC, showing a greater sensitivity to MHSW (Figure 2b).

Dose Dependent Antibacterial Assay

Table 6 shows a dose-dependent antibacterial effect of MHSW against *S. aureus* (SA-1, SA-2) and *P. aeruginosa* (PA-3, PA-5). In the control group (TSB without MHSW), bacterial growth remained high (8 log10 CFU/mL). At 0.75× MIC, a significant reduction was observed, with *P. aeruginosa* counts decreasing to 6.5–6.8 log10 CFU/mL and *S. aureus* to 6.2–6.3 log10 CFU/mL. At 0.5× MIC, inhibition was not as significant, with *P. aeruginosa* showing a greater reduction (PA-3: 7.5, PA-5: 7.0 log10 CFU/mL) compared to *S. aureus* (SA-1, SA-2: 7.8–7.9 log10 CFU/mL). However, at 0.25× MIC, bacterial counts were almost identical to the control.

Dose Dependent Bacterial Attachment Assay

Table 7 shows that MHSW reduces bacterial attachment to coverslips in a dose-dependent manner. In the control group, bacterial counts were high (7.7–9.4 log10 CFU/mL). At 0.75× MIC, there was a significant decrease in attachment, with counts dropping to 6.1–6.8 log10 CFU/mL, indicating strong antibacterial activity. At 0.5× MIC, bacterial attachment was moderately reduced (7.4–7.8 log10 CFU/mL), showing a weaker effect. At 0.25× MIC, bacterial counts were similar to the control, suggesting little to no inhibition. Overall, MHSW effectively reduces bacterial attachment at higher concentrations, with *S. aureus* showing slightly higher resistance than *P. aeruginosa*.

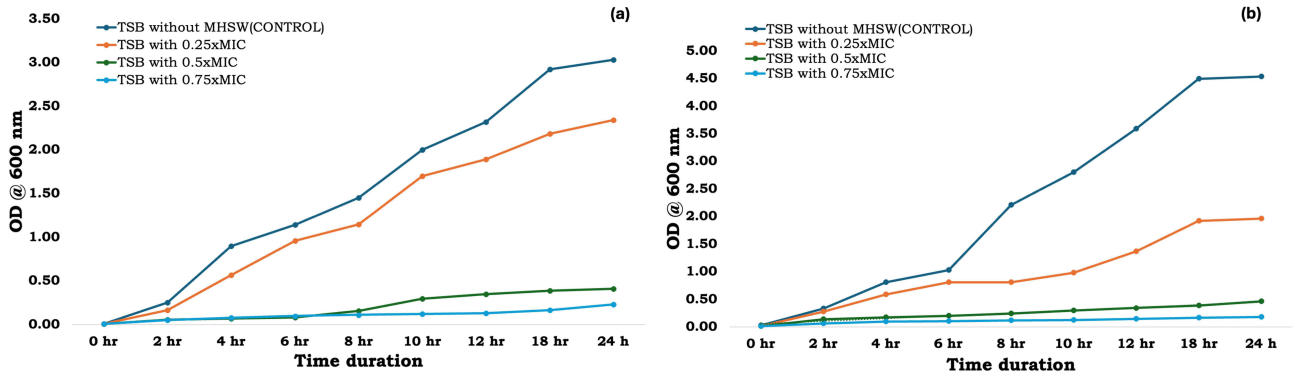

Figure 2 Growth pattern of standard (**a**) ***S. aureus*** (ATCC 25923) and (**b**) ***P. aeruginosa*** (ATCC 27853), with different MIC fractions of MHSW.

Table 6 Colony Count of *S. aureus* and *P. aeruginosa* in TSB with Variable Concentrations of MHSW (0.75× MIC, 0.5× MIC and 0.25× MIC)

Medium with Variable Conc. of MHSW	***Log10 CFU/mL (Mean ± S.D.)***			
	PA-3	**PA-5**	**SA-1**	**SA-2**
TSB without MHSW (CONTROL)	8.0 ± 0.1	7.9 ± 0.1	8.1 ± 0.1	8.2 ± 0.1
TSB with 0.75× MIC	6.5 ± 0.2	6.8 ± 0.2	6.2 ± 0.15	6.3 ± 0.15
TSB with 0.5× MIC	7.5 ± 0.1	7.0 ± 0.1	7.8 ± 0.1	7.9 ± 0.1
TSB with 0.25× MIC	8.0 ± 0.05	7.95 ± 0.05	8.1 ± 0.05	8.2 ± 0.05

