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*Research article*

# Antimicrobial activity of *Ziziphus mauritiana* and *Acacia nilotica* against multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae*

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**Abstract:** Multidrug-resistant (MDR) strains such as *Escherichia coli* and *Klebsiella pneumoniae,* resistant to broad-spectrum antibiotics, are a modern global issue. Plant extracts offer promising antimicrobial activity for such pathogens. This study evaluated the efficacy of aqueous and hydro-ethanolic extracts from leaves and bark of two cheaply and easily available plants, i.e., *Ziziphus mauritiana* (ber) and *Acacia nilotica* (gum Arabic or Kikar), against MDR *E. coli* and *K. pneumoniae*. Antibiotic susceptibility testing confirmed resistance in both strains to all tested antibiotics (ampicillin, ciprofloxacin, streptomycin, and tetracycline), with inhibition zones ≤ 10 mm. Antimicrobial assays revealed that hydro-ethanolic extracts of *Z. mauritiana* leaves exhibited the highest activity, with inhibition zones of 20 mm (*E. coli*) and 19 mm (*K. pneumoniae*) at 100 mg/mL, outperforming aqueous extracts. The minimum inhibitory concentration (MIC) for all the tested extracts was notably low (6 mg/mL for *E. coli* and *K. pneumoniae*). Growth curve analysis demonstrated significant suppression of bacterial proliferation by all extracts at MIC, with optical density ($OD_{600}$) values remaining below 0.25 over 24 h compared to controls ($OD_{600} \geq 2.20$). Additionally, biofilm formation was completely inhibited at MIC as well as sub-inhibitory concentrations (sub-MIC) of ½ MIC, highlighting that the antibiofilm activity of these extracts is not due to antimicrobial activity. Findings highlight the efficacy of extracts from different parts and solvents against MDR pathogens, suggesting their potential as an adjunct therapy. Findings suggest that hydro-ethanolic extracts perform significantly better than aqueous extracts, while no significant difference in antimicrobial activity was found in extracts from different parts of plants. Lastly, it was found that *Z. mauritiana* extracts have greater antimicrobial

potential than *A. nilotica* extracts.

**Keywords:** antimicrobial resistance; antibiofilm activity; *Klebsiella*; *Escherichia*; plant extract

## 1. Introduction

A plant extract is a concentrated bioactive compound derived from a plant material for specific applications [1]. Plant-based extracts are natural, multi-component mixtures known for their broad-spectrum biological effects, such as antifungal, antimicrobial, antioxidant, anti-inflammatory, and medicinal properties [1].

Antimicrobial activity is defined as the capacity of a chemical compound to inhibit microbial growth. Antimicrobial agents are essential for treating infections, preventing pathogen transmission, and protecting high-risk populations. However, the rise of antimicrobial resistance (AMR), where pathogens evolve to resist antibiotics and other antimicrobial agents, presents a severe global threat. Two prevalent resistant pathogens are *Escherichia coli* and *Klebsiella pneumoniae* [2,3]. *E. coli* is a Gram-negative bacterium that frequently colonizes the intestinal tracts of humans and animals, causing severe diarrhea and being the primary cause of acute urinary tract infections (UTIs) and urosepsis [2,3]. Avian pathogenic *E. coli* (APEC) is particularly concerning, as it primarily infects poultry but shares genetic similarities with human-pathogenic strains [4]. *K. pneumoniae* is a non-motile, non-spore-forming bacterium that also typically colonizes the human gastrointestinal tract and nasopharynx [5]. However, it can become pathogenic, leading to respiratory and urinary tract infections, surgical wound infections, and bloodstream infections [5]. Its ability to cause severe conditions, including pyogenic liver abscesses, necrotizing pneumonia, endocarditis, septicemia, and endophthalmitis, is particularly concerning [5].

Plants are a source of potent antimicrobial and bioactive compounds capable of effectively eliminating bacteria, offering a promising alternative in combating antibiotic resistance. These bioactive phytochemicals exert their effects by disrupting critical bacterial functions, such as cell wall synthesis and metabolic pathways, and can also overcome existing resistance mechanisms [6,7].

*Z. mauritiana*, a member of the Rhamnaceae family, is commonly known as Chinee Apple, Indian Jujube, or Ber. It is widely cultivated and naturalized in South Asia, including China, India, and Pakistan. This species is valued horticulturally and ecologically but is also considered a weed in some pastoral contexts [8]. Its spreading management often involves synthetic herbicides or mechanical clearing [8]. This versatile species produces fruits containing high levels of vitamin C, protein, phosphorus, and iron [9]; its leaves are traditionally used in treating urinary tract infections, and its roots for dysentery [10]. Additionally, the root bark demonstrates valuable anti-inflammatory, anti-allergic, and analgesic properties, further establishing *Z. mauritiana* as an important medicinal plant in traditional healthcare systems [9]. *Z. mauritiana* contains several bioactive compounds, notably flavonoids such as quercetin, which have demonstrated significant neuroprotective and therapeutic potential. In silico studies have shown that quercetin from *Z. mauritiana* strongly binds to the GABA-A receptor, comparable to diazepam, suggesting its potential use in treating neurological disorders like postpartum depression by modulating neurotransmission and neuroinflammation [11,12]. Endophytic fungi isolated from *Z. mauritiana* roots have demonstrated antibacterial activity against bacteria causing skin infections, including *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The bioactive

compounds identified in these fungi include flavonoids, alkaloids, and anthraquinones, which contribute to the antibacterial effects [11,12].

