

Article

Antimicrobial Activity and Antibiotic Synergy of Saponin-Enriched Bark Extracts from *Argania spinosa*: Influence of Ecogeographical Origin

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Abstract

Antimicrobial resistance represents a major global health challenge, highlighting the urgent need for alternative bioactive compounds from natural sources. This study investigated the phytochemical composition and antimicrobial potential of saponin-enriched extracts from the trunk bark of *Argania spinosa* (L.) Skeels, collected from two contrasting Algerian regions: the coastal area of Stidia (ES) and the Saharan region of Tindouf (ET). Extraction yields were comparable (approximately 12.6%). UHPLC-MS analysis revealed distinct phytochemical profiles, with ES enriched in oleanane-type saponins and flavonoids, whereas ET showed a higher abundance of bayogenin-type derivatives. Key compounds included arganine C, E, and J, as well as catechin and quercetin. Antimicrobial activity was evaluated using agar well diffusion and broth microdilution assays against clinically relevant microorganisms, including the reference strains *Staphylococcus aureus* and *Listeria innocua*, together with *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus mirabilis*, and *Candida albicans*. Both extracts exhibited broad-spectrum antimicrobial activity, although ES consistently showed lower Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal, Fungicidal Concentration (MBC)/(MFC) values than ET. MIC values ranged from 25 to 50 mg/mL for ES and from 50 to 100 mg/mL for ET. Synergistic interactions were observed between ES and gentamicin against *S. aureus* and between both extracts and kanamycin against *K. pneumoniae*. Membrane permeability assays demonstrated that both extracts increased bacterial membrane permeability, with ET producing a stronger permeabilizing effect. Atomic force microscopy of ES-treated cells revealed marked alterations in bacterial surface morphology, while molecular docking supported strong interactions of mi-saponin B and arganine derivatives with key bacterial targets. Collectively, these findings highlight the potential of *A. spinosa* bark saponins as natural antimicrobial agents and promising antibiotic adjuvants against multidrug-resistant pathogens.



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Keywords: *Argania spinosa*; antimicrobial resistance; triterpenoid saponins; antimicrobial activity; antibiotic synergy; membrane permeability; atomic force microscopy; molecular docking; phytochemical profiling; UHPLC-MS

1. Introduction

The risk of antibiotic resistance has been recognized since the advent of the first antibiotics following the discovery of penicillin [1,2]. Today, antimicrobial resistance (AMR) is recognized as one of the most serious threats to global health. Recent global estimates indicate that bacterial AMR was associated with nearly 5 million deaths worldwide in 2021, including more than 1 million deaths directly attributable to resistant infections, highlighting its growing impact on public health and healthcare systems worldwide [3]. A major driver of this phenomenon is the misuse of antibiotics in clinical and agricultural settings, which accelerates the emergence and dissemination of multidrug-resistant (MDR) organisms [4]. These pathogens, defined as microorganisms resistant to at least three classes of antimicrobials, are associated with increased morbidity, mortality, and healthcare expenditures [5]. The challenge is particularly severe in low- and middle-income countries, where self-medication, limited healthcare access, and economic constraints promote inappropriate antibiotic use [6]. Compounding this issue, the development of new synthetic antibiotics has slowed markedly, widening the therapeutic gap and highlighting the urgent need for alternative antimicrobial strategies [7].

Plant-derived secondary metabolites have gained increasing attention due to their structural complexity, chemical diversity, and broad pharmacological potential [8]. According to the World Health Organization (WHO), medicinal plants represent valuable sources of bioactive molecules or precursors for drug development, and approximately 80% of the global population relies on phytotherapy for primary healthcare [9]. Their richness in polyphenols, flavonoids, alkaloids, terpenoids, and saponins underlies a wide range of biological activities, including antimicrobial, antioxidant, and anti-inflammatory effects [9,10]. Plant-derived metabolites often act on multiple bacterial targets rather than a single pathway, a multitarget mechanism that may enhance antimicrobial efficacy while limiting resistance development [11]. In addition, many phyto-compounds have been reported to enhance antibiotic efficacy through synergistic interactions, thereby reducing the required therapeutic dose and potentially limiting the emergence of resistance [12]. However, despite these promising properties, the therapeutic application of plant-derived compounds remains limited by variability in chemical composition, insufficient standardization, and the lack of extensive in vivo validation.

Among medicinal plants, *Argania spinosa* (L.) represents a North African endemic species, naturally confined to southwestern Morocco and western Algeria [13]. In the Tindouf region (southwestern Algeria), argan populations occupy approximately 50,000 to 56,000 hectares [14], and the species has also been introduced into the coastal area of Stidia (Mostaganem) [15]. Beyond the widely commercialized argan oil [16], various organs, including leaves, roots, pulp, and bark, have long been used in traditional medicine [17]. *A. spinosa* is recognized for its richness in secondary metabolites such as triterpenoid saponins, sterols, flavonoids, and phenolic compounds [18]. Saponins, in particular, represent one of the most structurally diverse groups. Their amphiphilic architecture, composed of a hydrophobic triterpenoid backbone linked to hydrophilic sugar moieties, governs their ability to interact with biological membranes and alter permeability [19]. These molecules can interact with lipopolysaccharides (LPS) in Gram-negative bacteria and may facilitate the activity of co-administered antimicrobials [20,21]. Such properties make saponins

promising candidates for the development of novel antimicrobial or antibiotic-adjuvant strategies [22].

Limited studies have focused on saponin-enriched bark extracts of *Argania spinosa* and their combined antimicrobial and mechanistic evaluation. The present study aimed to evaluate the antimicrobial activity and antibiotic-adjuvant potential of saponin-enriched bark extracts of *Argania spinosa* by integrating phytochemical profiling, membrane permeability assays, atomic force microscopy (AFM), and molecular docking analyses. Reference strains (*Staphylococcus aureus* and *Listeria innocua*), together with clinically relevant Gram-positive and Gram-negative multidrug-resistant isolates and a fungal pathogen, were selected to assess broad-spectrum antimicrobial activity. This integrative approach provides a multi-scale perspective linking phytochemical composition to biological activity and potential mechanisms of action.

2. Materials and Methods

The experimental workflow adopted in this study is summarized in Figure 1.

