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# Characterization and Evaluation of Clay Samples Against Bacterial Pathogens

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**Abstract**

The rise of antimicrobial resistance (AMR) necessitates the development of new antimicrobial agents. This study explores the antibacterial properties of thirteen clay samples (S1-S13) from diverse geological locations against S. aureus, S. epidermidis, E. coli, K. pneumoniae, and P. aeruginosa using the disc diffusion method. Bulk and mineral suspensions of the most active clay leachate samples were then tested for their growth-inhibiting activities against the tested pathogens. Chemical analysis, conductivity, pH measurements, and quantification of iron, aluminium, silver, and copper in selected samples with strong antibacterial effects were performed. They were evaluated and correlated with their antibacterial activities. Inhibition ranges were 60-90% for bulk minerals and 15-65% for mineral suspensions. S2, S6, and S8 clay leachates exhibited significant bactericidal effects, with S8 being the most active. Their activity decreased after three months. Elemental analysis revealed silicon, iron, and aluminium as crucial components. Modified clay samples with hemoglobin (Hb) and sodium borohydride (NaBH4) increased antibacterial activity. Notably, direct contact between clay mineral solutions and bacterial cells was just as successful as dialysis tube separation. These results suggest the potential of clay minerals as alternative agents against AMR pathogens, emphasizing the need for further research into their mechanisms and clinical applications.



**Keywords:** *Antibacterial Activity, Elemental Analysis Clay, and Clay Leachates*

## 1. Introduction

The surge of human illnesses that are antibiotic-resistant spurred the investigation into alternative antibacterial agents (Friedlander et al., 2015; Lemire et al., 2013). Conventional antibiotics that affect DNA replication, protein synthesis, and cell wall production induce antimicrobial resistance (AMR) (Walsh, 2000). Diverse bacteria have derived resistance to numerous first-line and last-resort antimicrobials as a result of the selective pressure of antibiotic exposure in medicine and animal husbandry (Bartlett et al., 2013; Gross, 2013; Michael et al., 2014; Spellberg & Gilbert, 2014). The group of microorganisms known as "ESKAPE" (Enterococcus faecalis, Methicillin-resistant Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli) is generally correlated with the development of multi-drug resistance and virulence (Kirsner, 2016). The O'Neill study predicted that by 2050, AMR might cause 10 million annual deaths globally (O'Neill, 2014). This suggests significant worries about the paucity of innovative antimicrobial medicines under development for different regulatory and economic reasons (Piddock, 2012).

Clays have been applied for curative purposes for decades; however, their practices and reported health advantages are limited (Carretero, 2002; Ferrell, 2008). "Clay" is attributed to minerals of any sort that are less than 2 µm in size. They include smectite, illite, and kaolinite, which, once hydrated, give a massive surface area (100′s $m^2/g$), allowing for cation exchange. Only a few clays have been noted to possess potent antibacterial activity against a collection of human ailments (Azmi et al., 2021; B. Williams et al., 2008; Morrison et al., 2014; Williams et al., 2011). The French green clay was used in the treatment of Buruli ulcer, a necrotizing cutaneous infection caused by Mycobacterium ulcerans (Williams et al., 2014). Antibacterial potency of clay leachates was also shown against Escherichia coli and methicillin-resistant Staphylococcus aureus (MRSA). The clay deposits can come from hydrothermally changed volcaniclastic settings, either altered pyroclastic material or bentonite, i.e., volcanic ash. However, each deposit is mineralogically unique. Some clays were also used to treat cancer (Cervini-Silva et al., 2016), dermatitis (Fowler, 2001; Sandri et al., 2014), irritable bowel syndrome (Ducrotte et al., 2005), kidney diseases (Zhang et al., 2009), detoxification (Williams et al., 2009), and even many other purposes (Moosavi, 2017).

Clays display a collection of distinctively desirable qualities that could work in their favor for AMR applications. They are widespread and are considered to operate through mechanisms in bacteria that have not yet developed resistance (Williams et al., 2014). Uncertainty surrounds the methods through which some clays appear to be antibacterial. These antibacterial actions may be related to direct contact between the charged surfaces of the clay grains and the bacterial cell surface, resulting in cell lysis. The bacterial cell membrane may also become more permeable when exposed to reactive cations, such as aluminium ion ($Al^{3+}$) and iron ion ($Fe^{2+}$), in an aqueous solution, leading to the production of production of reactive oxygen species (ROS) (Londono et al., 2017;

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Morrison et al., 2016; Otto & Haydel, 2013; Zhao et al., 2005).

Many of the antimicrobial clays that have already been characterized evolved in volcaniclastic settings that have undergone hydrothermal alteration (Williams et al., 2011) such as the Eifel region of West Germany (Zarate-Reyes et al., 2018), the Amazon Rainforest (Londono & Williams, 2016), and the Cascade Mountain range in Douglas County, Oregon (Morrison et al., 2016). Even though the origin of the green clay, known as Argiletz (also known as Illite or Sea Clay) and/or associated clay combinations, is a trade secret, it is most likely from the Massif Central area of France, close to the Chaîne des Puys or the Cantal stratovolcano (Incledion et al., 2021).

Studies on the antibacterial properties of clays shows that the Arab region is scarce, However, Jordanian soils demonstrated significant potential to inhibit certain pathogenic bacteria (Ibbini et al., 2018). In this study, we attempted to bridge this knowledge gap by characterizing the geological and biological properties of 13 soil samples, obtained from Arabic regions including Saudi Arabia, Egypt, Palestine, Syria, Jordan, and Lebanon, and we assessed their antibacterial activity. Given their historical usage as traditional "healing clays," these soil samples may have distinct antibacterial characteristics that need further examination. By assessing their antibacterial activity against MDR organisms, this study adds to the rising interest in natural clay-based medicines as a viable solution to worldwide AMR epidemic.