Abbreviations: *PA, P. aeruginosa; SA, S. aureus.*

Table 7 Colony Counts of Strains That Attached on Coverslips After Incubation for 24 h (n = 5) with Variable Concentrations of MHSW (0.75× MIC, 0.5× MIC, and 0.25× MIC)

Medium with Variable Conc. of MHSW	***Log10 CFU/mL (Mean ± S.D.)***			
	PA-3	**PA-5**	**SA-1**	**SA-2**
TSB without MHSW(CONTROL)	8.1 ± 0.2	7.7 ± 0.2	9.1 ± 0.15	9.4 ± 0.2
TSB with 0.75× MIC	6.1 ± 0.1	6.3 ± 0.1	6.8 ± 0.25	6.5 ± 0.15
TSB with 0.5× MIC	7.7 ± 0.2	7.4 ± 0.1	7.8 ± 0.15	7.6 ± 0.2
TSB with 0.25× MIC	8.2 ± 0.1	7.6 ± 0.1	8.4 ± 0.18	8.1 ± 0.15

Abbreviations: *PA, P. aeruginosa; SA, S. aureus.*

Plasma Coagulation by S. aureus

Table 8 shows that MHSW affects the ability of *S. aureus* and its cultured supernatant to induce plasma coagulation. At the highest concentration (0.7× MIC), coagulation was completely inhibited for both *S. aureus* cells and their supernatant. At 0.5× MIC, *S. aureus* cells from strain SA2 were able to induce coagulation and strain SA1's supernatant was also active. At the lowest concentration (0.25× MIC), both strains and their supernatants induced coagulation.

Table 8 Plasma Coagulation Induction by *S. aureus* Strains or Their Cultured Supernatant After Incubation for 24 h in Medium (Plasma) with Variable MIC of MHSW

Strain No	**Concentration of MHSW**	**Cultured Supernatant of S. aureus Cells**	**S. aureus Cells**
SA-1	0.7× MIC	Negative	Negative
	0.5× MIC	Positive	Negative
	0.25× MIC	Positive	Positive
SA-2	0.7× MIC	Negative	Negative
	0.5× MIC	Negative	Positive
	0.25× MIC	Positive	Positive

Abbreviations: *SA, S. aureus.*

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Table 9 Effect of MHSW on Metallo-Beta-Lactamase (MBL) Production in *P. aeruginosa*

	PA-3	PA-5
Without MHSW	Positive	Positive
With 1× MIC	Negative	Negative
With 0.75× MIC of MHSW	Positive	Negative
With 0.5× MIC of MHSW	Positive	Positive
With 0.25× MIC of MHSW	Positive	Positive

Abbreviations: *PA, P. aeruginosa.*

Metallo-Beta-Lactamase (MBL) in P. aeruginosa

Table 9 demonstrates that MHSW affects the production of metallo-beta-lactamase (MBL) in *P. aeruginosa*, but only at higher concentrations. Without MHSW, both strains (PA-3 and PA-5) tested positive for MBL production. When treated with 1× MIC of MHSW, MBL production was inhibited in both strains. At 0.75× MIC, PA-5 was negative, but PA-3 still produced MBL. At lower concentrations (0.5× MIC and 0.25× MIC), both strains were positive.

Effect of MHSW Sub-MIC on Biofilms Formation and Exopolysaccharides Production

All isolates and both combination treatments showed statistically significant biofilm reductions at every tested sub-MIC (Figure 3(a)). At 0.25× MIC significance varied from very strong to strong (PA3: $p < 0.0001$; SA1, SA2 and the PA3 + SA1 combination: $p < 0.001$; PA5 and the PA5 + SA2 combination: $p < 0.01$). At 0.50× and 0.75× MIC the effects were uniformly highly significant for all isolates and both combinations (mostly $p < 0.0001$). All isolates and both combination treatments produced statistically significant inhibition of EPS at every tested sub-MIC (Figure 3(b)). At 0.25× MIC significance ranged from $p < 0.0001$ to $p < 0.01$ (with most single isolates and one combination at $p < 0.0001$ and one combination at $p < 0.001$). At both 0.50× and 0.75× MIC the reductions were highly significant ($p < 0.0001$) in all isolates and combinations.