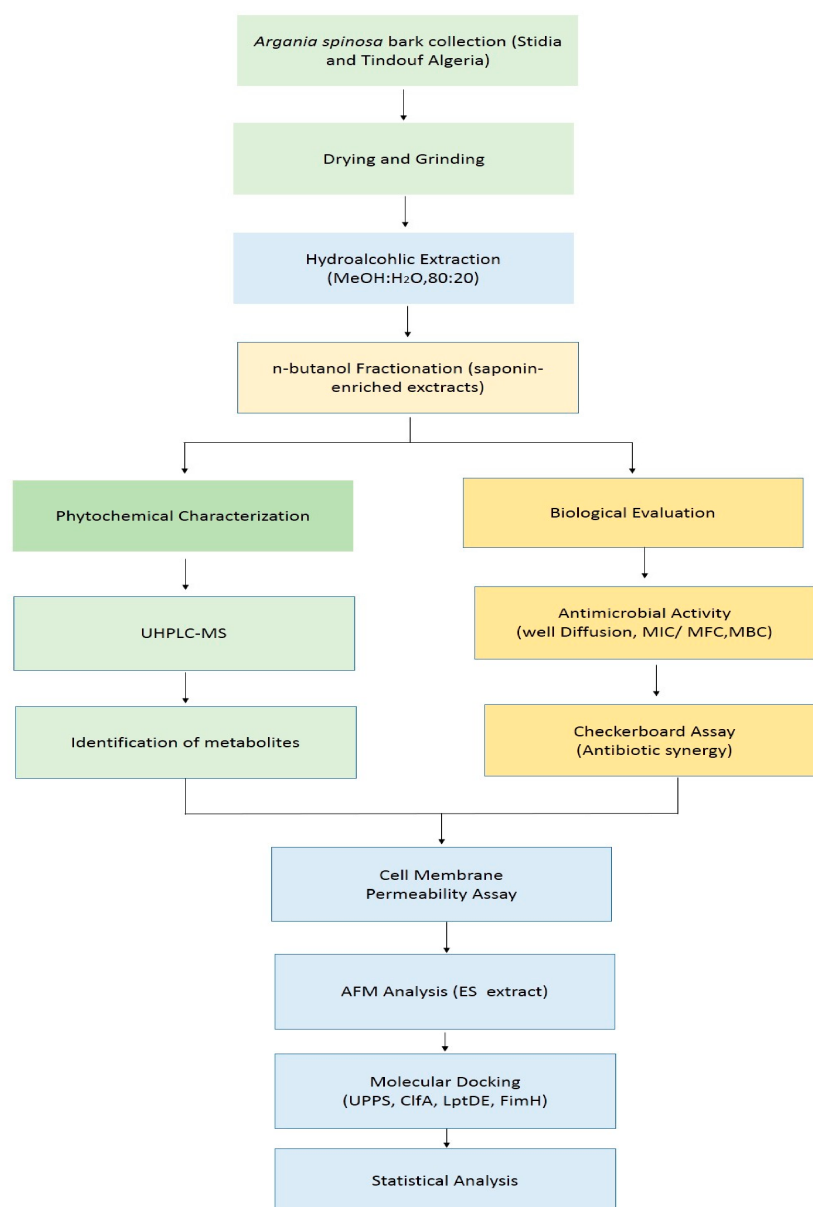


Figure 1. Experimental workflow for the phytochemical characterization and antimicrobial assessment of *Argania spinosa* bark extracts.

2.1. Plant Material

The trunk bark of *Argania spinosa* (L.) Skeels was collected from two locations in Algeria: the Tindouf region in the Sahara, situated between 27° and 29° North latitude and 7° to 9° West longitude, and Stidia in the northwest, positioned at 35°48' N latitude and 0°03' E longitude, with an elevation of 35 m. Botanical identification of the plant material was performed by Dr. Zahafi, Department of Biology, University of Mascara, Algeria. Following collection, the bark was dried at room temperature in a protected area and then ground through multiple steps to prepare it for later use.

2.2. Saponin Extraction

Twenty grams of *Argania spinosa* L. trunk bark powder were macerated in 200 mL of a methanol/water mixture (80:20, *v/v*) for 24 h at room temperature. The extract was filtered and concentrated under reduced pressure at 40 °C, then partitioned three times with saturated *n*-butanol (1:1, *v/v*). The combined butanolic fractions were evaporated at 40 °C, and the resulting residue was precipitated three times with 50 mL of ethyl ether. The final precipitate was dried to obtain a saponin-enriched fraction [23].

All solvents used for extraction were of analytical grade. Methanol, *n*-butanol, and diethyl ether were purchased from Honeywell Riedel-de Haën (Seelze, Germany).

2.3. Analysis of Secondary Metabolites of *Argania spinosa* Extracts

2.3.1. Determination of Total Phenolic Content (TPC)

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method [24,25], with minor modifications. Briefly, methanolic extract solutions (1 mg/mL) were reacted with diluted Folin–Ciocalteu reagent (1:10), followed by the addition of 7.5% Na₂CO₃. After incubation in the dark for 90 min at room temperature, absorbance was measured at 760 nm using a JENWAY 6400 spectrophotometer (Jenway, Stone, UK). Gallic acid (25–50 µg/mL) was used as the calibration standard, and results were expressed as mg gallic acid equivalents (GAE) per g dry extract.

2.3.2. Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was quantified using the aluminum chloride colorimetric assay [26,27]. Methanolic extract solutions (1 mg/mL) were reacted sequentially with NaNO₂, AlCl₃, and NaOH, and absorbance was measured at 510 nm. Catechin (200–400 µg/mL) was used as the calibration standard, and results were expressed as mg catechin equivalents (CE) per g extract.

2.3.3. Determination of Total Tannin Content (TTC)

Condensed tannin content (TTC) was determined using the vanillin–HCl colorimetric method [28,29]. Methanolic extract solutions (1 mg/mL) were mixed with vanillin reagent (4% vanillin in methanol containing 1% HCl) and incubated at 35 °C for 20 min. Absorbance was measured at 500 nm. Catechin (200–500 µg/mL) was used as the calibration standard, and results were expressed as mg catechin equivalents (CE) per g extract.

2.4. High-Performance Liquid Chromatographic Analysis of *Argania spinosa* Extracts

UHPLC–MS analysis was performed using an Agilent 1290 Infinity II system coupled to an Agilent 6546 QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), equipped with a Dual Jet Stream electrospray ionization (ESI) source. Chromatographic separation was achieved on a ZORBAX RRHD Eclipse plus C18 column (1.8 µm, 2.1 × 50 mm) maintained at 35 °C. A gradient elution was applied using water (0.1% formic acid, A) and acetonitrile (0.1% formic acid, B), from 5% to 95% B over 10 min at a flow rate of 0.4 mL/min. Data were acquired in positive and negative ESI modes using the following source parameters:

drying gas temperature, 350 °C; drying gas flow, 10 L/min; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; nebulizer pressure, 40 psi; capillary voltage, 3500 V; nozzle voltage, 500 V; fragmentor voltage, 120 V; and a mass acquisition range of m/z 100–1700.

The analysis was performed for qualitative metabolite profiling. Peak areas were used for semi-quantitative comparison only and do not reflect absolute concentrations due to differences in ionization efficiency. No HPLC–UV quantification using external calibration was performed; therefore, peak areas were used solely for the relative comparison of metabolite abundance.

Data processing was carried out using MassHunter software (Agilent Technologies, Santa Clara, CA, USA, Version 10.0.2).

2.5. Microbial Strains

Antimicrobial assays were performed against reference strains, including *Staphylococcus aureus* ATCC 25923 and *Listeria innocua* ATCC 33090, together with multidrug-resistant (MDR) clinical isolates obtained from the diagnostic microbiology laboratory of the University Hospital Center of Oran.

The clinical isolates were identified using standard microbiological procedures routinely applied in clinical microbiology laboratories. These included *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus mirabilis*, and *Candida albicans*, isolated from patients presenting with urinary, wound (pus), and vaginal infections.

The study protocol was approved by the institutional ethics committee of the University Hospital Center of Oran. No formal approval number was issued. All patient-related data were fully anonymized prior to analysis, and no personal identifying information was collected.

2.6. Isolation and Identification of Pathogenic Microorganisms

Further identification of the clinical isolates was performed as follows. Bacterial identification followed a standardized phenotypic workflow. Clinical specimens were cultured on Chapman agar for Gram-positive bacteria, and on EMB, MacConkey, and Hektoen agars for Gram-negative bacteria, as well as on Sabouraud dextrose agar (SDA) for yeast isolation [30]. Plates were incubated at 37 °C for 24–72 h.