*A. nilotica* (also known as *Vachellia nilotica*) is a multipurpose tree native to Africa, the Middle East, and the Indian subcontinent. It belongs to the *Fabaceae* family and is widely used in traditional medicine and for various economic applications such as tannin extraction, timber, fuel, and fodder [13–15]. *A. nilotica* is a medicinally important plant rich in bioactive secondary metabolites, particularly condensed tannins, flavonoids, and gums, which contribute to its significant ecological, economic, and therapeutic value [16,17]. The fruits and seeds of *A. nilotica* are rich in diverse bioactive molecules, including tannins, saponins, phytosterols, alkaloids, cyclitols, anticoagulant agents, mucilage, fibers, gums, proteins, amino acids, carbohydrates, terpenes, oils, fatty acids, and essential minerals like calcium, potassium, iron, zinc, selenium, and copper [13,14]. *A. nilotica* exhibits a wide range of medicinal properties. Traditionally, it has been used to treat numerous ailments such as various cancers, heart and liver diseases, diabetes, malaria, asthma, arrhythmia, immune disorders, respiratory infections, gastrointestinal issues, and skin diseases. Its fruits are considered safe and effective when used in appropriate doses and durations [13,14].

Recent studies have highlighted the plant's antioxidant, antimicrobial, anti-inflammatory, and wound-healing activities, making it a promising candidate for topical formulations in skin therapeutics. The bioactive compounds like tannins, flavonoids, alkaloids, and essential fatty acids contribute to these effects [13,18]. Although *A. nilotica* is widely used traditionally without reported adverse effects, toxicity can arise from overdose or prolonged use. The human body can detoxify many compounds, but careful formulation and dosing are necessary to ensure safety [14].

There exists a research gap regarding whether extracts prepared from different solvents and different parts of plants perform differently or similarly against multi-drug resistant (MDR) pathogens. The primary objective of this study is to explore alternative antimicrobial agents derived from plant sources, addressing the critical global challenge of antimicrobial resistance (AMR). The secondary objective of this study is to compare the antimicrobial activities of extracts prepared from different parts of plants, such as leaves and bark, as well as of extracts made using different solvents.

## 2. Materials and methods

### *2.1. Strains and culture conditions*

Two MDR strains, i.e., *E. coli* and *K. pneumoniae,* were obtained from the Institute of Microbiology, Government College University Faisalabad. These strains were well-characterized, having been previously isolated and reported in earlier research [19,20]. Both strains were streaked on LB agar medium prepared according to previous research [21] to obtain pure cultures. Inoculated agar plates were incubated for 24 h at 37°C. After 24 h, both agar LB plates were assessed for pure growth and then stored at 20°C.

### *2.2. Antibiotic susceptibility testing*

Antibiotic susceptibility testing (AST) was conducted with minor modifications to the protocol described in [22]. Fresh cultures of *E. coli* and *K. pneumoniae* were adjusted to a 0.5 McFarland standard optical density (OD) in sterile saline [23], and the suspensions were uniformly swabbed onto

Mueller–Hinton agar (MHA) plates made according to [24]. Antibiotic-impregnated discs (Thermo Fisher Scientific), with tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and streptomycin (10 µg), were aseptically placed on the spot-inoculated plates. A gentamicin disc (10 µg) was also placed there, which served as the positive control. The plates were incubated at 37°C for 24 h. After incubation, the zones of inhibition around each disk were measured in millimeters and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [25] to determine the susceptibility (S), intermediate (I), or resistance (R) of the bacterial strains to each antibiotic. The test was performed in triplicate to ensure reproducibility. A negative control plate with just sterile saline spotted in the center was prepared along with the testing plates.

### *2.3. Plant extract preparation*

Plant extracts were prepared according to the protocol outlined in [26] with slight modifications. Leaves and bark of *A. nilotica* and *Z. mauritiana* were collected from a cultivation site in Aminpur Bangla, Faisalabad (GPS coordinates: 31°29'21.2"N 72°50'58.3"E). Plant parts were washed, cut into small pieces, and shade-dried for 5 days before being ground into fine powder. For extraction, 25 g of each powdered sample was separately soaked in 100 mL of solvent: distilled water (both plants) or 70% ethanol (*Z. mauritiana* leaves only) for 24 h with periodic agitation. The mixtures were filtered through Whatman® grade 1 filter paper, and the filtrates were then concentrated using a rotary evaporator (90°C for aqueous, 70°C for ethanolic extracts) to obtain semi-solid extracts, which were stored in Eppendorf tubes at 20°C.

### *2.4. Antibacterial activity assay*

The antibacterial activity of *A. nilotica* and *Z. mauritiana* extracts against *E. coli* and *K. pneumoniae* was assessed using a modified agar well diffusion assay [27]. First, semi-solid plant extracts (100 mg each) were dissolved in 1 mL of dimethyl sulfoxide (DMSO). Tryptic soy broth (TSB) was prepared according to [28]. Subsequently, 10 mL of TSB was dispensed into two 20 mL glass tubes, which were then inoculated with 100 µL of each bacterial strain and incubated at 37 °C for 24 h. For the assay, bacterial lawns were prepared by streaking the TSB cultures onto MHA plates using a sterile swab. Four wells were then punched per plate with a sterile cork borer. Each well was loaded with 100 µL of the prepared plant extracts. As solvent controls, MHA plates supplemented with DMSO (at a concentration below 1%) were prepared for each bacterial strain. Plates were allowed to pre-diffuse for 1 h in a laminar airflow cabinet before being incubated at 37°C for 24 h. Following incubation, zones of inhibition were measured in millimeters using calipers.

### *2.5. Minimum inhibitory concentration (MIC) assay*

The MIC of the plant extracts was determined using a modified microdilution method based on [29]. For each plant extract, a 100 mg/mL stock solution was prepared by dissolving the extract in DMSO. This stock was subsequently serially diluted in TSB to achieve final concentrations of 12, 10, 8, 6, and 4 mg/mL. DMSO concentration was ensured not to exceed 1% (v/v) in any well, thereby preventing solvent toxicity. For the assay, 100 µL of each extract dilution was dispensed into a 96-well microplate, followed by the addition of 100 µL of bacterial inoculum from TSB cultures. Essential controls were

included on each plate: a growth control (TSB + bacteria), a solvent control (TSB + 1% DMSO + bacteria), and a sterility control (TSB only). The microplate was incubated at 37 °C for 24 h. Following incubation, the MIC was recorded as the lowest extract concentration that showed no visible bacterial growth. This was determined by measuring the absorbance ($OD_{600}$) of each well using a microplate reader. All tests were performed in triplicate to ensure reproducibility.