## 2. Materials and Methods

### *2.1 Clay Samples and Their Processing*

Table 1 summarizes the sources of the clay samples. Samples were collected at 0–20 cm depth using a 1.45 cm diameter soil core. Samples were packaged in airtight plastic bags, labelled as S1-S13. After that, the samples were air-dried at room temperature and gently disaggregated, using a sterile mortar and pestle to achieve a smooth consistency, allowing them to pass through a 250 mm sieve (Haydel et al., 2007). Fine bulk mineral particles were collected and, then, stored in plastic bags at 4ºC. Before use, all bulk clay mineral samples were sterilized in an autoclave (121°C, 15 psi (pounds per square) inch for 1 hr) to remove any microbial contamination.

**Table 1.** Clay sample locations and coordinates

| Sample reference number | Region and Location of sampling | Coordinates (Decimal Degrees) |
|---|---|---|
| S1 | Makka (Saudi Arabia). Sample collected from the entrance of Mecca, near the main road at the boundary of the Haram area. | 21.3639278, 39.6705242 |
| S2 | Madina East area (Saudi Arabia) | 24.4687291, 39.6426387 |
| S3 | Madina West area (Saudi Arabia) | 24.4635445, 39.5647931 |
| S4 | Madina Seven Mosques (Saudi Arabia) | 24.4768649, 39.5962940 |
| S5 | Madina Ohod Mountain (Saudi Arabia) | 24.5231679, 39.6268699 |
| S6 | Madina Awaly area (Saudi Arabia) | 24.7368447, 39.4605876 |
| S7 | Alkodus (Palestine) | 31.7705626, 35.2227902 |
| S8 | Egypt (Helwan) | 29.8415394, 31.3341513 |
| S9 | Coastline of Sidon (Saida-Lebanon) | 33.5582651, 35.3687324 |
| S10 | Al-Wastani area of Sidon (Saida-Lebanon) (Red Clay) | 33.5529660, 35.3903445 |
| S11 | Jordanian side of the Dead Sea (Jordan) | 31.5061133, 35.5597424 |
| S12 | White clay from Kashta'ar Village in northern Aleppo (Syria) | 36.1956659, 37.1095367 |
| S13 | Red clay from Kashta'ar Village in northern Aleppo (Syria) | 36.1956659, 37.1095367 |

### *2.2 Preparation of Clay Leachates, Mineral Suspension, and Modified Clay*

The samples, prepared for antibacterial susceptibility testing, included the bulk minerals, aqueous mineral leachates, mineral suspensions, and modified clay samples as follows.

***2.2.1 Clay Leachate Preparation:*** Bulk clay samples were ultrasonically treated with deionized water (100 mg/mL) for 2 minutes, followed by 24 hrs. of shaking, to produce clay mineral aqueous leachates. The mixture was, then, centrifuged for 30 minutes at 15,000 revolutions per minute (rpm). The supernatant, i.e., leachate, was filtered using a 0.22 µm membrane filter (Williams & Hillier, 2014).

***2.2.2 Clay Mineral Suspension:*** Sterilized bulk mineral samples were mixed in sterile deionized water, resulting in a 10% solution.

***2.2.3 Sample Modification with Hemoglobin:*** Sample modifications were performed on S2 from Madina and S8 from Egypt (Helwan) as they showed the most promising antibacterial action. To 4g of each clay sample S2 and S8, 50 mg of oxidized hemoglobin was added, and samples were named S2+ and S8+, respectively. The mixtures were pounded for 15 minutes in a mortar and pestle.

***2.2.4 Reduction:*** S2+ and S8+ (2 grams) were reduced with sodium borohydride ($NaBH_4$, 100 mg) through a 15 minutes mechanochemical grinding. Distilled water (10 ml) was then gradually added until the evolution of hydrogen gas ended. The pastes were then dried at 100°C. These samples were designed as S2++ and S8++ and used to assess their antimicrobial activities.

### *2.3 Elemental Analysis of Selected Bulk Minerals*

X-ray fluorescence (XRF) was performed for quantitative and qualitative determination of the elemental composition of selected bulk minerals. Experiments were performed on samples S2, S6, and S8 at the chemistry laboratory, the Lebanese American University (LAU), using XRF portable Niton (XL3t Thermo). Observing their broad-spectrum

antimicrobial activity, they demonstrated efficacy against all tested bacterial pathogens. This targeted approach ensures that the most promising samples are further analyzed to better understand their composition and potential mechanisms of action.

### *2.4 Conductivity and pH Measurement of Selected Clay Samples*

Measurements of pH and electrical conductivity were made for selected clay samples (S2, S6, and S8). Samples were suspended at a ratio of 1 g of clay to 20 mL in distilled water, and measurements were done at 25ºC. The mixtures were first ultrasonicated and shaken for 24 hrs. The pH was measured using a Mi 151 Bench meter, and the electrical conductivity (EC) was measured using a Mi 170 conductivity meter from Martini.