The isolates were then examined microscopically following Gram staining, with evaluation of cell morphology and motility [31]. Identification was further confirmed by biochemical profiling using API systems (bioMérieux) and the VITEK automated system [32].

2.7. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was evaluated using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, and the inhibition zones were interpreted according to the recommendations of the EUCAST (CA-SFM/EUCAST, 2023) [33]. For yeast strains, antifungal susceptibility testing was additionally performed using the disk diffusion method following the standardized guidelines of the Clinical and Laboratory Standards Institute (CLSI M44-A2, 2009) [34], ensuring a reliable assessment of antifungal activity.

Gram-negative isolates were tested against a broad panel of antibiotics, while *Staphylococcus epidermidis* was evaluated against selected antibiotics. *Candida albicans* was tested against two antifungal agents.

The antibiotic panel included β -lactams: amoxicillin–clavulanic acid (AMC, 30 μ g), cefotaxime (CTX, 30 μ g), ceftazidime (CAZ, 30 μ g), piperacillin (PRL, 100 μ g), and oxacillin (OX, 1 μ g); aminoglycosides: gentamicin (GEN, 10 μ g), kanamycin (KAN, 30 μ g), neomycin (N, 30 μ g), and tobramycin (TOB, 10 μ g); fluoroquinolones: ciprofloxacin (CIP, 5 μ g) and pefloxacin (PEF, 5 μ g); tetracyclines: doxycycline (DO, 30 μ g); macrolides: erythromycin

(E, 15 µg); and other antibiotics: fusidic acid (FA, 10 µg), chloramphenicol (C, 30 µg), nitrofurantoin (F, 50 µg), and sulfamethoxazole–trimethoprim (SXT, 25 µg).

The antifungal agents included nystatin (NYS, 50 µg) and terbinafine (TER, 30 µg).

2.8. Antimicrobial Activity of Crude Bark Extracts Enriched in Saponins

2.8.1. Agar Well Diffusion Method

The dried saponin-enriched extracts were dissolved in dimethyl sulfoxide (DMSO; BDH Chemicals Ltd., Poole, UK) to obtain a stock solution of 200 mg/mL. For the agar well diffusion assay, the extracts were diluted to a final concentration of 100 mg/mL.

Antimicrobial activity was evaluated using agar well diffusion and broth microdilution methods. For the diffusion assay, bacterial suspensions were adjusted to a 0.5 McFarland standard ($\approx 1 \times 10^8$ CFU/mL; $OD_{625} = 0.08\text{--}0.10$). Wells (6–8 mm in diameter) were punched into Mueller–Hinton agar, and 30 µL of each extract solution (100 mg/mL) was added to each well. Plates were pre-incubated at 4 °C for 2 h to facilitate diffusion, followed by incubation at 37 °C for 24 h [35].

2.8.2. Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method as previously described [36], with minor modifications. Briefly, 50 µL of Mueller–Hinton (MH) broth (or Sabouraud dextrose broth for fungi) was dispensed into 96-well microplates, followed by the addition of 50 µL of ES or ET extracts (200 mg/mL in DMSO).

Two-fold serial dilutions were performed to obtain final concentrations ranging from 100 to 0.78 mg/mL. Each well was inoculated with 5 µL of microbial suspension adjusted to 0.5 McFarland standard.

Positive control wells contained inoculated broth without extract, while negative control wells contained broth and TTC reagent without microorganisms. After incubation at 37 °C for 24 h, 40 µL of 2 mg/mL 2,3,5-triphenyltetrazolium chloride (TTC) was added to each well, followed by an additional incubation for 1 h.

The final concentration of DMSO did not exceed 1% (*v/v*) and showed no inhibitory effect on microbial growth.

2.8.3. Minimum Bactericidal and Fungicidal Concentrations (MBC/MFC)

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by subculturing 5 µL aliquots from wells showing no visible growth after the MIC assay onto Mueller–Hinton agar (for bacteria) or Sabouraud dextrose agar (for fungi), followed by incubation at 37 °C for 24 h, according to established protocols [37].

2.9. Checkerboard Assay

Bacterial suspensions were adjusted spectrophotometrically to the 0.5 McFarland standard ($OD_{625} = 0.08\text{--}0.10$), corresponding approximately to 1×10^8 CFU/mL, and subsequently diluted in Mueller–Hinton broth to obtain a final inoculum density of approximately 5×10^5 CFU/mL in each well. The assay was performed in sterile 96-well microplates using the checkerboard microdilution method, as previously described [38]. Gentamicin (4–256 µg/mL) was tested against *Staphylococcus aureus* ATCC 25923, while kanamycin (4–256 µg/mL) was tested against *Klebsiella pneumoniae*, either alone or in combination with crude saponins (0.78–100 mg/mL), prepared by serial two-fold dilutions from the highest to the lowest concentration. These strains were selected as representative models of Gram-positive and Gram-negative bacteria, respectively, due to their clinical relevance and frequent involvement in multidrug-resistant infections. Plates were incubated

at 37 °C for 24 h, and the fractional inhibitory concentration index (FICI) was calculated to evaluate the interaction between antibiotics and extracts, as previously described [38]. Interactions were interpreted as synergistic ($FICI \leq 0.5$), additive ($0.5 < FICI \leq 1$), indifferent ($1 < FICI \leq 4$), or antagonistic ($FICI \geq 4$).

2.10. Bacterial Cell Permeability

Bacterial suspensions ($OD_{625} < 0.1$) were prepared in 30 mL of mineral medium, prepared as previously described by [39], and used for all permeability assays. In 96-well microplates, 100 μ L of bacterial suspension was mixed with 100 μ L of each extract in wells containing the same medium. To assess cell membrane permeability, 20 μ L of 0.1% crystal violet solution was added to each well, and the plates were incubated at 30 °C for 15 min, as previously described [38]. The samples were then centrifuged at $4000 \times g$ for 10 min at 20 °C, and 70 μ L of the supernatant was transferred to a new microplate. Absorbance was measured at 590 nm. Crystal violet uptake was expressed as a percentage relative to untreated control cells, reflecting changes in bacterial membrane permeability.

2.11. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was used to investigate nanoscale alterations in bacterial cell surface morphology following exposure to the extracts. The *Stidia* extract (ES, 100 mg/mL in DMSO) was tested against *Staphylococcus aureus* ATCC 25923 and a clinical isolate of *Klebsiella pneumoniae*. This concentration was selected based on the MIC value (50 mg/mL) to ensure detectable alterations in bacterial cell surface morphology.

Bacterial suspensions were incubated with the extract for 30 min at 37 °C, while untreated cells served as controls. After incubation, cells were gently washed twice with phosphate-buffered saline (PBS, pH 7.4) and immobilized on poly-L-lysine-coated mica substrates.

AFM imaging was performed in liquid tapping mode using silicon nitride cantilevers (spring constant $k = 0.03$ – 0.1 N/m, tip radius ≤ 10 nm) [40]. Topographic and deformation images were acquired at scan sizes ranging from 0.5 to 2 μ m with a resolution of 512×512 pixels. The acquired images were used to evaluate changes in bacterial surface morphology and deformation patterns induced by the extract. Representative images were selected for qualitative comparison between treated and untreated cells.

2.12. Molecular Docking Analysis

Molecular docking was performed to evaluate the antibacterial potential of phytochemicals identified in the *Argania spinosa* bark extract from *Stidia* (ES) against key bacterial proteins involved in the survival and pathogenicity of *Staphylococcus aureus* and *Klebsiella pneumoniae*.