### *2.6. CV biofilm assay*

This assay was adapted from the method described in a previous study [30], with some modifications. The purpose of this assay was to determine whether the MIC of each plant extract specifically inhibited biofilm formation and/or if antibiofilm activity was solely due to general antibacterial activity. Before the test, the MIC and sub-inhibitory concentrations (sub-MICs) such as ½ MIC and ¼ MIC determined for each plant extract were supplemented with Luria-Bertani (LB) broth in separate containers at 1% (v/v). Two sterile 96-well polyvinyl chloride (PVC) plates (BioPro) were used, one for each bacterial strain. 200 µL of extract-containing LB broth [21] was added to the wells of both plates, followed by inoculation with the respective bacterial strains. Afterward, plates were incubated at 37 °C for 48 h in a static incubator. After incubation, the PVC plates were decanted and rinsed three times with distilled water to remove planktonic bacteria. Subsequently, 200 µL of 0.01% crystal violet (CV) dye was pipetted into the wells of all plates, followed by incubation for 15 min. Plates were then decanted again and rinsed three times with distilled water until excess CV dye was drained. Finally, 200 µL of 95% ethanol was pipetted into the wells of all plates, and the absorbance was recorded at 570 nm ($OD_{570}$) using a microplate reader. The assay was performed in triplicate for reproducibility.

### *2.7. Growth curve assessment method*

To evaluate the impact of plant extracts on the growth kinetics of *E. coli* and *K. pneumoniae*, growth curves were generated in LB broth supplemented with the respective plant extracts as described in [31].

First, a stock solution was prepared for each plant extract (*A. nilotica* leaves and bark extracts, *Z. mauritiana* leaves and bark extracts), equivalent to its previously determined MIC. Separate sterile Erlenmeyer flasks, each containing 50 mL of sterile LB broth, were then prepared. Each flask was supplemented with the respective plant extract at 1% (v/v). A separate flask containing 50 mL of sterile LB broth, without any supplementation, served as the positive control. Each flask was then inoculated with the respective bacterial strain (*E. coli* or *K. pneumoniae*), ensuring an initial optical density of approximately 0.05 at 600 nm ($OD_{600}$). The flasks were incubated at 37 °C under continuous shaking at 150 rpm. Bacterial growth was monitored by measuring the $OD_{600}$ of the medium using a UV-Vis spectrophotometer (Thermo Fisher Scientific) at hourly intervals for 24 h. For each treatment and control, measurements were taken in triplicate. The mean $OD_{600}$ values were then plotted against time to generate the growth curves. This experiment was conducted independently for both *E. coli* and *K. pneumoniae*.

### *2.8. Statistical analysis*

Statistical analysis was performed using Welch's t-test, which accounts for unequal variances [32]. For endpoint analyses, the mean absorbance values of each plant extract were compared against the growth or positive control (uninoculated LB). In all analyses, absorbance data from three independent replicates (n = 3) were utilized for each extract and control condition. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant. All statistical computations were conducted using GraphPad Prism 9.

## 3. Results

### *3.1. Antibiotic susceptibility testing*

Both *E. coli* and *K. pneumoniae* displayed complete resistance to all tested antibiotics (Figure 1), with extremely small zones of inhibition ranging from 6 to 10 mm (Table 1). Specifically, *E. coli* exhibited resistance to tetracycline (8 mm), ampicillin (6 mm), ciprofloxacin (7 mm), and streptomycin (9 mm). Similarly, *K. pneumoniae* showed resistance to tetracycline (10 mm), ampicillin (7 mm), ciprofloxacin (6 mm), and streptomycin (8 mm). In contrast, both bacterial strains were susceptible to the positive control, gentamicin, demonstrating zones of inhibition greater than 16 mm. These findings strongly indicate a multidrug-resistant (MDR) phenotype to broad-spectrum antibiotics in both bacterial strains.

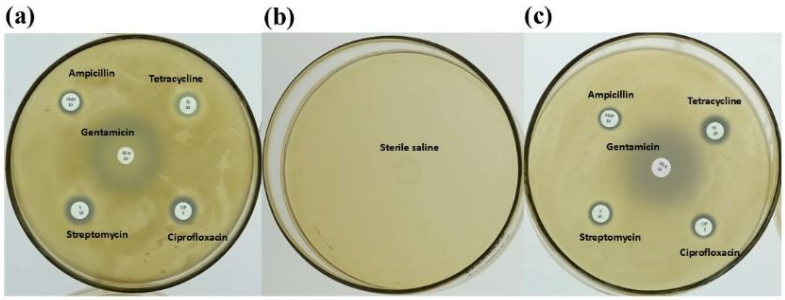


**Figure 1.** Antibiotic susceptibility testing results for *Escherichia coli* (a) and *Klebsiella pneumoniae* (c) alongside a negative control using normal saline (b). The tested antibiotic discs included tetracycline (30 µg), ampicillin (10 µg), ciprofloxacin (5 µg), and streptomycin (10 µg), with gentamicin (30 µg) serving as the positive control.

**Table 1.** Antibiotic resistance profiles of bacterial strains showing the zone of inhibition diameter (mm). R indicates resistant, whereas S indicates susceptible phenotypes.

| Bacterial strains | Tetracycline (30 µg) | Ampicillin (10 µg) | Ciprofloxacin (5 µg) | Streptomycin (10 µg) | Gentamicin (10 µg) |
|---|---|---|---|---|---|
| *Escherichia coli* | R (8.0 ± 0.5 mm) | R (6.0 ± 0.7 mm) | R (7.0 ± 0.6 mm) | R (9.0 ± 0.4 mm) | S (16.5 ± 0.7 mm) |
| *Klebsiella pneumoniae* | R (10.0 ± 0.6 mm) | R (7.0 ± 0.5 mm) | R (6.0 ± 0.8 mm) | R (8.0 ± 0.7 mm) | S (17 ± 0.8 mm) |

*3.2. Antimicrobial activity of extracts against bacterial strains*

All tested extracts from *A. nilotica* and *Z. mauritiana* exhibited significant antibacterial activity against *E. coli*, as evidenced by clear zones of inhibition (Figure 2). Among these, the hydro-ethanolic extract of *Z. mauritiana* leaves demonstrated the strongest effect, producing a 20 mm inhibition zone at 100 mg/mL (Table 2). The aqueous extract of *A. nilotica* leaves showed the second highest activity with a 17 mm zone, followed by its bark extract at 15 mm. The aqueous extract of *Z. mauritiana* bark displayed the lowest activity, yielding a 14 mm zone. Against *K. pneumoniae*, plant extracts also revealed significant antimicrobial variation. The hydro-ethanolic extract of *Z. mauritiana* leaves (100 mg/mL) again showed the highest efficacy, forming a 19 mm inhibition zone, closely followed by its aqueous bark extract with a 17 mm zone. *A. nilotica* extracts generally exhibited slightly lower activity, with the bark extract (16 mm) outperforming the leaf extract (15 mm).