### *2.5 Antimicrobial Susceptibility Test*

The antimicrobial susceptibility of the clay samples was assessed using the agar diffusion method and counting the Colony Forming Unit (CFU). Kirby-Bauer Disk Diffusion Method was used to evaluate the antimicrobial activity of the aqueous clay leachates (Biemer, 1973). Bacterial inoculum (100 µL, 1.5 x $10^8$ CFU/ml) was streaked on Mueller Hinton (MH) Agar plates. Sterile filter paper discs of 5 mm diameter, containing 50 µL of the clay leachate solution, were positioned on the surface of the agar. The plates were placed at 4 °C for 2 hours to allow the diffusion of leachates onto the medium, then they were incubated at 35 °C ± 2 °C for 24 hours. Each sample was analyzed in triplicate. In this study, an initial screening of 13 leachate samples was conducted to assess their antibacterial efficacy against three specific bacteria strains: S. aureus, S. epidermidis, and E. coli. From these screenings, the top three samples exhibiting the most promising outcomes were selected for further analysis (S2, S6, and S8). These chosen samples were meticulously preserved for a period of three months in sterile tubes and maintained at a temperature of 4ºC to prevent any alteration in their antibacterial properties. Following the preservation period, the antibacterial activities of these selected samples were systematically re-evaluated, using the disk diffusion method against S. aureus, S. epidermidis, and E. coli. Additionally, the modified samples S2+, S2++, S8+, and S8++ were subjected to rigorous testing against a broader spectrum of bacteria, including P. aeruginosa and K. pneumoniae.

All microbial strains used were clinical isolates, kindly provided by the Microbiology Laboratory at Beirut Arab University. These isolates were previously identified, using 16S rRNA sequencing. To ensure consistency and reliability in the evaluation process, the results obtained from these modified samples were meticulously compared with those from the unaltered clay leachates, specifically S2 and S8, as well as negative controls, utilizing the same disk diffusion method.

### *2.6. Screening Antibacterial Activity*

#### *2.6.1 Screening Antibacterial Activity of Selected Bulk Minerals and Mineral Suspension Against Bacterial Pathogens Using the Plate Counting Method*

The quantitative antimicrobial activities of selected bulk minerals and mineral suspensions (S2, S6, and S8) were determined on the tested bacterial pathogens. Determining bacterial growth inhibition vs. bactericidal activity of samples was achieved by plating serial dilutions of bulk minerals/mineral suspensions with pathogens alongside negative controls. CFU/ml was determined for S. aureus, S. epidermidis, and E. coli according to Hedge (Hedges, 2002). 10% clay mineral suspensions in sterile $dH_2O$ were prepared for S2, S6, and S8. Bacterial cells were suspended in 10% mineral suspension, or sterile dH2O (as control), at an initial concentration equivalent to 0.5 McFarland (1.5 x $10^8$ CFU/ml). The suspensions were incubated at 37˚C for 24 hrs in a shaker at 150 rpm. Serial dilutions of the mixtures (10 dilutions) were plated onto MH Agar plates and incubated at 37°C for 24 hrs. Colony-forming units (CFU/ml) were determined. All antimicrobial assays were performed in triplicate.

#### *2.6.2 Comparing the Antibacterial Activity of Clay Samples Against E. Coli Directly Mixed with Mineral Suspension and Separated Using a Dialysis Tube*

Standard inoculum from E. coli (1.5 × $10^8$ CFU/ml) was divided into two parts. The first part was mixed with 10% mineral suspension of S2, S6, and S8 in a ratio of 1:1. The second part was placed in a sterile dialysis tube suspended in 10% mineral suspension of S2, S6, and S8. The two sets, along with negative controls, were incubated at 37 ºC for 24 hrs. E. coli survival was determined post-CFU enumeration.

### *2.7 Statistical Analysis*

The obtained data were presented as mean ± standard deviation. The difference between the groups was statistically determined by t-test and ANOVA, where relevant. Significance set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA).

## 3. Results

### *3.1 Antibacterial Effects of Clay Leachates*

Leachates of clay samples were assayed against S. aureus, S. epidermidis, E. coli, K. pneumoniae, and P. aeruginosa to evaluate their antimicrobial activities. As shown in Figure 1, clay leachate samples S2, S6, and S8 showed effects against all tested bacteria. The other samples show activity against at least one pathogen. The highest activity was achieved by the sample S8, obtained from Helwan, Egypt, with zone of inhibition (ZOI) of 17.33 ± 1.53, 11.67 ± 0.58, and 11.00 ± 1.00 against S. epidermidis, S. aureus, and E. coli, respectively. Clay leachate samples S2 and S6 from Madina, KSA, showed also antibacterial capabilities against all bacterial pathogens with ZOI of 5.67 ± 0.58, 8.00 ± 0.00, and 6.00 ± 0.00 for S2 against S. aureus, S. epidermidis, and E. coli, respectively and where S2 showed the highest inhibition among tested leachates against K. pneumoniae, and P. aeruginosa with ZOI of 7.33 ± 1.15, and 7.67 ± 0.58, respectively. As for S6, ZOI were 7.00 ± 0.58, 5.67 ± 0.58, 7.67 ± 0.58, 6.67 ± 0.58, and 6.67 ± 0.58 were determined for S. aureus, S. epidermidis, E. coli, K. pneumoniae, and P. aeruginosa, respectively. Clay leachate of sample S11 from the Dead Sea in Jordan gave a ZOI of 11.67± 0.58 against S. epidermidis, lower values were recorded for S. aureus and E. coli with no effect seen against the other 2 pathogens. Almost all clay leachate samples showed an inhibition activity against E. coli, except for S12

and S13 which showed inhibitory activity only against S. aureus and S. epidermidis. S2, S6, and S8 clay leachates were the most active against the tested pathogens, therefore they were selected for further studies.

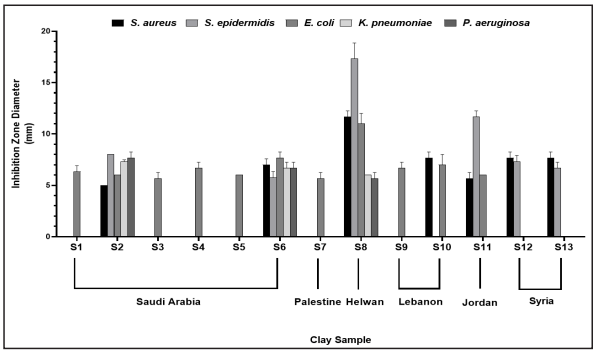


**Figure 1.** Comparison of antibacterial activities of clay leachates. Results are represented as the mean of triplicate ± SD.