The three-dimensional structures of the target proteins were retrieved from the Protein Data Bank (PDB) [41]:

- Undecaprenyl diphosphate synthase (UPPS, PDB ID: 4H8E);
- Clumping factor A (ClfA, PDB ID: 1N67);
- Lipopolysaccharide transporter (LptDE, PDB ID: 5IV9);
- Fimbrial adhesin (FimH, PDB ID: 9AT9).

Ligand structures (phytochemicals) were obtained from the PubChem database [42]. Reference antibiotics (ampicillin, gentamicin, and kanamycin) were included for comparison.

Protein preparation was carried out using AutoDock Tools v.1.5.7. [43], including removal of water molecules, addition of polar hydrogen atoms, assignment of charges, and optimization of side-chain orientations. Molecular docking simulations were performed using AutoDock Vina v.1.2.0 [44,45]. For each ligand, up to eight conformations were generated, and the conformation with the lowest binding energy (kcal/mol) was selected

for further analysis. Protein–ligand interactions were visualized and analyzed using Discovery Studio Visualizer v. 21.1.

2.13. Statistical Analysis

All experiments were performed in triplicate, and results are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the Shapiro–Wilk test. Differences between the ES and ET extracts were analyzed using Student's *t*-test, while comparisons among strains were performed using one-way analysis of variance (ANOVA). For membrane permeability assays, the effects of extract type, concentration, and their interaction were evaluated using two-way ANOVA. Post hoc multiple comparisons were performed using Tukey's test. Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Phytochemical Composition of *Argania spinosa* (L.) Bark Extracts

In this study, the extraction yield and phytochemical composition of the ES and ET extracts obtained from the trunk bark of *Argania spinosa* (L.) Skeels were evaluated. The total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) were quantified and expressed as mg per gram of dry extract (mg/g). Results are presented as mean $\pm$ standard deviation (Table 1). The extraction yields were comparable between ES and ET, reaching $12.61 \pm 0.06\%$ and $12.65 \pm 0.03\%$, respectively. Similarly, TPC values showed no notable variation, with values of 45.20 ± 0.03 and 44.07 ± 0.03 mg GAE/g, respectively. TTC values were also comparable, reaching 509.13 ± 0.13 mg CE/g for ES and 503.27 ± 0.13 mg CE/g for ET. In contrast, TFC was markedly higher in ES (455.15 ± 0.17 mg CE/g) than in ET (187.85 ± 0.10 mg CE/g), indicating a substantial difference in flavonoid accumulation between the two extracts. No significant differences were observed for extraction yield, TPC, or TTC ($p > 0.05$), whereas TFC was significantly higher in ES than in ET ($p < 0.05$).

Table 1. Extraction yields and phytochemical contents of the ES and ET saponin-enriched *A. spinosa* bark extracts.

Extracts	Yield (%)	TPC (mg/g)	TFC (mg/g)	TTC (mg/g)
ES	12.61 ± 0.06	45.20 ± 0.03	455.15 ± 0.171	509.13 ± 0.126
ET	12.65 ± 0.03	44.07 ± 0.03	187.85 ± 0.101	503.27 ± 0.128

3.2. Comparative UHPLC–MS Analysis of *Argania spinosa* Bark Extracts

UHPLC–MS analysis revealed qualitative differences in the phytochemical composition of the ES and ET bark extracts. The corresponding chromatographic profiles are presented in Figures 2 and 3. Several metabolites were detected in both extracts, including the triterpenoid saponins arganine C, arganine J, mi-saponin B, and arganine E, as well as the flavonoid catechin. Differences in the relative abundance of these compounds were observed between the extracts.

The ET extract was characterized by the presence of several metabolites that were not detected in ES, including the flavonoids quercetin-O-pentoside and quercetin-3-O-galactoside (hyperoside), as well as the triterpenoid saponins 3-O-bayogenin, mi-saponin A, and arganine G.

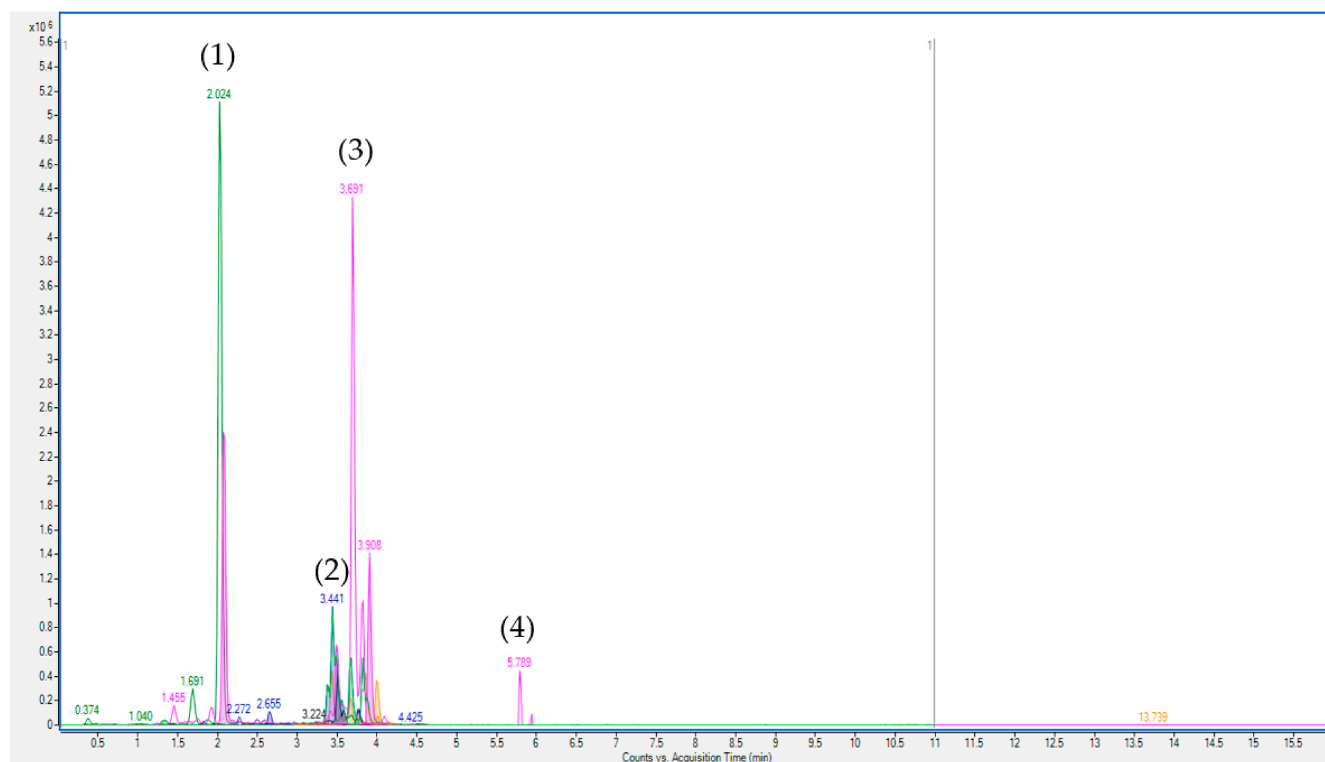


Figure 2. UHPLC–MS chromatogram of the ES extract acquired in positive and negative ESI modes. Major annotated peaks correspond to (1) Catechin, (2) Myricetin, and (3) Mi-saponin B, (4) Arganine J. Peak areas are presented as relative abundances and were used for semi-quantitative comparison of the detected metabolites.