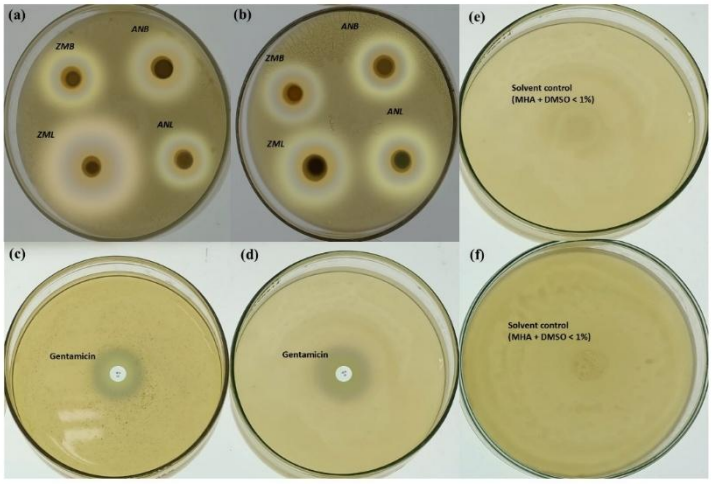


**Figure 2.** Antimicrobial activity of plant extracts (100 mg/mL) against *Escherichia coli* (a) and *Klebsiella pneumoniae* (b). Gentamicin served as the positive control for both *E. coli* (c) and *K. pneumoniae* (d). A solvent control (Muller–Hinton agar supplemented with DMSO at less than 1% concentration) was also included for *E. coli* (c) and *K. pneumoniae* (d), demonstrating no antimicrobial effects. The tested extracts are ZMB (*Ziziphus mauritiana* bark), ZML (*Ziziphus mauritiana* leaves), ANB (*Acacia nilotica* bark), and ANL (*Acacia nilotica leaves*). The zones of inhibition for each extract illustrate their efficacy against the bacterial strains.

**Table 2.** Antibacterial activity of *Acacia nilotica* and *Ziziphus mauritiana* leaves and bark extracts (100 mg/mL) against *Escherichia coli* and *Klebsiella pneumoniae* represented by inhibition zone diameters (mm).

| Extracts | *Escherichia coli* inhibition zone (mm) | *Klebsiella pneumoniae* inhibition zone (mm) |
|---|---|---|
| *Acacia nilotica* leaves extract | 17 ± 0.5 | 15 ± 0.6 |
| *Ziziphus mauritiana* leaves extract | 20 ± 0.4 | 19 ± 0.5 |
| *Acacia nilotica* bark extract | 15 ± 0.6 | 16 ± 0.4 |
| *Ziziphus mauritiana* bark extract | 14 ± 0.5 | 17 ± 0.6 |
| *Positive control (Gentamicin)* | 16 ± 0.6 | 17.5 ± 0.6 |
| *Solvent/negative control (LB+DMSO)* | 0 | 0 |

### *3.3. MIC of extracts against* E. coli *and* K. pneumoniae

The MIC of various plant extracts against *E. coli* and *K. pneumoniae* was thoroughly evaluated across concentrations from 4 to 12 mg/mL (Table S1), consistently demonstrating the antibacterial activity of all tested extracts (Figure 3). For *E. coli* (Table S2), complete growth inhibition was observed at 6 mg/mL (MIC) for all extracts ($OD_{600} \leq 0.05$), with significant bacterial growth resuming at 4 mg/mL ($OD_{600} \geq 0.3$). Similar results were obtained against *K. pneumoniae* (Table S3), with all extracts showing an MIC of 6 mg/mL, except for *Z. mauritiana* bark extract, which exhibited slightly higher residual growth ($OD_{600} = 0.01 \pm 0.0027$) at this concentration before complete loss of inhibition at 4 mg/mL ($OD_{600} = 0.5 \pm 0.004$). The majority of the recorded $OD_{600}$ values are statistically highly significant ($p \leq 0.001$, $p \leq 0.01$) with t-values ranging from −113.23 to −1.86 (df = 2.08–3.98). Control experiments validated these findings: growth controls (TSB + bacteria) showed robust growth ($OD_{600} = 0.52 \pm 0.014$ for *E. coli*; $0.62 \pm 0.019$ for *K. pneumoniae*), while solvent controls (TSB + 1% DMSO + bacteria) demonstrated comparable growth ($OD_{600} = 0.56 \pm 0.016$ and $0.68 \pm 0.020$, respectively), confirming the absence of solvent toxicity. Sterility controls (TSB only) remained negative ($OD_{600} = 0.02 \pm 0.0021$ and $0.02 \pm 0.0013$). Triplicate experiments produced consistent results, establishing the reliability of the observed MIC values and confirming the concentration-dependent antibacterial effects of all plant extracts.