Clay leachate samples S2, S6, and S8 were then preserved at 4 ºC and were tested again after 3 months to determine their stability with time. They were tested against the most susceptible pathogens S. aureus, S. epidermidis, and E. coli. As shown in Table 2, the three clay leachate samples exhibited decreased activity against the tested pathogens. S2 seemed to lose all its activity (98%, 97.5%, and 98.3% against S. aureus, S. epidermidis, and E. coli, respectively while S6 and S8 showed a significant reduction in activity.

**Table 2.** Effects of selected clay leachates against S. aureus, S. epidermidis, and E. coli following three months of preservations. Results are recorded as the mean of triplicate ± SD.

| | Sample number | Inhibition zone (mm) | Reduction in activity (%) |
|---|---|---|---|
| ***S. aureus*** | S2 | 5.67±0.58 | 98 |
| | S6 | 7.00±0.58 | 14 |
| | S8 | 11.67±0.58 | 42 |
| ***S. epidermidis*** | S2 | 8.00 ± 0.00 | 98 |
| | S6 | 5.67 ± 0.58 | 96 |
| | S8 | 17.33 ± 1.53 | 62 |
| ***E. coli*** | S2 | 6.00 ± 0.00 | 98 |
| | S6 | 7.67 ± 0.58 | 22 |
| | S8 | 11.00 ± 1.00 | 27 |

### *3.2 Determining pH and Conductivity Measurements of Selected Clay Samples*

The pH and conductivity of the 5% sample leachates of S2, S6, and S8 were determined at 25 ºC (Table 3). The pH values ranged from 8.05 to 8.45, while the conductivities of the leachates ranged from 695 ± 4.24 µS/cm to 1458.5 ± 94 µS/cm. The pH of the most active leachate S8 was 8.05 ± 0.49 with a mean conductivity of 1458.5 ± 94 µS/cm.

**Table 3.** Conductivity and pH measurements of selected clay leachates (S2, S6, and S8)

| Sample number | pH | EC (µS/cm) |
|---|---|---|
| **S2** | 8.35 ± 0.21 | 695 ± 4.24 |
| **S6** | 8.45 ± 0.07 | 6745 ± 134 |
| **S8** | 8.05 ± 0.49 | 1458.5 ± 94 |

### *3.3 Elemental Analysis of Most Effective Clay Samples*

The results of the elemental analysis are shown in Table 4. Silicon (Si), Fe, and Al were found in higher percentages compared to the others. The highest percentages of Si, Fe, and Al were found in S2 with values of 63.725, 25.022, and 10.600 %, respectively. S8 showed the highest Silver (Ag) content with 3.460 %, as well as the highest amount of Zinc (Zn) and Copper (Cu) with 0.888 and 0.759 %, respectively. The highest amount of Zirconium (Zr) was detected in S6 with 1.215%.

**Table 4.** Elemental analysis of selected clay samples using X-ray Fluorescence

| Element | S2 (%) | S6 (%) | S8 (%) |
|---|---|---|---|
| Mo | 0.022 | 0.014 | 0.024 |
| Zr | 0.430 | 1.215 | 0.624 |
| Sr | 0.012 | 0.008 | 0.050 |
| W | 0.013 | 0.013 | 0.023 |
| Zn | 0.003 | 0.003 | 0.888 |
| Cu | 0.002 | 0.002 | 0.759 |
| Ni | 0.004 | 0.004 | 0.025 |
| Co | 0.009 | 0.009 | 0.010 |
| Fe | 25.022 | 23.683 | 8.194 |
| Mn | 0.037 | 0.032 | 0.026 |
| Cr | 0.013 | 0.028 | 0.039 |
| V | 0.028 | 0.028 | 0.122 |
| Ti | 0.166 | 0.165 | 0.113 |
| Al | 10.600 | 8.407 | 9.290 |
| Si | 63.725 | 28.348 | 47.887 |
| Sb | 0.005 | 0.004 | 4.085 |
| Ag | 0.009 | 0.005 | 3.460 |

### *3.4 Antibacterial Effect of Modified Clay Samples*

Modified Clay leachate samples S2$^{+}$, S2++, S8+, and S8++ were prepared for testing their antibacterial activities against all five tested pathogens. The results were compared with those of unmodified clay leachates (S2 and S8), using the Kirby Bauer method. Figure 2 shows the results. The addition of Hb to S2 significantly increased the inhibition of S. aureus and E. coli by 44%, and 25%, respectively. A slighter increase in the inhibition of P. aeruginosa and K. pneumoniae by 12% and 10%, respectively was obtained. The addition of Hb + NaBH4 to S2 led to a significant increase in inhibition of S. aureus, K. pneumoniae, P. aeruginosa, and E. coli by 59 %, 41 %, 38 %, and 22 %, respectively.

Concerning S8 (Figure 3), the addition of Hb significantly increased the inhibition of P. aeruginosa and K. pneumoniae by 53% and 41%, respectively. Also, the addition of Hb + NaBH4 to S8 led to a significant increase in inhibition of P. aeruginosa and K. pneumoniae by 52%, and 51%, respectively. For S. aureus and E. coli, the increase in inhibition was by 17% and 15%, respectively when compared with the addition of Hb alone. It should be noted that the addition of Hb or Hb + NaBH4 to S2 did not increase the growth inhibition of S. epidermidis and the same modification of S8 significantly decreased the inhibition of S. epidermidis by approximately half.