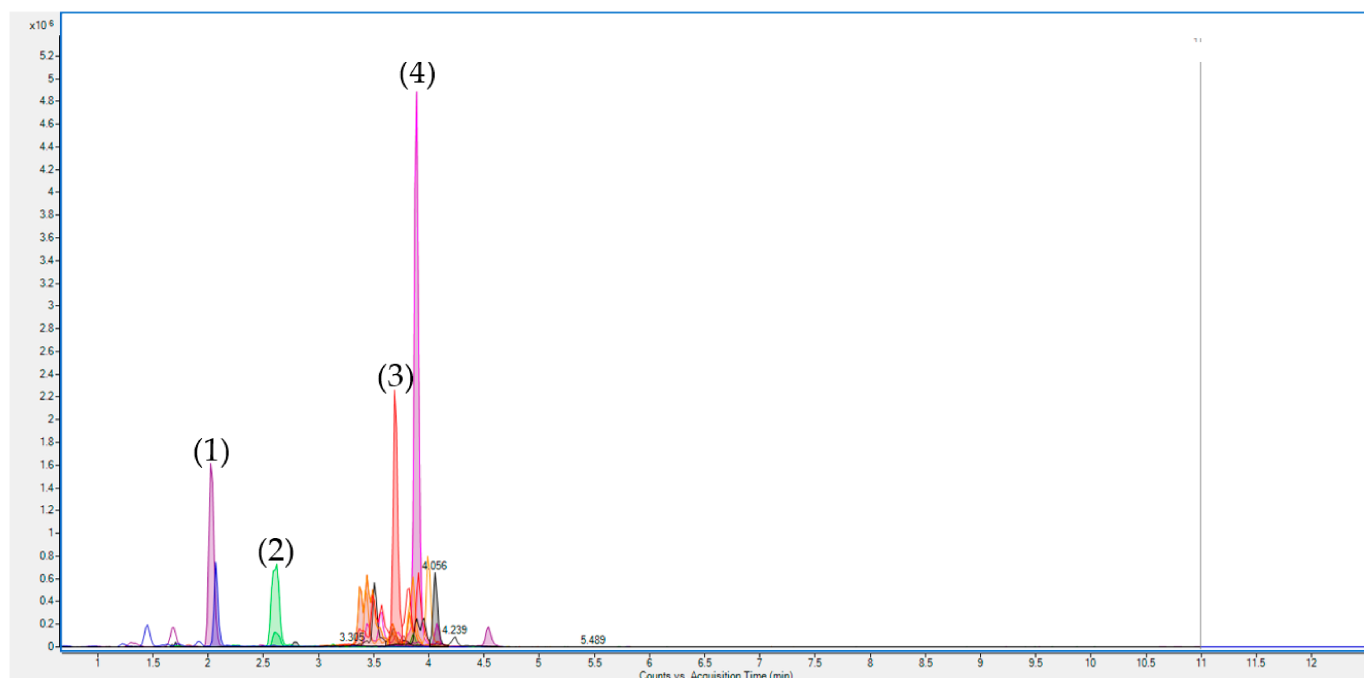


Figure 3. UHPLC–MS chromatogram of the ET extract acquired in positive and negative ESI modes. Major annotated peaks correspond to (1) Catechin, (2) Hyperoside, (3) Arganine E, and (4) Mi-saponin A. Peak areas are presented as relative abundances and were used for semi-quantitative comparison of the detected metabolites.

In contrast, the ES extract was characterized by the presence of myricetin, which was not detected in ET. In addition to these qualitative differences, variations in the relative abundance of metabolites common to both extracts were observed. The complete list of identified compounds, together with their retention times, molecular masses, and relative peak areas, is presented in Table 2.

Table 2. Comparative semi-quantitative UHPLC–MS analysis of selected flavonoids and triterpenoid saponins detected in the ES and ET bark extracts.

Compound	Formula	ES RT (min)	ES Peak Area	ET RT (min)	ET Peak Area
Catechin	C ₁₅ H ₁₄ O ₆	1.34	284,658	2.07	2,155,092
Quercetin	C ₁₅ H ₁₀ O ₇	3.77	454,101	2.62	689,811
Myricetin	C ₁₅ H ₁₀ O ₈	2.84	71,252	ND	ND
Quercetin-O-pentoside	C ₂₀ H ₁₈ O ₁₁	ND	ND	2.79	224,706
Hyperoside	C ₂₁ H ₂₀ O ₁₂	ND	ND	2.61	3,883,985
Arganine G	C ₄₇ H ₇₆ O ₁₉	ND	ND	3.86	545,631
3-O-Bayogenin	C ₅₇ H ₉₂ O ₂₆	ND	ND	4.06	2,280,195
Mi-saponin A	C ₅₈ H ₉₄ O ₂₇	ND	ND	3.89	1.50 × 10 ⁷
Arganine C	C ₅₈ H ₉₄ O ₂₈	3.52	2,223,418	3.67	1,090,914
Arganine J	C ₆₂ H ₁₀₀ O ₃₀	4.01	1,466,815	3.86	2,631,685
Mi-saponin B	C ₆₃ H ₁₀₂ O ₃₁	3.91	5,163,479	3.71	9,286,538
Arganine E	C ₆₃ H ₁₀₂ O ₃₂	3.46; 3.67	2,132,088; 5,660,354	3.44	5,454,717

Footnote: Data were acquired in positive and negative electrospray ionization (ESI) modes. Retention times (RT) are expressed in minutes. Peak areas are reported as relative abundances. Two isomeric peaks were detected in ES. ND: not detected.

3.3. Antibiotic Susceptibility and Multidrug Resistance Profiles of the Tested Pathogenic Isolates

The antibiotic susceptibility profiles of the tested pathogenic isolates are presented in Table 3.

Table 3. Antibiotic and Antifungal Susceptibility Profiles of Tested Pathogenic Microorganisms.

Antibiotic Tested	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>S. marcescens</i>	<i>P. mirabilis</i>	<i>S. epidermidis</i>	<i>C. albicans</i>
KAN, 30 µg	S	S	NI	S	NT	NT
AMC, 30 µg	R	R	R	R	NT	NT
AMP, 10 µg	R	R	R	S	NT	NT
CTX, 30 µg	R	R	R	I	NT	NT
C, 30 µg	R	I	S	S	NT	NT
CIP, 5 µg	R	R	R	R	NT	NT
DO, 30 µg	NT	NT	NT	NT	S	NT
E, 15 µg	NT	NT	NT	NT	R	NT
FE, 50 µg	NT	NT	NT	NT	NI	NT
GEN, 10 µg	R	R	R	R	NT	NT
NA, 30 µg	R	R	S	R	NT	NT
NYS, 50 µg	NT	NT	NT	NT	NT	NI
OX, 1 µg	NT	NT	NT	NT	R	NT
PEF, 5 µg	R	R	S	S	NT	NT
PRL, 100 µg	R	R	R	S	NT	NT
SXT, 25 µg	R	R	R	R	NT	NT
TER, 30 µg	NT	NT	NT	NT	NT	NI
TOB, 10 µg	R	S	S	S	NT	NT

Footnote: Antibiotic abbreviations are defined in Section 2.7; S: susceptible; I: susceptible, increased exposure; R: resistant; NI: not interpreted; NT: not tested. Interpretations were assigned according to EUCAST (2023) [33] breakpoints specific to each organism-antimicrobial combination, where applicable. *Staphylococcus aureus* ATCC 25923, used in the gentamicin synergy assays, is a quality-control reference strain with a documented gentamicin-susceptible phenotype.