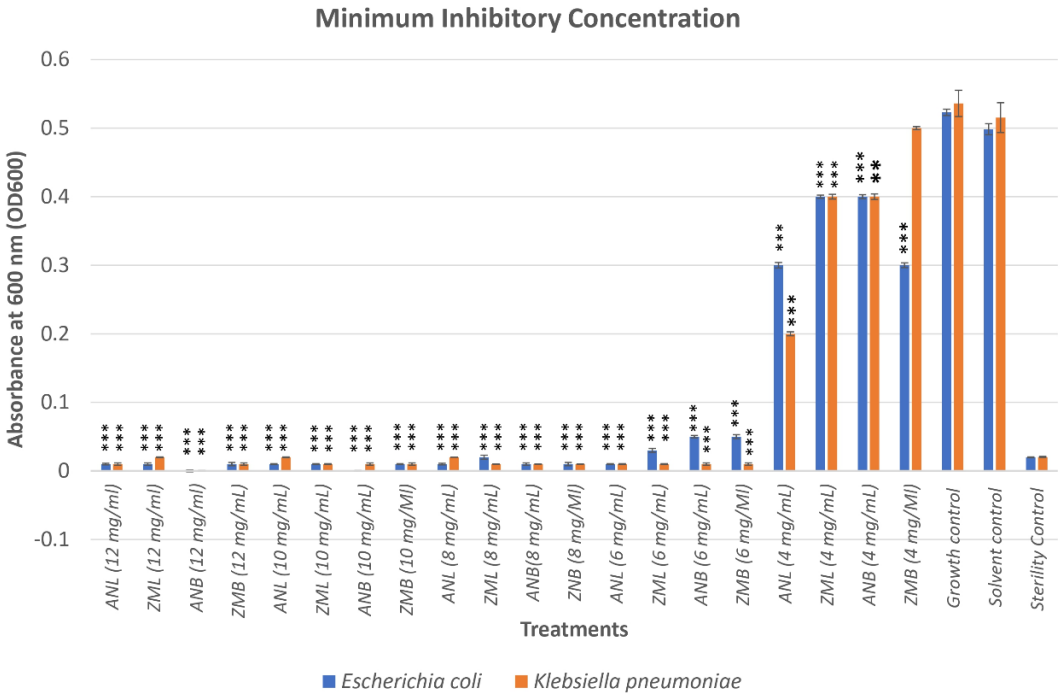


**Figure 3.** Minimum inhibitory concentrations of extracts of *Acacia nilotica* leaves (ANL), *Ziziphus mauritiana* leaves (ZML), *Acacia nilotica* bark (ANB), and *Ziziphus mauritiana* bark (ZMB) on *Escherichia coli* and *Klebsiella pneumoniae*. The absorbance at 600 nm ($OD_{600}$) indicates bacterial growth, with lower absorbance values suggesting higher inhibition with statistical significance (***$p \leq 0.001$; **$p \leq 0.01$). Each treatment was tested at concentrations of 12, 10, 8, 6, and 4 mg/mL. Growth control represents bacterial growth without inhibitors. Solvent control accounts for the effect of the solvent. Sterility control confirms the sterility of the medium.

### *3.4. Biofilm inhibition by MIC and sub-MICs of extracts*

The impact of the MIC and sub-MICs, such as ½ MIC and ¼ MIC, of plant extracts on the biofilm formation of *E. coli* and *K. pneumoniae* was evaluated after a 48-h incubation period by measuring absorbance at 570 nm. The positive control, LB broth without any plant extract, exhibited substantial biofilm formation, with mean absorbance values of 2.53 for *E. coli* (Table S4) and 3.20 for *K. pneumoniae* (Table S5). At ¼ MIC, the plant extracts demonstrated an effect on biofilm formation that was approximately equivalent to the positive control, since biofilm formation values ranged from 2.52 to 2.59 for *E. coli* and from 3.23 to 3.31 for *K. pneumoniae*. In contrast, all tested plant extracts at MIC and ½ MIC demonstrated a complete inhibition of biofilm development for both bacterial strains (Figure 4) with a high degree of statistical significance ($p \leq 0.001$) and t-values from −57.21 to −41.63 (df = 2.24–3.95). For both *E. coli* and *K. pneumoniae*, the biofilm inhibition in the presence of each plant extract was above 99% compared to the positive control (Table 3). Furthermore, the biofilm inhibition observed at sub-MICs revealed that the antibiofilm activity of plant extracts is distinct from their antibacterial activity, as concentrations lower than MIC did not inhibit bacterial growth (in MIC assay) but prevented biofilm formation. These findings strongly suggest that the minimum inhibitory concentration of each plant extract effectively impeded the development of biofilms by both *E. coli*

and *K. pneumoniae*, showing a significant difference from the unimpeded biofilm formation observed in the positive control.

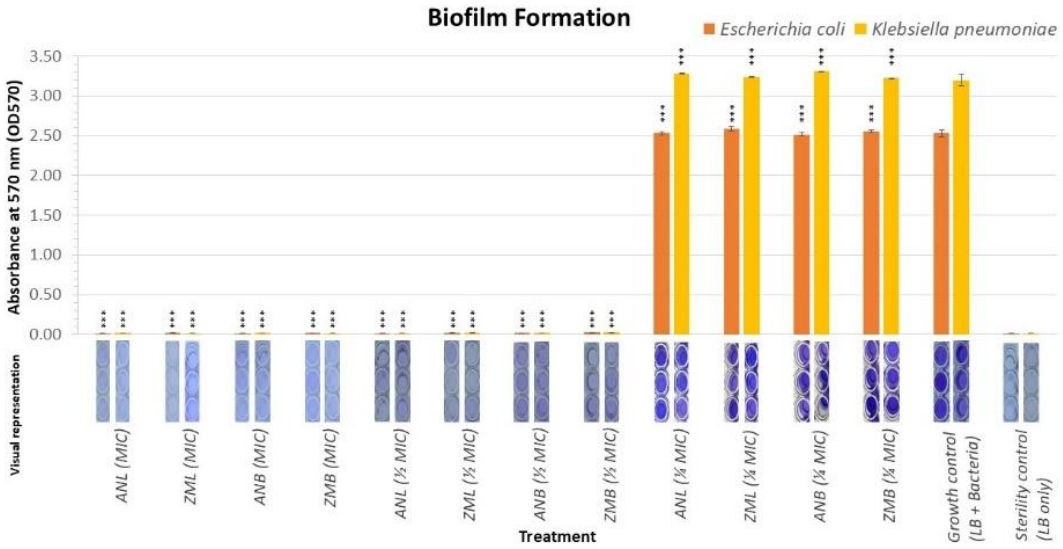


**Figure 4.** Biofilm formation at $OD_{570}$ (***$p \leq 0.001$) as demonstrated by *Escherichia coli* and *Klebsiella pneumoniae* after treatment with extract of *Acacia nilotica* leaves (ANL), *Ziziphus mauritiana* leaves (ZML), *Acacia nilotica* bark (ANB), and *Ziziphus mauritiana* bark (ZMB) at MIC, ½ MIC, and ¼ MIC.