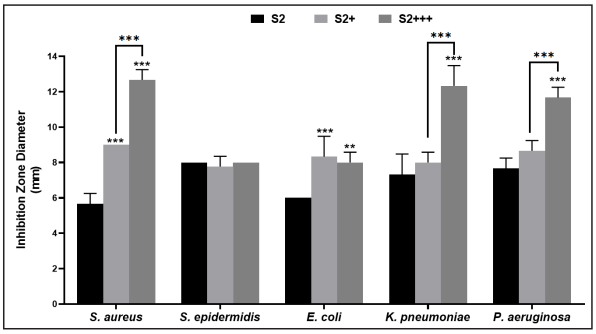


**Figure 2.** Effect of S2 modifications on bacterial growth. Results are recorded as the mean of triplicate ± SD. The difference in the inhibition zone was analyzed using two-way ANOVA. Results were compared against S2 unless indicated on the graph.
**P<0.01, *** P<0.001. +: Hb, ++: Hb + NaBH4

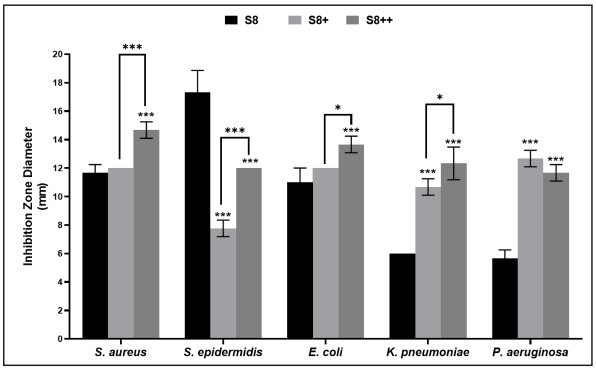


**Figure 3.** Effect of S8 modifications on bacterial growth. Results are recorded as the mean of triplicate ± SD. The difference in the inhibition zone was analyzed using two-way ANOVA. Results were compared against S2 unless indicated on the graph.
*P<0.05, ***P<0.001. +denotes Hb only, ++ denotes Hb and NaBH4.

### *3.5 Growth Inhibition of Bacterial Pathogens Treated with Mineral Suspensions and Bulk Minerals*

The effects of the clay samples S2, S6, and S8 as mineral suspensions (10%) and bulk minerals on the growth of S. aureus, S. epidermidis, and E. coli are shown in Figure 4. The bulk minerals of the tested samples (S2, S6, and S8) showed a significant reduction in CFU for S. aureus, S. epidermidis, and E. coli when compared to the effect of mineral suspensions of the tested samples. The mean increase in percent reduction was 50.75% ±5, except for S8 which was tested against S. epidermidis, where bulk minerals showed a decrease in reduction in CFU by 5% compared to the effect of mineral suspension. S2 bulk minerals showed the highest reduction in E. coli CFU with a mean percent reduction of 90%, followed by S. epidermidis with a mean percentage reduction of 85% ± 5, then S. aureus with a mean percentage reduction of 83.33% ± 5.77.

### *3.6 Antibacterial Activity of Mineral Suspensions Separated from Bacteria by Dialysis*

The activity of the clay samples S2, S6, and S8 on E. coli, suspended in solution, was determined directly when mixed with mineral suspensions and after separating them from the mineral suspensions, using sterile dialysis tubes. The results were compared with those obtained with non-treated bacteria. The results are shown in Figure 5 where inhibitory activities were still recorded on E. coli bacterial suspension even when separated from mineral suspensions by dialysis. No difference was obtained when the % reduction in CFU of E. coli mixed with S2 mineral suspension or separated by dialysis tube was compared. However, a reduction in CFU by 10 and 6% was observed when E. coli was treated with S6 and S8, respectively, and separated by dialysis tubes. No significant statistical difference in % reduction in CFU of E. coli subjected directly to mineral suspensions or separated by dialysis tubes (p>0.05) was also noted.