Effect of MHSW Sub-MIC on Cell Surface Hydrophobicity and Alginate Production

Significance varied by isolate and concentration when it came to cell surface hydrophobicity (Figure 4(a)). At 0.25× MIC, all isolates and combinations reached significance ($p < 0.01$ for PA3, PA5, SA1, SA2; $p < 0.0001$ for PA3 + SA1; $p < 0.001$ for PA5 + SA2). At 0.50× MIC significance was weaker but present (PA3 $p < 0.05$; PA5 and SA1 $p < 0.01$; SA2 $p < 0.05$;

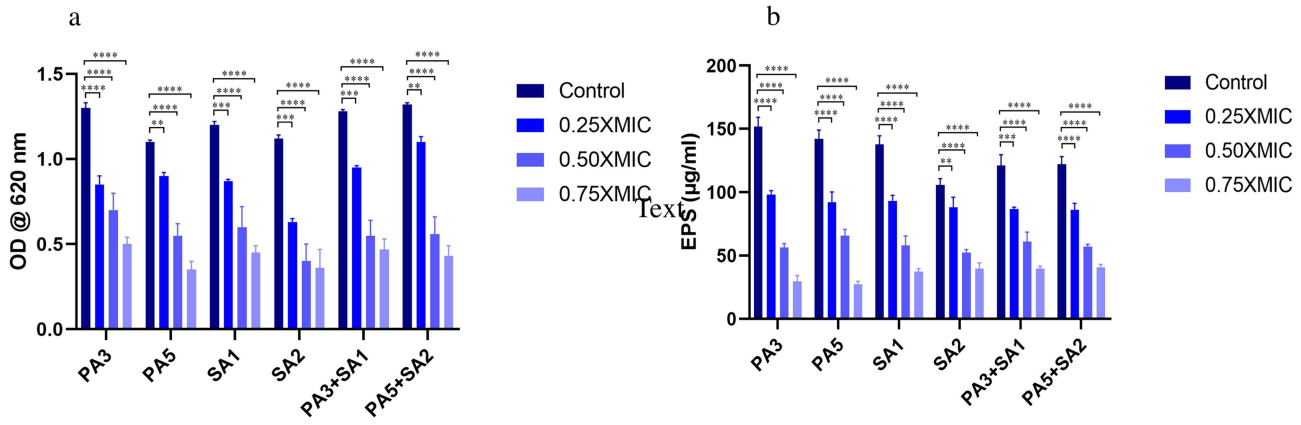

Figure 3 Effect of MHSW Sub-MIC on (**a**) Biofilms formation, (**b**) Exopolysaccharides production (Asterisks indicate levels of statistical significance: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

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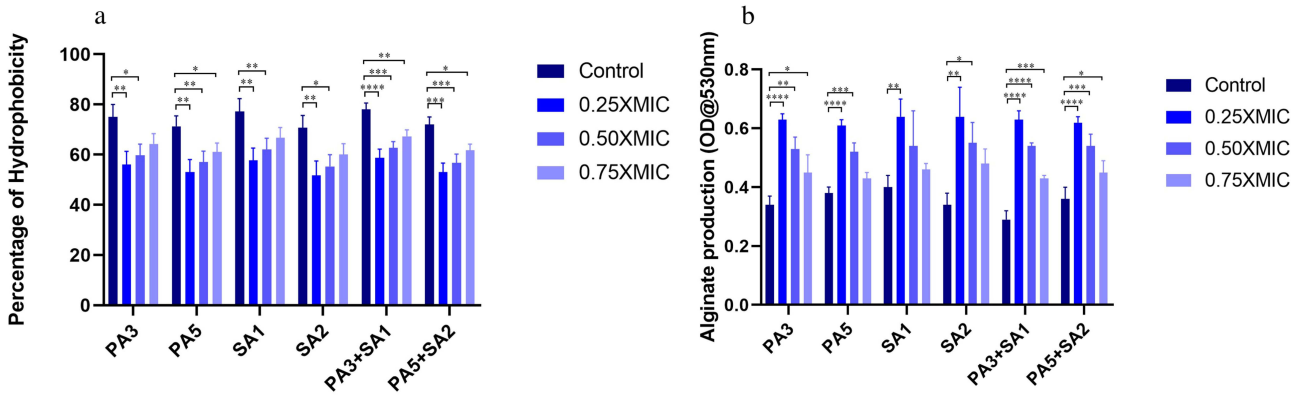