As shown in Table 3, the tested Gram-negative isolates (*E. coli*, *K. pneumoniae*, *S. marcescens*, and *P. mirabilis*) exhibited a high level of resistance to multiple antibiotics, confirming their classification as multidrug-resistant (MDR) strains. Resistance was consistently observed against β -lactams, including amoxicillin/clavulanic acid and ampicillin, as well as against trimethoprim/sulfamethoxazole.

Among aminoglycosides, Kanamycin remained effective against *E. coli*, *K. pneumoniae*, and *P. mirabilis*, whereas gentamicin showed a resistant profile across all tested isolates. Tobramycin retained activity against *K. pneumoniae* and *P. mirabilis*. Resistance to fluoroquinolones was also observed, as indicated by the resistance profiles to ciprofloxacin and nalidixic acid in most isolates.

For the Gram-positive isolate (*S. epidermidis*), erythromycin and oxacillin exhibited resistant phenotypes, whereas fosfomycin could not be categorically interpreted.

Regarding the fungal isolate (*C. albicans*), inhibition zones were observed for nystatin and terbinafine. The corresponding inhibition diameters are presented in Table 3.

3.4. Comparative Antimicrobial Activity of Argania spinosa Bark Extracts Against Tested Microbial Strains

3.4.1. Antimicrobial Activity Assessed by Agar Well Diffusion Method

Marked differences in antimicrobial activity were observed according to both the microbial strain and the extract tested. At a concentration of 100 mg/mL, both extracts inhibited the growth of Gram-positive bacteria, Gram-negative bacteria, and *C. albicans*, although with varying inhibition zone diameters. The antimicrobial activity of ES and ET extracts against the tested microbial strains is presented in Table 4.

Table 4. Antimicrobial activity of ES and ET extracts against Gram-positive bacteria, Gram-negative bacteria, and *Candida albicans*, expressed as inhibition zone diameters (mm).

Strain	ES (100 mg/mL)	ET (100 mg/mL)
<i>S. aureus</i> ATCC 25923	34 $\pm$ 1	24 $\pm$ 1
<i>L. innocua</i> 33090	34.66 $\pm$ 0.57	14.66 $\pm$ 0.57
<i>S. epidermidis</i>	30.33 $\pm$ 0.57	17.66 $\pm$ 1.15
<i>E. coli</i>	18.33 $\pm$ 0.57	25.33 $\pm$ 0.57
<i>P. mirabilis</i>	14 $\pm$ 1	25 $\pm$ 1
<i>S. marcescens</i>	13.66 $\pm$ 0.57	23 $\pm$ 1
<i>K. pneumoniae</i>	16 $\pm$ 1	20.33 $\pm$ 1.52
<i>C. albicans</i>	35.66 $\pm$ 0.57	17.33 $\pm$ 0.57

Footnote: Inhibition zone diameters are expressed in millimeters (mm). All measurements were performed at an extract concentration of 100 mg/mL.

The ES extract exhibited the strongest activity against *Staphylococcus aureus* ATCC 25923, *L. innocua* ATCC 33090, *S. epidermidis*, and *C. albicans*, with inhibition zones ranging from 30.33 $\pm$ 0.57 to 35.66 $\pm$ 0.57 mm. Notably, ES also showed measurable activity against Gram-negative bacteria, including *E. coli*, *P. mirabilis*, *S. marcescens*, and *K. pneumoniae*.

The ET extract displayed antimicrobial activity against all tested strains and produced larger inhibition zones against several Gram-negative bacteria, particularly *E. coli*, *P. mirabilis*, *S. marcescens*, and *K. pneumoniae*, compared with the ES extract. However, activity varied among individual strains, indicating differences in susceptibility rather than a strict Gram-positive/Gram-negative selectivity pattern. ES exhibited significantly larger inhibition zones than ET against *S. aureus*, *L. innocua*, *S. epidermidis* and *C. albicans* ($p < 0.05$), whereas ET showed significantly greater activity against *E. coli*, *P. mirabilis*, *S. marcescens* and *K. pneumoniae* ($p < 0.05$).

3.4.2. Determination of Minimum Inhibitory, Bactericidal, and Fungicidal Concentrations of *Argania spinosa* Bark Extracts

To further evaluate their antimicrobial potential, MIC, MBC, and MFC values were determined for both extracts against the tested microorganisms (Table 5).

Table 5. MIC, MBC, and MFC values (mg/mL) of *Argania spinosa* bark extracts from Stidia (ES) and Tindouf (ET) against the tested microbial strains.

Strain	ES MIC	ES MBC/MFC	ET MIC	ET MBC/MFC
<i>L. innocua</i> ATCC 33090	25	50	50	100
<i>S. aureus</i> ATCC 25923	50	100	50	100
<i>S. epidermidis</i>	25	50	50	100
<i>E. coli</i>	25	50	50	100
<i>P. mirabilis</i>	50	100	50	100
<i>S. marcescens</i>	50	100	100	200
<i>K. pneumoniae</i>	50	100	100	200
<i>C. albicans</i>	50	100	50	100

Footnote: MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration. Values are expressed in mg/mL. MBC/MFC values correspond to bactericidal or fungicidal endpoints depending on the tested microorganism.

The ES extract exhibited MIC values ranging from 25 to 50 mg/mL and MBC/MFC values ranging from 50 to 100 mg/mL against the tested microorganisms. In contrast, the ET extract showed MIC values ranging from 50 to 100 mg/mL and MBC/MFC values ranging from 100 to 200 mg/mL, with the highest values recorded for *S. marcescens* and *K. pneumoniae*. Comparison of the antimicrobial parameters revealed significantly lower MIC and MBC/MFC values for ES than for ET ($p = 0.021$).

3.5. Checkerboard Microdilution Analysis of *Argania spinosa* Extracts in Combination with Aminoglycosides

The combined antibacterial effects of ES and ET extracts with aminoglycosides were evaluated using the checkerboard microdilution assay against *S. aureus* ATCC 25923 and *K. pneumoniae*. The results are presented in Table 6.

Table 6. Interactions between the extracts and aminoglycosides determined by checkerboard microdilution assay against *S. aureus* ATCC 25923 and *K. pneumoniae*.

Bacterial Strain	Extract	Antibiotic	MIC-E (mg/mL)	MIC-A (μg/mL)	MIC-EC (mg/mL)	MIC-AC (μg/mL)	FICI	Interaction
<i>S. aureus</i> ATCC 25923	ES	GEN	50	256	25	64	0.50	Synergistic
<i>S. aureus</i> ATCC 25923	ET	GEN	50	256	50	64	1.004	Additive
<i>K. pneumoniae</i>	ES	KAN	100	128	50	64	0.50	Synergistic
<i>K. pneumoniae</i>	ET	KAN	100	128	25	64	0.50	Synergistic

Footnote: GEN, gentamicin; KAN, kanamycin; MIC-E, MIC of extract alone; MIC-A, MIC of antibiotic alone; MIC-EC, MIC of extract in combination; MIC-AC, MIC of antibiotic in combination; FICI, fractional inhibitory concentration index. Interactions were classified as synergistic ($FICI \leq 0.5$), additive ($>0.5-1.0$), indifferent ($>1.0-4.0$), or antagonistic (≥ 4.0).