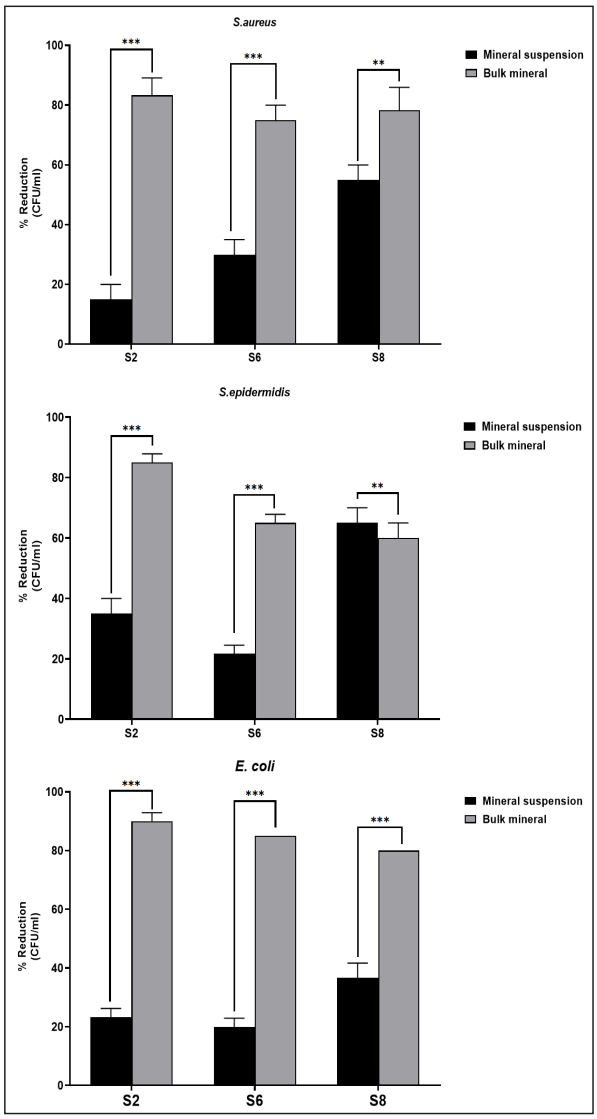


**Figure 4.** Percent reduction in bacterial pathogens counts (CFU/ml) using mineral suspensions and bulk minerals. Comparison of the effect of selected clay samples (Mineral suspensions) and (Bulk minerals) against bacterial pathogens. Results are mean of triplicates ± SD. The difference between Clay Mineral Suspensions and Bulk Minerals was assessed using a paired sample t-test.
** P<0.01, ***P<0.001.

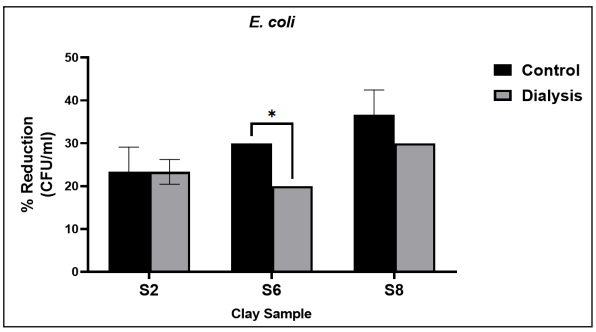


**Figure 5.** Percent reduction in E. coli CFU/ml using mineral suspensions with and without dialysis. Results are mean of triplicate ± SD. The difference was assessed using a paired sample t-test.
* p<0.05

## 4. Discussion

In 2021, AMR was expected to cause 4.71 million deaths worldwide, with 1.14 million fatalities directly attributed to AMR (Naghavi et al., 2024). According to projections, by 2050, AMR would cause 1.91 million attributable deaths and 8.22 million related fatalities globally, with South Asia and Latin America anticipated to have the greatest rates (Naghavi et al., 2024). These alarming results underline the critical need for new antibiotic treatments to tackle resistant diseases. In this context, our research looks at the potential of clay-based materials as a new antibacterial method, including their efficiency against resistant microorganisms and their potential function as an alternative or supplement to traditional antibiotics. In the present investigation, antibacterial susceptibility testing was performed on aqueous mineral leachates, modified mineral leachates, bulk mineral, and mineral suspension samples. Leachates of all collected clay samples have been tested against two Gram-positive bacteria (S. aureus & S. epidermidis) and three Gram-negative (E. coli, K. pneumoniae & P. aeruginosa) as they belong to the "ESKAPE" group. These bacteria showed various levels of tolerance to metal ions leached, and this tolerance could be attributed to the difference in the cell wall structure (Kirsner, 2016). Initially, the primary focus of this research was on evaluating the leachates' effectiveness against the well-known and prevalent pathogens, S. aureus, S. epidermidis, and E. coli. Encouraged by the promising results obtained in this phase, the study's scope was broadened to encompass additional bacterial strains, namely P. aeruginosa and K. pneumoniae. This expansion aimed to comprehensively assess the leachates' antibacterial potential across a wider range of pathogens, enhancing the study's overall significance and applicability.

Clay leachates S2, S6, and S8 were observed to possess broad-spectrum antimicrobial capabilities, displaying activities against all tested bacterial pathogens. The highest activity resulted from S8 (Egypt - Helwan) against S. epidermidis followed by S. aureus, and finally E. coli. Both clay leachate S2 (Madina - Saudi Arabia) and S6 (Madina-Saudi Arabia) showed broad-spectrum antimicrobial capabilities against all bacterial pathogens. The results were consistent with the study done on two types of French green clays sold as "healing clays" (Williams et al., 2008b). Clay leachate labeled S11 (Dead Sea- Jordan) showed an effect against S. epidermidis, with a lower effect against S. aureus and E. coli; whereas clay leachate S9 (Saida-Lebanon) showed no activity against any of the pathogens tested in the current study except against E. coli. Many of the tests of the leachates inhibited the E. coli strain, as well as other pathogenic strains of S. aureus, S. epidermidis, K. pneumoniae, and P. aeruginosa but in different degrees of activity. The present study revealed no significant difference in the tolerance of metal ions between Gram-positive and Gram-negative bacteria; however, E. coli exhibited greater susceptibility to clay leachates compared to S. aureus. Consequently, each microbial strain may possess a distinct inhibitory control that interacts with its antimicrobial mechanism (Ibbini et al., 2018).

The highest activity recorded was attained against S. epidermidis which was inhibited using S8. Clay mineral surfaces are naturally negatively charged thus allowing free exchange of positively charged species, such as metal cations. In a hydrated suspension, these cations can be released from the surface of the minerals into the surrounding medium (Cunningham et al., 2010; Otto & Haydel, 2013; Williams & Haydel, 2010). It can also be suggested that the clay leachates inhibit the activity of cells due to the soluble species that react with bacteria (Londono & Williams, 2016; Morrison et al., 2014). The activity of metals in solution may be influenced by the surface chemistry of the clays that alter their potential energy or reactivity

Williams and et al. found that the antibacterial French green clay leachates lose their bactericidal effect after sitting in a test tube for 6 months (Williams et al., 2008b). Whether this is due to a change in oxidation state or another chemical effect regulated by the clay surface should be further evaluated. To verify this hypothesis, the activity of clay leachates preserved for 3 months was tested on the bacterial pathogens. The results showed that almost all leachates exhibited decreased activity against the tested pathogens. S2 seemed to lose almost all its activity.