**Table 3.** Biofilm inhibition percentages of MIC, ½ MIC, and ¼ MIC of *Acacia nilotica* and *Ziziphus mauritiana* leaves and bark extracts against *Escherichia coli* and *Klebsiella pneumoniae*.

| Extracts | *Escherichia coli* biofilm inhibition observed | *Klebsiella pneumoniae* biofilm inhibition observed |
|---|---|---|
| *Acacia nilotica* leaves extract (MIC) | 100% | 99% |
| *Ziziphus mauritiana* leaves extract (MIC) | 99% | 100% |
| *Acacia nilotica* bark extract (MIC) | 100% | 99% |
| *Ziziphus mauritiana* bark extract (MIC) | 99% | 100% |
| *Acacia nilotica* leaves extract (½ MIC) | 100% | 100% |
| *Ziziphus mauritiana* leaves extract (½ MIC) | 99% | 99% |
| *Acacia nilotica* bark extract (½ MIC) | 99% | 99% |
| *Ziziphus mauritiana* bark extract (½ MIC) | 99% | 99% |
| *Acacia nilotica* leaves extract (¼ MIC) | 0% | −3% |
| *Ziziphus mauritiana* leaves extract (¼ MIC) | −2% | −1% |
| *Acacia nilotica* bark extract (¼ MIC) | −1% | −3% |
| *Ziziphus mauritiana* bark extract (¼ MIC) | −1% | −1% |
| **Growth/positive control (LB + bacteria)** | 0% | 0% |
| **Sterility/negative control (LB only)** | 100% | 100% |

*3.5. Growth curve validates the consistent antimicrobial activity of the extracts*

The growth inhibitory effects of plant extracts on *E. coli* and *K. pneumoniae* were evaluated by monitoring their growth kinetics over a 24 h period. The growth curves, represented by $OD_{600}$ values recorded hourly, revealed a stark contrast between the untreated control and the extract-treated cultures. In the positive control (LB broth), both bacterial species exhibited typical growth patterns, reaching the stationary phase by approximately 11–14 h with peak $OD_{600}$ values of 2.20 for *E. coli* (Table S6) and 2.30 for *K. pneumoniae* (Table S7), followed by a gradual decline in the subsequent hours.

Conversely, the presence of all tested plant extracts significantly suppressed the growth of both bacterial pathogens throughout the 24-h incubation (Figure 5). For *E. coli*, the maximum $OD_{600}$ values recorded after 11 h in the presence of the extracts remained below 0.25 (Table S8), while the final $OD_{600}$ values after 24 h were below 0.12 (Table S9). Similarly, for *K. pneumoniae*, the corresponding maximum absorbance values were below 0.25 (Table S10), while the final $OD_{600}$ values were below 0.15 (Table S11). For all treatments involving both bacterial strains, the maximum and final absorbance values exhibited a high degree of statistical significance ($p \leq 0.001$), demonstrating t-values from−90.91 to −79.11 (df = 2.19 – 3.91) for both maximum ($11^{th}$) and final absorbance ($24^{th}$) values. The sustained suppression of growth over the entire 24-h period suggests that the tested plant extracts possess compounds with significant antibacterial activity against both *E. coli* and *K. pneumoniae*.

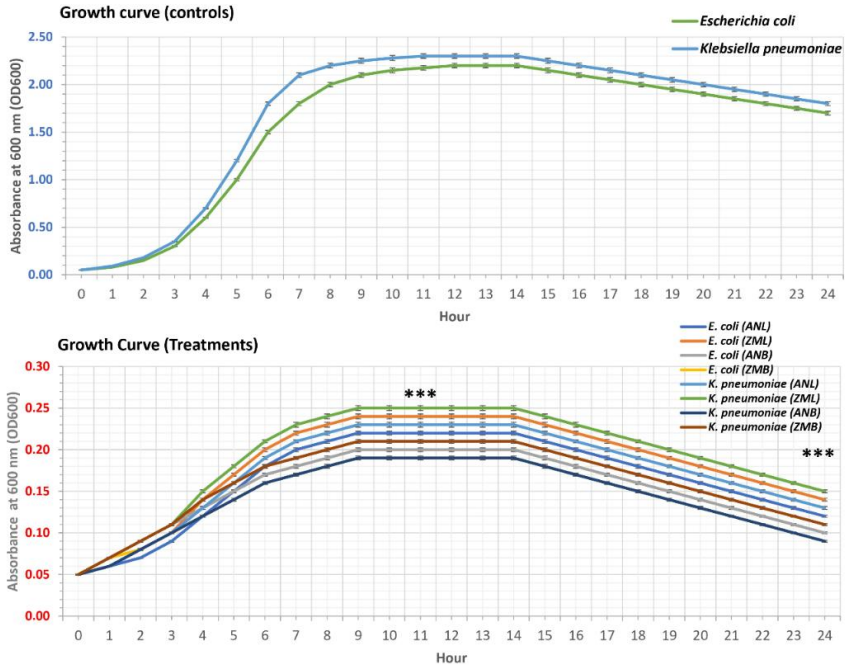


**Figure 5.** Growth kinetics of *Escherichia coli* and *Klebsiella pneumoniae* reduced dramatically by treatment with extracts of *Acacia nilotica* leaves (ANL), *Ziziphus mauritiana* leaves (ZML), *A. nilotica* bark (ANB), and *Z. mauritiana* bark (ZMB), at the respective minimum inhibitory concentration (MIC), compared to LB broth controls, over 24 h. Values represent absorbance at 600 nm ($OD_{600}$) at each time point, with statistical significance (***$p \leq 0.001$), demonstrating the inhibitory effects of the extracts on bacterial growth.