The checkerboard assay demonstrated a synergistic interaction between ES and gentamicin against *S. aureus* ATCC 25923 ($FICI = 0.50$), whereas the ET–gentamicin combination showed an additive effect ($FICI = 1.004$). Against *K. pneumoniae*, both ES–kanamycin and ET–kanamycin combinations exhibited synergistic interactions, with $FICI$ values of 0.50.

A reduction in aminoglycoside MIC values was observed in all tested combinations. For *S. aureus*, the MIC of gentamicin decreased from 256 to 64 μg/mL in the presence of either ES or ET. Similarly, for *K. pneumoniae*, the MIC of kanamycin decreased from 128

to 64 µg/mL when combined with either extract. However, despite the similar reduction in gentamicin MIC, only the ES–gentamicin combination met the criterion for synergism, whereas the ET–gentamicin combination produced an additive effect.

Collectively, these results indicate that *A. spinosa* bark extracts can enhance the antibacterial activity of aminoglycosides, with the magnitude of the interaction depending on the extract–antibiotic combination tested.

3.6. Effect of *Argania spinosa* Bark Extracts on Bacterial Cell Membrane Permeability

The effect of ES and ET extracts on bacterial membrane permeability was evaluated against *S. aureus* ATCC 25923 and *K. pneumoniae*. The results are presented in Figure 4.

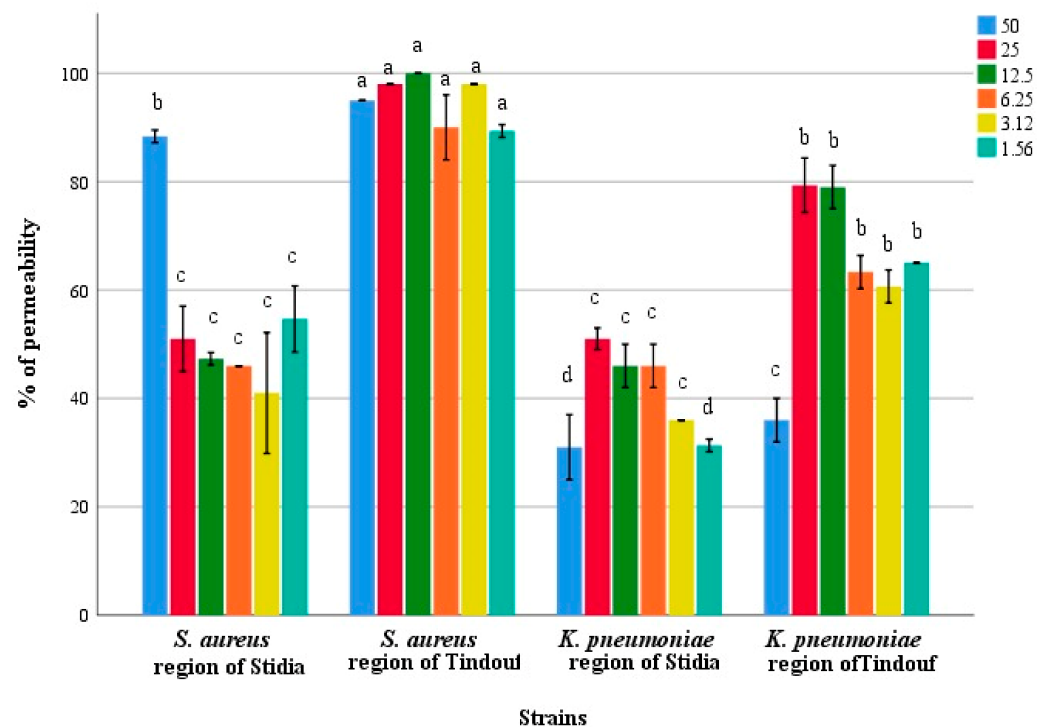


Figure 4. Comparative analysis of bacterial cell membrane permeability induced by *Argania spinosa* bark extracts in Gram-positive and Gram-negative bacteria. Data are expressed as mean $\pm$ SD ($n = 3$). Different lowercase letters indicate statistically significant differences among treatments according to the post hoc test ($p < 0.05$), whereas treatments sharing the same lowercase letter are not significantly different.

For *S. aureus* ATCC 25923, both extracts increased membrane permeability across the tested concentration range. Permeability values ranged from 42% to 89% for ES and from 89% to 100% for ET, indicating a stronger membrane-permeabilizing effect of the ET extract. In *K. pneumoniae*, permeability values were generally lower for ES than for ET. The ES extract induced permeability values ranging from 31% to 51%, whereas ET produced values between 36% and 79%. Unlike *S. aureus*, the permeability response of *K. pneumoniae* did not exhibit a clear concentration-dependent trend, with moderate fluctuations observed among the tested concentrations. Two-way ANOVA revealed significant effects of extract type, concentration, and their interaction on membrane permeability for both bacterial strains ($p < 0.0001$ for all factors). Post hoc comparisons identified significant differences among treatments, as indicated by different letters above the bars in Figure 4. Overall, membrane permeability was significantly influenced by extract type, concentration, and bacterial strain, with ET generally producing higher values than ES. However, these effects were not directly associated with antibacterial potency, since ES consistently exhibited

lower MIC values against most tested microorganisms despite inducing lower permeability levels. These findings suggest that membrane permeabilization alone may not fully explain the antimicrobial activity of the extracts and that additional mechanisms may contribute to bacterial growth inhibition. Given its superior antibacterial activity, the ES extract was selected for further nanoscale characterization by atomic force microscopy (AFM).

3.7. AFM Visualization of Membrane Damage in *Staphylococcus aureus* and *Klebsiella pneumoniae* Induced by the ES Extract of *Argania spinosa*

Atomic force microscopy (AFM) was employed to investigate the nanoscale effects of the ES extract on the bacterial cell envelope. Under control conditions, both *S. aureus* and *K. pneumoniae* exhibited smooth and homogeneous surfaces, indicative of intact envelope integrity. Figure 5A showed a relatively uniform mechanical response, consistent with preserved cellular structure. Similarly, untreated *K. pneumoniae* cells (Figure 5D) displayed regular surface morphology without evident structural abnormalities.