Figure 4 Effect of MHSW Sub-MIC on (**a**) Cell surface hydrophobicity, (**b**) Alginate production (Asterisks indicate levels of statistical significance: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

PA3 + SA1 and PA5 + SA2 $p < 0.001$). At 0.75× MIC single isolates were not significant for PA3, SA1 and SA2, whereas PA5 showed $p < 0.05$, and both combinations remained significant at 0.75× MIC (PA3 + SA1 $p < 0.01$; PA5 + SA2 $p < 0.05$).

Figure 4(a) shows reductions in alginate productions upon exposure to sub-MICs of MHSW. At 0.25× MIC most reductions were highly significant (PA3, PA5, PA3 + SA1 and PA5 + SA2: $p < 0.0001$; SA1 and SA2: $p < 0.01$). At 0.50× MIC significance varied (PA3 $p < 0.01$; PA5 $p < 0.001$; SA2 $p < 0.05$; PA3 + SA1 $p < 0.0001$; PA5 + SA2 $p < 0.001$; SA1 not significant). At 0.75× MIC several single isolates were not significant (PA5, SA1, SA2: not significant) while PA3 remained significant at $p < 0.05$ and both combinations were significant (PA3 + SA1 $p < 0.001$; PA5 + SA2 $p < 0.05$), indicating strongest and most consistent significance for the combination treatments across concentrations.

Effect of MHSW Sub-MIC on Preformed Biofilm Reduction and Immature Biofilm Reduction

All strains and combinations showed very strong significance in preformed biofilm reduction (Figure 5(a)). At 0.25× and 0.50× MIC all p values were 0.0001. At 0.75× MIC, the significance was slightly lower but still statistically meaningful. PA5 remained highly significant ($p < 0.0001$), PA3, SA1 and the PA3 + SA1 combination were significant at $p < 0.001$, and SA2 and the PA5 + SA2 combination were significant at $p < 0.01$.

Figure 5(a) shows how immature biofilm biomass decreased when exposed to sub-MIC levels of MHSW. At 0.25× MIC, no isolates showed significant reduction. At 0.50× MIC, all treatments significantly inhibited biofilm formation. PA3, SA2, and both combinations had $p < 0.001$, whereas PA5 and SA1 had $p < 0.01$. At 0.75× MIC, inhibition was also strong. PA3, PA5, and PA3 + SA1 showed $p < 0.001$, and SA1, SA2, and PA5 + SA2 showed $p < 0.01$.

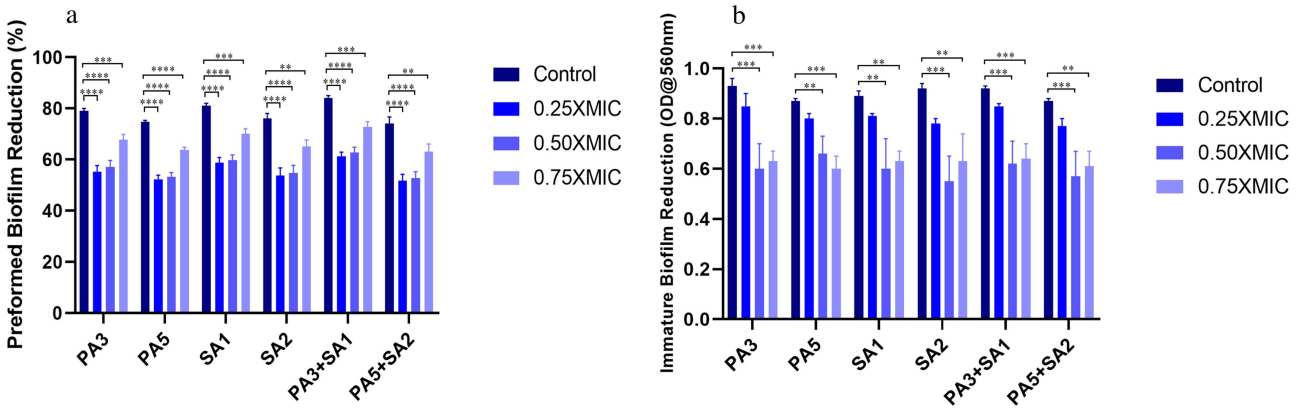

Figure 5 Effect of MHSW Sub-MIC on (**a**) Preformed biofilm reduction, (**b**) Immature biofilm reduction (Asterisks indicate levels of statistical significance: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