## 4. Discussion

This study evaluated the antimicrobial activity of *A. nilotica* and *Z. mauritiana* aqueous and hydro-ethanolic extracts against *E. coli* and *K. pneumoniae*. The efficacy varied significantly depending on the extract type, plant part, and concentration. Hydro-ethanolic extract showed greater antimicrobial and antibiofilm activity than aqueous extracts, due to its ability to dissolve a wider range of bioactive compounds [33,34], such as essential oils and phenolic compounds, known for their antimicrobial properties [33–36]. Several studies have reported that hydro-ethanolic extracts consistently outperform aqueous extracts in antimicrobial assays [37]. This indicates that different plant parts and extraction methods can significantly influence antimicrobial activity.

Both *Z. mauritiana* and *A. nilotica* have been investigated for their antimicrobial potential against significant pathogens like *E. coli* and *K. pneumoniae*. *A. nilotica* consistently demonstrates robust and broad-spectrum antimicrobial activity, effectively inhibiting extended-spectrum beta-lactamase (ESBL)-producing strains and significantly reducing biofilm activity in MDR pathogens like *E. coli* and *K. pneumoniae* [38–43]. Its extracts show impressive inhibition zones (20–39 mm) and low MIC values (≤12.5 mg/mL) against these resistant bacteria, with some pod lysates even exhibiting heat-resistant bactericidal properties [43,39]. In contrast, research on *Z. mauritiana* yields more varied results; while some methanolic and ethanolic leaf extracts show notable inhibition zones (up to 101.47 $mm^2$) and activity against MDR strains [44–48], and while related species like *Ziziphus lotus* also demonstrate efficacy [49], other studies report no activity against clinical isolates [43,50]. The presence of endophytic actinobacteria in *Z. mauritiana* that exhibit antibacterial activity against MDR strains [47] further complicates a direct comparison but points to an indirect antimicrobial role. The antimicrobial efficacy of *Z. mauritiana* and *A. nilotica* extracts against *E. coli* and *K. pneumoniae* varies significantly by extract type, plant part, and bacterial strain. For *Z. jujuba*, methylene chloride and n-butanol fractions showed the strongest activity against *E. coli* (0.01–0.025 mg/mL), while its ethanolic fruit extract was notably effective (0.65 ± 0.22 mg/mL), having been cited as the most susceptible among the tested microorganisms [51,52]. *Z. mauriti*ana var. *spontanea*'s ethyl-acetate extract achieved the lowest MIC against *K. pneumoniae* (2 µg/mL), with its ethanolic extract at 6 ± 0.55 µg/mL [53]. *A. nilotica*'s leaf extracts inhibited *E. coli* at 1.56–3.12 mg/mL, but strain-specific differences were observed: pod extracts required 3.12 mg/mL for *E. coli* E1/E3 and 6.25 mg/mL for E2, while bark extracts consistently showed 6.25 mg/mL across all strains [54]. In stark contrast, *A. nilotica* exhibited no detectable inhibition of *K. pneumoniae* in ethanolic or chloroform leaf extracts [55], and its gum aqueous solution required an impractically high MIC (200 mg/mL) [56]. These disparities underscore the importance of strain selection, plant part, and solvent in evaluating antimicrobial potential.

Both *Z. mauritiana* and *A. nilotica* exert their antimicrobial effects through a diverse array of phytochemicals, including common classes such as flavonoids, saponins, terpenoids, and tannins [57–59]. A predominant mechanism among phytochemicals is the disruption of bacterial cell membrane integrity, leading to the leakage of essential intracellular components and ultimately cell death [60]. This has been clearly documented for *A. nilotica* through the release of electrolytes, proteins, and nucleic acids and observed morphological damage [61,62], with specific compounds like oleic acid contributing to this effect [63]. Similarly, saponins in *Z. mauritiana* are hypothesized to increase membrane permeability [64,65], and flavonoids also cause direct membrane damage [66]. Beyond membrane effects, *Z. mauritiana* flavonoids have demonstrated unique activity by inhibiting DNA gyrase in *E. coli*, halting nucleic acid synthesis [67]. In contrast, *A. nilotica* stands out for its robust

antibiofilm activity, significantly reducing biofilm formation in *E. coli* and *K. pneumoniae* by disrupting biofilm structure and interfering with quorum sensing [62–68]. A very similar mechanism has been reported in a study where bioactive compounds halted the synthesis of polysaccharide components of biofilm by disrupting gene expression in *P. aeruginosa* [69]. Similarly, another study reported that bioactive compounds in species like *Zingiber officinale* demonstrate antimicrobial activity by disrupting peptidoglycan synthesis, which is an essential component of the bacterial cell wall [70].

While some compounds found in *A. nilotica* may contribute to efflux pump inhibition [71], direct evidence specific to *A. nilotica* in the provided literature is limited, with related studies referring to *Acacia macrostachya* [72,73]. Both plants' phytochemicals may also contribute to the inhibition of protein synthesis and DNA damage [71], as well as enzyme inhibition, affecting critical cellular processes [61,71]. The complex, multi-component nature of their extracts suggests potential synergistic effects, which could make it more challenging for bacteria to develop resistance [47]. The identification of specific bioactive compounds in *A. nilotica*, such as 3-Cyclohexane-1-Carboxaldehyde and oleic acid [62], represents a significant step toward targeted pharmaceutical development. Similarly, a study found that baicalein, a bioflavonoid, synergistically enhances tetracycline's effectiveness against *S. aureus* by inhibiting efflux pumps and key bacterial processes, i.e., bacterial growth, DNA synthesis, biofilm formation, and cellular invasiveness, suggesting it as a promising adjunctive therapy for multidrug-resistant *S. aureus* [74].