The selection of samples S2, S6, and S8 for elemental analysis and chemical modifications was based on their broad-spectrum antimicrobial activity, as they demonstrated efficacy against all tested bacterial pathogens. The quantitative and qualitative XRF analyses of mineral mixtures of selected clay samples (S2, S6, and S8) revealed the presence of Si, Fe, Al, Ag, Cu, and Zn. The highest percentage of elements was obtained for Si > Fe > Al. The highest percentages of Si, Fe, and Al were present in S2. S8 and showed the highest Ag content as well as the highest amount of Zn and Cu. Ag primarily exert their antimicrobial action by binding to thiol (sulfhydryl) groups in bacterial proteins and enzymes, disrupting their function (Bragg & Rainnie, 1974; Fuhrmann & Rothstein, 1968; Furr et al., 1994). This interaction impairs essential cellular processes and leads to bacterial death. Other sulfur-containing compounds do not neutralize silver's activity, highlighting the significance of thiol group binding. Additionally, silver ions can disrupt bacterial membranes by causing the release of potassium ions ($K^+$), further contributing to its bactericidal effect (Fuhrmann & Rothstein, 1968; Rayman et al., 1972; Schreurs & Rosenberg, 1982). As for Cu, it can initiate cell damage through several mechanisms. The first involves oxidative damage, where copper ions produce reactive oxygen species (ROS) that disrupt membrane integrity and lead to cell death. This death occurs via non-enzymatic oxidative damage to membrane phospholipids (Santo et al., 2012). Additionally, copper ions penetrate the bacterial cell, causing oxidative stress and DNA degradation through redox cycling, which results in the destruction of genetic material (Dalecki et al., 2017; Santo et al., 2011). However, the exact sequence of events in "contact killing" is debated, with some studies suggesting that copper's antibacterial effect could also involve inhibition of cellular respiration and DNA damage rather than direct membrane degradation (Weaver

et al., 2010).

The highest amount of Zn was detected in S6. Other elements such as Sb, Mo, Sr, Ni, Ti, and V, were present in small quantities. It is well known that the antibacterial properties vary with the metal toxicity and the type of bacteria (Yasuyuki et al., 2010). Some metals display specific effects on bacteria due to their chemical or physical properties (Morrison et al., 2016; Williams & Haydel, 2010).

The crystalline structure of metal elements disrupts bacterial cell envelopes, and/or impairs the efflux of bacterial metabolites (Ghadiri et al., 2015). Morrison and et al. showed that a clay derived from volcanogenic hydrothermal alteration destroys bacteria through the synergistic actions of Fe and Al (Morrison et al., 2016). Metals can interact synergistically to produce a toxicity level greater than that predicted by adding their toxicities (Preston et al., 2000). For example, lower concentrations of Fe can be toxic to E. coli in the presence of Al (Fatima Camoes Amador, 1999), also Zn and Cu have been shown to act synergistically against E. coli (Preston et al., 2000).

pH enhances the solubility of metals in leachate, thus elevating their concentration and possibly their toxicity to organisms within their scope of influence (Antoniadis et al., 2006). pH and conductivity measurements for clay leachate samples S2, S6, and S8 were conducted in the present study to determine their influence on antibacterial activities. The most active leachate S8 recorded a mean pH measurement of 8.05 ± 0.49, which was slightly alkaline similar to pH values recorded for S2 and S6 (8.35, and 8.45, respectively). The mean conductivity determined for S8 was 1458.5 ± 94 µS/cm. Conductivities of the other leachate samples ranged from 695 ± 4.24 µS/cm for S2 to 6747 ± 134 µS/cm for S6. Many bacteria can adapt to varying external pH conditions; however, pH impacts the rate of chemical reactions that affect the flow of nutrients through the membrane. A study done by Borquaye et al. measured both the pH and conductivities of aqueous clay leachates collected from Ghana to examine if they played any role in the observed antimicrobial activities (Borquaye et al., 2016) and found the most active antibacterial clay leachate had a pH of 2.81 and a conductivity of 54.2 µS/cm.