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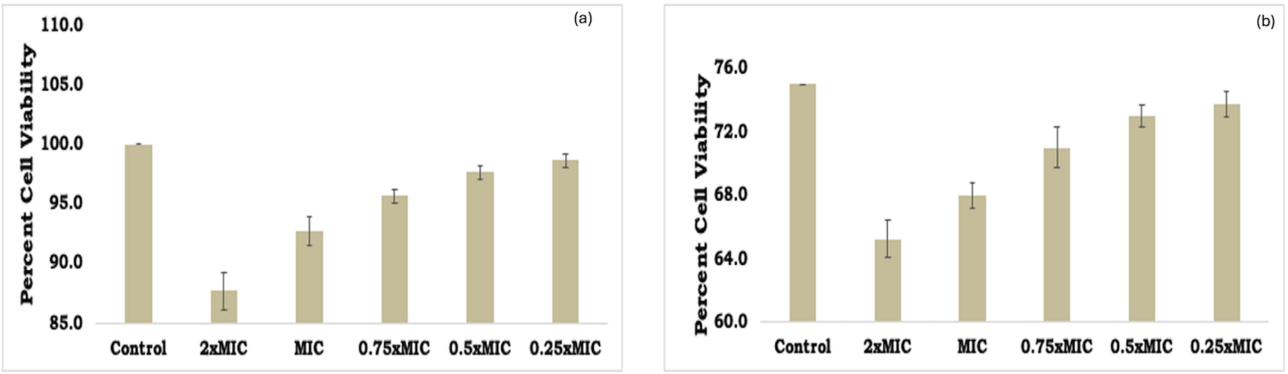

Figure 6 Percent cell viability of (**a**) L929 fibroblasts and (**b**) human PBMCs (*peripheral blood mononuclear cells*) after 24 hour exposure to MHSW at different concentrations.

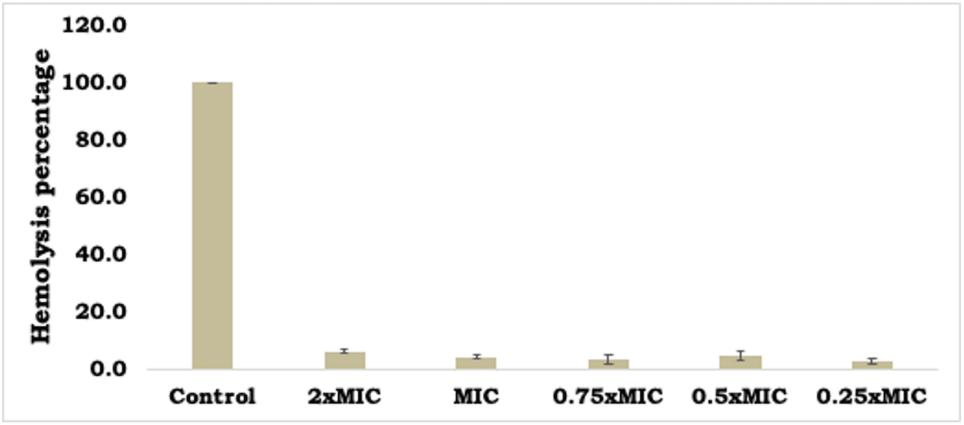

Figure 7 Percent homolysis of human erythrocytes after exposure to MHSW at different concentrations.

Cytotoxicity Tests

Cell Viability Assay

Exposure of L929 fibroblasts and human peripheral blood mononuclear cells (PBMCs) to MHSW for 24 h produced only a small, concentration-dependent reduction in cell viability (Figure 6a and b). Compared to untreated controls (100.0 ± 0.0%), the highest test concentration (2× MIC) showed the greatest decrease in viability but remained high overall (L929: 87.7 ± 2.5%; PBMCs: 87.0 ± 4.0%). At 1× MIC, viability was a little higher (L929: 92.7 ± 2.5%; PBMCs: 90.7 ± 2.6%), and values at 0.75×, 0.5× and 0.25× MIC were similar to control levels (L929: 95.7 ± 0.6%, 97.7 ± 0.6%, 98.7 ± 0.6%; PBMCs: 94.7 ± 1.5%, 97.3 ± 2.1%, 98.3 ± 1.5%, respectively).