The conflicting results regarding the direct antimicrobial activity of *Z. mauritiana* extracts, particularly against *E. coli* and *K. pneumoniae* [41,50], underscore a critical limitation in the current research. This variability is likely due to several factors: differences in the specific plant part used, which contain varying phytochemical concentrations [44]; variations in extraction methods and solvents, significantly influencing the profile of bioactive compounds [57]; the concentration and dosage of the extract [57]; the heterogeneity of bacterial strains, as different clinical isolates possess distinct resistance mechanisms [47]; and variations in assay methodologies, such as disc diffusion versus broth microdilution, or differences in incubation conditions [47,57]. These factors collectively emphasize the urgent need for standardization in research methodologies for plant material collection, extraction, phytochemical characterization, and antimicrobial testing in future studies to ensure reproducibility and comparability of findings.

The toxicity profiles of both *Z. mauritiana* and *A. nilotica* extracts are highly dependent on the specific plant part and extraction solvent. Acute toxicity studies revealed high safety margins for certain extracts: an aqueous leaf extract of *Z. mauritiana* was deemed practically non-toxic with an $LD_{50}$ > 5000 mg/kg BW (body weight) [71], and an aqueous root extract of *A. nilotica* showed a high safety margin with an oral $LD_{50}$ (the dose at which a substance is lethal for 50% of animals tested) of 5000 mg/kg BW [72]. However, sub-acute (28-day) studies reveal more nuanced safety concerns, particularly regarding hepatotoxicity. Repeated administration of *A. nilotica* aqueous root extract at 500 mg/kg BW resulted in significant elevations of liver enzymes (ALT, AST, ALP, LDH) and altered lipid metabolism, suggesting potential hepatotoxicity, with a No Observable Adverse Effect Level (NOAEL) below 250 mg/kg BW [72]. Similarly, *Z. mauritiana* aqueous leaf extract at 400 mg/kg BW showed increased white blood cell (WBC) concentration, serum gamma-glutamyl transferase (GGT) activity (indicating hepatocyte damage), and alterations in liver-to-body weight ratio and mild liver inflammation. Kidney function was also affected, with changes in creatinine and electrolytes [71]. Furthermore, a methanolic root extract of *Z. mauritiana* had a considerably lower $LD_{50}$ (375 mg/kg)

and caused mortality at higher sub-acute doses, along with slight vacuolation and hepatic/tubular necrosis in the liver and kidney, even when biochemical markers remained stable [73]. This stark difference underscores the critical influence of extract type on safety. Importantly, information on chronic toxicity studies (beyond 28 days) for *Z. mauritiana* extracts is currently lacking [71].

Beyond their primary antimicrobial focus, both *Z. mauritiana* and *A. nilotica* demonstrate a wide spectrum of other pharmacological activities. Both exhibit anti-inflammatory, antioxidant, and antidiabetic/antihyperglycemic properties [65–67]. *Z. mauritiana* additionally shows anticancer, hepatoprotective, wound healing, antiulcer, neuroprotective, sedative, anxiolytic, analgesic, and immunomodulatory functions [65,66,68,73,74]. *A. nilotica*, in turn, offers glucose and lipid-lowering, neuroprotective, analgesic, antipyretic, antihypertensive, antispasmodic, antiplatelet, anti-protozoan, antiviral, antidiarrheal, anthelmintic, and antiulcer/healing effects [75,76]. A significant advantage for *A. nilotica* is the existence of promising translational findings from human clinical trials, where its extracts have been effective in treating gingivitis and bacterial vaginosis, reducing salivary *Mutans streptococci*, and promoting healing in oral ulcers and UTI pathogens [77–79]. This human data positions *A. nilotica* with a more advanced potential for direct clinical application than *Z. mauritiana*. Both *Z. mauritiana* and *A. nilotica* represent compelling candidates for the development of novel antimicrobial agents, particularly in the face of escalating antibiotic resistance. *A. nilotica* stands out for its consistently robust efficacy against multidrug-resistant and biofilm-forming *E. coli* and *K. pneumoniae*, with identified specific bioactive compounds paving the way for more targeted drug development. *Z. mauritiana*, while showing promise with its multifaceted mechanisms, necessitates further investigation to resolve conflicting antimicrobial activity reports. Furthermore, the varying toxicity profiles dependent on plant part and extraction solvent emphasize the need for comprehensive toxicological assessments, especially chronic studies, to fully ascertain safety for potential therapeutic applications. Future research should also focus on elucidating synergistic effects of compounds within crude extracts and conducting more human clinical trials to validate the observed in vitro and in vivo efficacies of both plants. Ultimately, a deeper understanding of their precise mechanisms and safety will unlock their full therapeutic potential in addressing the global challenge of antimicrobial resistance.

While these findings are promising, variations in extraction methods, plant sources, and bacterial strains limit direct comparisons. Further research should standardize extraction protocols, assess in vivo efficacy, and isolate specific bioactive compounds for drug development. Additionally, toxicity studies are needed to ensure safety for clinical use.

## 5. Conclusions

The emergence of multidrug-resistant pathogens such as *E. coli* and *K. pneumoniae* highlights an urgent need for alternative antimicrobial strategies. This study found that extracts from various plant parts, prepared with different solvents, offer a promising solution to this problem. Hydroethanolic extracts consistently demonstrated superior antimicrobial activity compared to aqueous extracts, suggesting that solvent polarity plays a crucial role in extracting effective compounds from plant tissues. However, the specific plant part used had a minimal impact on the range of antimicrobial activity. The extracts not only inhibited bacterial growth but also prevented biofilm formation. This research reinforces the potential of natural products in combating antimicrobial resistance and supports their integration into healthcare as adjunct therapies, lessening our dependence on synthetic antibiotics.

## Author contributions

Conceptualization: ARR, AA; Data curation: ARR, AA; Formal Analysis: ARR, AA; Funding acquisition: ARR, AA; Investigation: ARR, AA; Methodology: ARR, AA; Project administration: ARR, AA; Resources: ARR, AA; Supervision: ARR, AA; Validation: ARR, AA; Visualization: ARR, AA; Writing – original draft: ARR, AA; Writing – review & editing: ARR, AA.

## Use of Generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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## Conflict of interest

The authors declare they have no conflict interest in this paper.

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