Sorokina and et al. demonstrated that in bacterial cells, iron is known to be present as $Fe^{2+}$ and $Fe^{3+}$, which may be converted to one another (Sorokina et al., 2013). However, iron is important for biological systems; its concentration in the cells should be maintained at a definite low level. At high concentrations, iron has a toxic effect. The ferrous iron cations generate ROS, which cause peroxidation of the cell membrane lipids, as well as protein and DNA damage (Storz & Imlayt, 1999). As the chemical composition of the tested clay samples in the current study indicated the presence of Fe in the most potent antibacterial samples from Madina (S2) and Helwan (S8), therefore, modifications of these clays via solid-state mechanochemical addition of hemoglobin, the most abundant hemeprotein in biological systems, was done. The expected results must answer the following questions: a) Does hemoglobin with iron in the bound state act as an inhibitor of bacterial pathogens? b) In what oxidation state is the inhibition more effective? c) What is the effect of structure on the magnitude of inhibition? and d) How do other metals, e.g., silver, influence the magnitude of inhibition? Modified Clay leachate samples $S2^{+}$, $S2^{++}$, $S8^{+}$, and $S8^{++}$, were examined for their antibacterial activities against all bacterial pathogens using the disk diffusion method. The addition of hemoglobin (Hb) was referred to as oxidized hemoglobin samples (OHB) and the addition of Hb and NaBH4 was referred to as reduced hemoglobin samples (RHB). Zones of inhibitions detected for bacterial pathogens treated with (S2 and S8) and modified samples (S2+, S2++, S8+, and S8++) were compared. The addition of Hb to clay samples (S2 and S8) resulted in varying degrees of increased inhibition against bacterial pathogens, ranging from 10% to 53%, while further addition of $NaBH_4$ to these samples led to even higher inhibition percentages, ranging from 22% to 59%. OHB showed an increase in antibacterial effect compared to the unmodified clay against selected pathogens. Reduced modified clay RHB showed appreciable bioactivity enhancement compared to OHB, as well as to that of the unmodified clay against the same selected pathogens. The ferric ion in oxidized hemoglobin (in its high spin state [$3d^5$, S=5/2]) is displaced from the heme plane as compared to that in the reduced low spin state [$3d^6$, S=0], where the ferrous ion lies in the heme plane. The topology/morphology of the tested pathogens can be thought to exist in a 2D dimensional state, making it easy to interact more with the iron in the plane of the heme ring than when it is displaced. It can be concluded that Hb with Fe in the bound state showed increased activity as an inhibitor of selected pathogens, and the low spin state enhanced the interaction between the reducing agent and selected pathogens in a way that increased its inhibition. Haydel and et al. added that the combination of elevated levels of reduced iron in French green clay samples and excessive free radical production in the presence of oxygen could cause oxidative stress and damage to bacterial cells, resulting in cell death (Haydel et al., 2007). As a result, the chemical structure and stereochemistry of the active clay-modified materials seem to play a major role in the bioactivity process. In the current study, chemical analysis of the clay sample of Helwan (S8) revealed the presence of Ag, and the highest antibacterial activity was achieved by this clay leachate sample against S. epidermidis, S. aureus, and E. coli. Miyoshi et al. prepared Ag nanoparticles on a type of clay mineral in n-hexanol by chemical reduction with $NaBH_4$ and they studied the antibacterial effect of samples against E. coli incubated in light and the dark. Results showed that action in the light was higher probably due to the formation of ·OH radicals. Antibacterial action was observed even after several years (Miyoshi et al., 2010). The effect of reduction on other metals, such as Ag, can be related to nanoparticle silver and light plasmonic antipathogenic effect, leading to the rupture of the double-helical DNA structure. The results showed that the antibacterial properties varied significantly with different metals.

The bulk mineral of the tested samples (S2, S6, and S8) showed a significant reduction in CFU for S. aureus, S. epidermidis, and E. coli. The mean increase in percent

reduction was 50.75% compared to the effect of mineral suspensions of the same tested samples. S2 bulk minerals showed the highest reduction in E. coli CFU followed by a reduction in S. epidermidis CFU and finally S. aureus. Results were consistent with the conclusion presented by Morrison et al.; who showed that the freely exchangeable metal ions bound to the surface of the pathogens might be responsible for the antibacterial activity of clay mixtures (Morrison et al., 2016). Williams and et al. declared that when antibacterial clays are hydrated, a series of spontaneous reactions occur emphasizing the importance of bacterial-clay contact for efficient contact killing (Williams et al., 2008b).

A study done by Cohn and et al. to test for the physical effect of clay on bacteria showed that the bacteria were killed without physically contacting the clay mineral surfaces (Cohn et al., 2006). In the present study determination of clay samples activities (S2, S6, and S8) against E. coli was evaluated by plate counting of the bacterial culture directly mixed with mineral suspensions and separated by dialysis. Inhibitory activities were still recorded on the bacterial suspension of E. coli, even when suspended in the dialysis tube. Comparing the % reduction of CFU of E. coli mixed with mineral suspensions and separated from mineral suspensions by dialysis tubes showed no significant difference. Results verified that physical contact as well as chemical transfer of chemicals between the clay and bacteria were required for antibacterial activity.

Clay's future medicinal uses offer significant potential, especially as an alternative to antibiotics in certain situations. Given its broad-spectrum antibacterial action, clay could be applied in topical therapies for skin conditions such as wound healing or dermatitis, providing a safer, non-toxic alternative to conventional antibiotics (Incledion et al., 2021). Clay could also be incorporated into medicinal formulations like ointments, lotions, or wound dressings, where it may act as a carrier for other therapeutic substances or promote healing by reducing bacterial loads and inflammation (Viseras et al., 2019). Additionally, clay-based formulations could be utilized as mouthwashes or toothpastes to address oral infections, even those resistant to conventional antibiotics (Borges de Macedo et al., 2022).

However, different limitations must be overcome before these prospective uses may be realised. One major problem is the chemical stability of clay formulations, since the antibacterial activity of clay leachates might diminish with time, as shown in this work. The long-term stability of these compounds in medicinal formulations must be extensively examined, including any possible changes in chemical composition and activity while kept or exposed to environmental conditions. Furthermore, environmental conditions such as pH, temperature, and humidity may have a major impact on the activity of clay-based formulations, necessitating the establishment of standardised techniques for clay preparation and preservation. Furthermore, although clays have shown promising results in vitro, their clinical use would demand extensive safety and toxicity testing, as well as investigations to identify their biocompatibility and possible negative effects in humans. Despite these obstacles, continuous research and development may someday allow clays to be employed in medical therapies, providing a unique method to fighting infections, especially in light of rising worries about antimicrobial resistance.

## 5. Conclusion

The study highlights the complexity of natural clay's antibacterial process and urges more investigation to find new clay deposits with potent antibacterial properties. Furthermore, emphasis is placed on the necessity of doing additional research on active chemical substances and their mechanisms of action. Although the antibacterial activity of clay leachates has been shown in vitro, more investigation—including animal models and clinical trials—is required to confirm the potential medicinal applications of this material.

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## Declaration

The authors declare that no similar work has been published by another journal, and the paper is the original work of the author(s) and not copied (in whole or in part) from any other works.

## Data Availability

The datasets utilized or analyzed in the present study can be obtained from the corresponding author upon reasonable request.

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