Haemolysis Assay

Exposure of human erythrocytes to MHSW produced little hemolysis in all concentrations tested (Figure 7). At 2× MIC hemolysis averaged 6.3 ± 0.6%, at 1× MIC 4.3 ± 0.6%, at 0.75× MIC 3.7 ± 1.5%, at 0.5× MIC 4.7 ± 1.5% and at 0.25× MIC 3.0 ± 1.0%, compared with the control set at 100.0 ± 0.0%. The low percentages and small standard deviations indicate reproducible, negligible erythrocyte membrane disruption under the assay conditions.

Discussion

This study explored the antimicrobial and anti-biofilm properties of Ma'in Hot Spring Water (MHSW) against *S. aureus* and *P. aeruginosa*, two major pathogens involved in diabetic foot ulcers (DFUs).[33] The results indicate that MHSW demonstrates antibacterial activity and strong anti-biofilm effects in a dose-dependent manner.

MHSW is rich in minerals such as calcium, magnesium, terpenoids, and phenolics, as shown by LC-MS analysis. Minerals are potential sources of antimicrobial activity in hot springs.[34] Ca^{2+} and Mg^{2+} are known to disrupt model

S. aureus membranes and also kill stationary-phase *S. aureus* cells, demonstrating a membrane-targeting action.[35] Terpenes play an important role as efflux pump inhibitors which makes them excellent candidates for drug development in combating antibacterial resistance.[36] Other studies have suggested that phenolics possess quorum-sensing inhibiting properties as well as antimicrobial properties.[37]

In contrast to our study, alkaline hot springs in the Indian Himalayas, such as those in Sikkim, show a different mineral composition. The cations in these springs are found in the following order of abundance: $Ca^{2+} > Na^{+} > Mg^{2+} > K^{+}$. The dominance of bicarbonate minerals in the water is due to the weathering of silicate minerals.[38] Comparing MHSW's mineral profile to these other hot springs offers many advantages and limitations. MHSW's high calcium and magnesium content, along with terpenoids and phenolics, may contribute to its antimicrobial activity via different mechanisms.[35–37] However, the presence of heavy metals, such as chromium, could pose limitations due to potential toxicity.[39]

The UV-visible spectrum analysis shows the presence of compounds with strong electronic transitions, which may be responsible for antimicrobial activity. Studies have shown that Some transition metal complexes show greater antibacterial activity against various bacterial strains than clinically used antibiotics.[40] The detection of heavy metals such as Chromium could play a role in bacterial inhibition. Previous studies have shown hexavalent chromium exposure alters the morphology, metabolism, and growth of both Gram-positive and Gram-negative bacteria. It disrupts nucleic acid structure, cell membranes, enzyme activity, oxidative phosphorylation, lipid peroxidation, and osmotic balance, leading to cell elongation, enlargement, and inhibited division.[41] Hexavalent chromium (Cr(VI)) is easily absorbed by cells and is acknowledged for its genotoxic and cytotoxic effects. Once inside the cells, it undergoes reduction to its more stable trivalent form, chromium (Cr(III)). This trivalent state is known to interfere with cellular DNA polymerase activity and disrupt polymerase-mediated DNA replication.[42] However, the presence of heavy metals exceeding international standards raises concerns regarding the safety and therapeutic application of MHSW.

The rising threat of antibiotic resistance necessitates the exploration of the synergistic effect of MHSW with conventional antibiotics. MHSW could improve the efficacy of amikacin or ampicillin against *P. aeruginosa* and *S. aureus*, respectively. Previous studies have shown that natural products can reduce the MIC of antibiotics in both Gram-positive and Gram-negative bacteria.[43] Evaluating such combinations could offer new strategies to overcome bacterial resistance. The MIC determination experiments revealed that MHSW at 50% concentration completely inhibited bacterial growth. Lower concentrations were found to be less effective. Similar antibacterial effects of hot spring waters have been reported elsewhere, with waters showing potent antimicrobial activity due to mineral content, chemical compounds, and physical agents.[34] Other studies have shown that Ma'in hot springs water (MHSW) possess antimicrobial activity against certain Gram-positive bacteria, a property attributed to its distinctive chemical composition. The water contains elevated levels of inorganic compounds, such as chloride and sulfide, along with trace heavy metals including cadmium (Cd), chromium (Cr), nickel (Ni), and manganese (Mn).[11] Moreover, sediment samples revealed the presence of culturable, antibiotic-producing Actinobacteria, especially strains related to *Nocardiopsis, Streptomyces*, and *Nocardioides*. These findings demonstrate Ma'in's potential as a resource for bioprospecting and illucidate the need for further chemical characterization of its bioactive compounds.[12] While Shakhatreh et al[11] characterized the chemical composition of Ma'in hot springs and reported broad-spectrum antimicrobial activity and Hussein et al[12] identified antibiotic-producing Actinobacteria in sediments, the present study extends this work by evaluating MHSW against clinically relevant isolates from diabetic foot ulcers and by investigating anti-biofilm and sub-inhibitory effects.

Further experiments confirmed that MHSW inhibits bacterial growth in a dose-dependent manner. The OD measurements showed almost complete suppression at 0.75× MIC for both *S. aureus* and *P. aeruginosa* which shows that higher concentrations are necessary for more pronounced antimicrobial effects. CFU enumeration assays confirmed these findings and showed significant bacterial reductions at 0.75× MIC and moderate inhibition at 0.5× MIC. This is consistent with studies showing that heavy metals as found in hot spring waters can disrupt bacterial cell membranes and interfere with enzymatic activity.[44]

One of the most important findings is MHSW's ability to inhibit biofilm formation and reduce biofilm-related virulence factors. Strong biofilm producers (*P. aeruginosa* PA3, PA5; *S. aureus* SA1, SA2) were most affected by MHSW. Since biofilms are important for chronic infections,[45] the findings suggest that MHSW could be used for DFU management. Moreover, certain components of MHSW could aid in wound healing. For example, zinc is essential for

regulating various stages of the wound healing process, including oxidative stress management, angiogenesis, coagulation, tissue re-epithelialization, inflammation, immune defense, membrane repair, and fibrosis/scar formation.[46] However, its presence in high concentrations could also lead to cytotoxic risks.[47]

In mixed biofilms, *P. aeruginosa* and *S. aureus* interactions may influence each other's biofilm-forming abilities and response to MHSW. These interactions can be antagonistic, involving competition for nutrients and growth inhibition, or synergistic, leading to co-aggregation, metabolic cooperation, and increased resistance to antibiotics or immune responses.[48] For example, *S. aureus* might enhance *P. aeruginosa* biofilms by supplying nutrients or modulating gene expression. MHSW's antimicrobial effects could disrupt these dynamics by inhibiting both species or selectively targeting one which can influence interspecies competition or synergy.

The data indicate a reduction in biofilm formation, exopolysaccharide (EPS) production, alginate production, and cell surface hydrophobicity in a concentration-dependent manner. The reduction in exopolysaccharide (EPS) and alginate production in *P. aeruginosa* and *S. aureus* biofilms following MHSW treatment could be because of the disruption of important genetic pathways involved in biofilm formation. In *P. aeruginosa*, the *pelA* and *pslA* genes are responsible for the synthesis of extracellular polysaccharides that form the biofilm.[49] MHSW might be able to inhibit these genes which likely results in decreased EPS and alginate production ultimately weakening the biofilm structure.

MHSW also affected specific virulence factors associated with biofilm stability. Plasma coagulation by *S. aureus* was also inhibited at higher MHSW concentrations. Since clotting of plasma is a bacterial immune invasion mechanism,[50] these results suggest impairment of these mechanisms. Moreover, MHSW inhibited metallo-beta-lactamase (MBL) production in *P. aeruginosa*, a common mechanism of antibiotic resistance.[51] These findings suggest that MHSW, aside from inhibiting bacterial growth, also disrupts bacterial virulence factors. This supports its use as an alternative or adjunctive therapy for DFUs.

The cytotoxicity data indicate that MHSW is essentially non-toxic to mammalian cells. Even at the highest concentration (2× MIC), L929 fibroblasts and PBMCs retained 87% viability, and at 1× MIC and below viability was >90%. By international standards, this is within the non-cytotoxic range. ISO 10993 5 considers a >30% viability drop as cytotoxic.[52] Likewise, the hemolysis assay showed only minimal red-cell damage, far below common safety thresholds. In practice <10% hemolysis is considered acceptable,[53] and values under 10% are non-hemolytic.[54]

Previous research on hot spring waters has demonstrated their antimicrobial potential due to mineral composition and unique physicochemical properties. For instance, hot spring waters in India have demonstrated antimicrobial activity attributed to large quantities of minerals and alkaline conditions.[34] In contrast, other studies have attributed the antimicrobial activity of hot springs to acidic conditions and the presence of ions such as iodide and manganese.[55] Nevertheless, the antimicrobial efficacy observed in MHSW aligns with these studies, indicating the potential of hot spring waters in the treatment of DFUs.

The results suggest that MHSW has excellent antimicrobial and anti-biofilm properties, making it a good candidate for DFU management. However, the presence of heavy metals exceeding safety limits raises concerns regarding toxicity making it necessary for future research to focus on the safe application of MHSW for therapeutic use. Future research should also work on isolating the active antimicrobial compounds in MHSW and assessing their efficacy in in vivo DFU models. Moreover, strategies to minimize heavy metal toxicity while retaining the antimicrobial benefits should be explored. These results can also be used to facilitate its clinical application. Topical formulations such as hydrogels or wound dressings containing MHSW could be developed. However, challenges related to pH stability, penetration into biofilms, and compatibility with wound exudates must also be addressed to maximize its therapeutic efficacy.

This study has several limitations. Firstly, it is based entirely on in vitro experiments and therefore does not replicate the complex clinical environment of diabetic foot ulcers. Additional studies using relevant in vivo models are needed to provide further insights into efficacy and safety. Secondly, the study focused on specific strains of *S. aureus* and *P. aeruginosa*, and strain-to-strain variability in resistance and biofilm formation may affect the generalizability of the findings. Furthermore, the antimicrobial properties of MHSW could be influenced by natural fluctuations in geothermal activity which can lead to environmental variability in its composition over time. One solution for this is to standardize MHSW through lyophilization or synthetic replication of its mineral profile to ensure consistency in composition and efficacy. Moreover, longitudinal sampling of MHSW in different seasons should be conducted to determine natural

fluctuations in its chemical composition and their impact on antimicrobial activity. Furthermore, this study addresses only one aspect of DFU management: infection control, while wound healing is multifactorial and also depends on factors such as adequate vascularization, glycemic control, tissue regeneration, and host immune response. Lastly, although chemical analyses identified multiple organic constituents and elevated heavy metal levels, we did not perform bioassay-guided fractionation or metal-removal controls. Consequently, we cannot assign the observed antimicrobial/anti-biofilm activity to specific compounds nor exclude a contribution from heavy metals. Future studies should include a broader range of bacterial strains and collect multiple MHSW samples across different seasons to account for seasonal variations. They should also focus on fractionating the sample, removing or neutralizing heavy metals, and isolating individual bioactive compounds to clarify their role and assess their potential for safe therapeutic applications.

Conclusions

In this study, Ma'in hot springs water (MHSW) demonstrated dose-dependent antibacterial and anti-biofilm activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* under the in vitro conditions tested, with effects that correlate with the presence of bioactive constituents. Moreover, MHSW was also found to be non-cytotoxic. Given these preliminary data, MHSW may warrant further development as a topical adjunct for local infection control in diabetic foot ulcers. However, these results are limited to in vitro assays and a small number of bacterial strains. The study did not include bioassay-guided fractionation, metal-removal controls, or assessment of natural compositional variability in MHSW. As a result, substantial additional work, including expanded in vitro testing, standardization of MHSW, preclinical wound models, and phased clinical trials is required before any clinical application can be recommended.

Institutional Review Board Statement

Original study was approved by the Local Research Ethics Committee (LREC) - University of Tabuk under the rules and regulations of the National Committee of Bioethics (NCBE), Kingdom of Saudi Arabia with approval no No. UT-191-59-2022, where bacteria were stock at −80°C. No patient details, including blood and tissue, were collected and were not required in this study.

Data Sharing Statement

Data are contained within the article.

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Disclosure

The authors declare no conflicts of interest in this work.

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