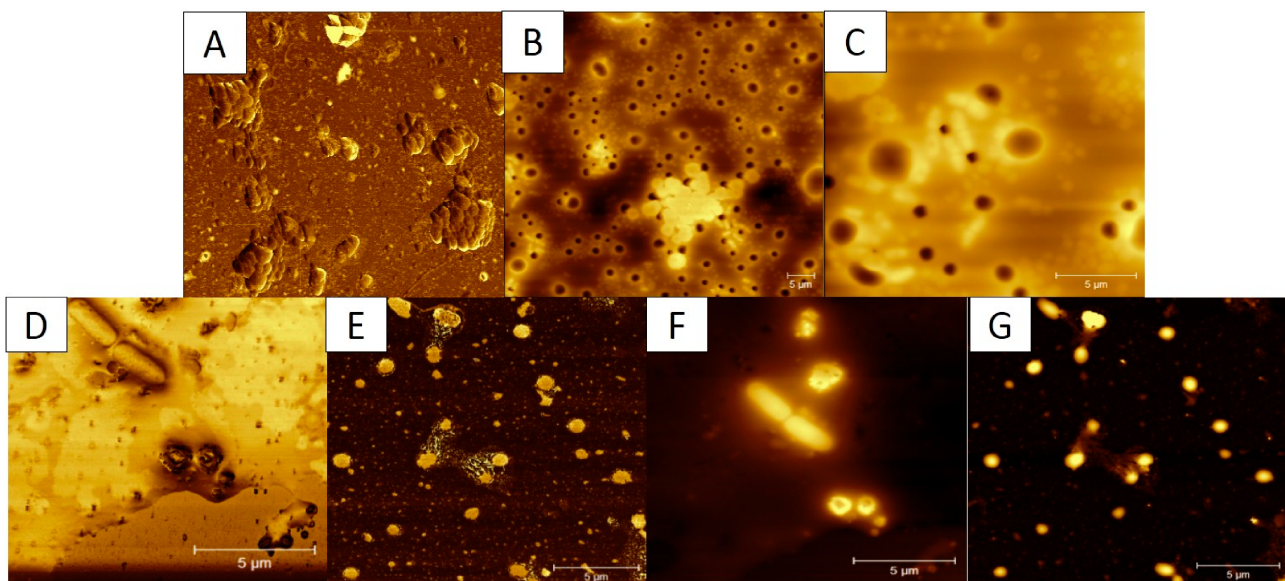


Figure 5. Representative AFM images of *Staphylococcus aureus* and *Klebsiella pneumoniae* before and after treatment with saponin-enriched extracts. (A) Nanomechanical deformation map of untreated *S. aureus*. (B,C) Height-mode AFM topographic images of treated *S. aureus*. (D) AFM topography of untreated *K. pneumoniae*. (E) Height-mode AFM image of treated *K. pneumoniae*. (F) Nanomechanical deformation map of treated *K. pneumoniae*. (G) AFM topographic image of treated *K. pneumoniae*, showing surface irregularities and envelope disruption.

Following treatment with the ES extract, AFM images revealed marked alterations in both bacterial species. In *S. aureus* (Figure 5B,C), surface roughening, irregular depressions, and pit-like structures were observed, suggesting disruption of the cell envelope. Treated *K. pneumoniae* cells (Figure 5E–G) displayed heterogeneous surface features, loss of surface regularity, and localized defects compared with untreated controls.

These observations were further supported by AFM deformation maps (Figure 5F), which revealed increased mechanical heterogeneity in treated cells. The observed nanoscale modifications indicate that exposure to the ES extract affected the structural organization of the bacterial envelope.

In summary, the AFM results support the hypothesis that disruption of envelope integrity contributes to the antibacterial activity of the ES extract.

3.8. Molecular Docking Analysis of Bioactive Compounds from *Argania spinosa*

Molecular docking was conducted using bioactive compounds identified in the *Argania spinosa* bark extract from Stidia (ES) to investigate their interactions with essential bacterial proteins.

For *S. aureus*, two targets were selected:

- UPPS, involved in cell wall biosynthesis;
- ClfA, a surface adhesion protein mediating host colonization.

For *K. pneumoniae*, the following targets were analyzed:

- LptDE, essential for outer membrane biogenesis;
- FimH, involved in bacterial adhesion.

The docking scores of all compounds and reference antibiotics are presented in Table 7, while detailed molecular interactions are summarized in Table 8.

Table 7. Binding energies (kcal/mol) of phytochemicals identified in the *Argania spinosa* Stidia extract (ES) and reference antibiotics against selected bacterial targets.

Compounds	Binding Energy (Kcal/mol)			
	<i>S. aureus</i>		<i>K. pneumoniae</i>	
	UPPS (PDB: 4H8E)	ClfA (PDB: 1N67)	LptDE (PDB: 5IV9)	FimH (PDB: 9AT9)
Arganine E	−7.7	−9.9	−11.4	−7.7
Arganine C	−8.8	−9.2	−12.1	−7.5
Arganine J	−7.7	−10.0	−12.0	−7.8
Myricetin	−7.3	−9.0	−8.0	−6.0
Catechin	−6.8	−8.1	−7.6	−5.8
Quercetin	−7.3	−8.9	−8.1	−6.0
Mi-saponin B	−9.5	−11.2	−12.8	−8.4
Gentamicin	−6.7	−9.2	-	-
Ampicillin	−7.6	−8.2	−7.7	−5.7
Kanamycin	-	-	−8.1	−5.7

Binding energies are expressed in kcal/mol. UPPS: undecaprenyl diphosphate synthase; ClfA: clumping factor A; LptDE: lipopolysaccharide transporter; FimH: fimbrial adhesion; (-) indicates that docking was not performed for the corresponding compound–target protein combination.

Table 8. Key molecular interactions of the best-docked compound (mi-saponin B) identified in the *A. spinosa* Stidia extract (ES) with selected bacterial targets and reference antibiotics.

Targets	Compounds	Binding Energy (Kcal/mol)	H-Bonds	Other Interactions
UPPS (PDB: 4H8E)	Mi-saponin B	−9.5	Gln247, Arg249, Glu220, Trp214, Tyr218	Leu158, Trp214
	Ampicillin	−7.6	Arg248, Ser217, Tyr218, Asp195, Trp214, Asn185, Ser219	Trp214
ClfA (PDB: 1N67)	Mi-saponin B	−11.2	Glu453, Glu456, Pro452, Phe455, Ser290, Asp340, Asn284, Ile339, Val450, Glu342	Ile488, Val288, Pro251, Tyr338, Phe455, Pro452
	Ampicillin	−8.2	Arg395, Thr397, Val450	Phe449, Pro341, Val288, Tyr399
LptDE (PDB: 5IV9)	Mi-saponin B	−12.8	Gly324, Arg748, Asp85, Ala305, Asn292, Asp306, Ser336, Arg302, Asn304, Lys322, Asp85, His239, Asn292	Lys84
	Kanamycin	−8.1	Lys322, Tyr290, Tyr323, Thr308, Thr332, Tyr330, Tyr98, Asp328, Asn750, Ile751	-
FimH (PDB: 9AT9)	Mi-saponin B	−8.4	Thr40, Thr25, Asn33, Asn23, Tyr21	Phe43, Ala6, Asn23
	Kanamycin	−5.7	Gln41, Thr7, Asp37, Val35, Asn23	Val35

Binding energies are expressed in kcal/mol. Hydrogen bonds and other interactions were identified based on docking analysis. (-) indicates that docking was not performed for the corresponding compound target protein combination.

The docking results suggested that all compounds may exhibit favorable interactions with the selected targets. Among them, saponins displayed the lowest binding energy values compared to flavonoids and reference antibiotics.

Mi-saponin B showed the lowest binding energy values across all targets, with docking scores of -11.2 kcal/mol and -9.5 kcal/mol against *S. aureus* ClfA and UPPS, respectively. It also exhibited strong predicted interactions with *K. pneumoniae* proteins, with values of -12.8 kcal/mol for LptDE and -8.4 kcal/mol for FimH. Arginine derivatives (C, J, and E) also showed notable docking scores, particularly against LptDE (-12.1 , -12.0 , and -11.4 kcal/mol, respectively) and ClfA (-9.2 to -10.0 kcal/mol). In contrast, flavonoids (catechin, quercetin, and myricetin) exhibited comparatively higher binding energy values across all targets. Taken together, saponins exhibited lower binding energy values than the reference antibiotics, including kanamycin and ampicillin, suggesting a higher predicted binding stability. Following the identification of mi-saponin B as the best-docked compound, a detailed interaction analysis was performed to characterize its binding modes. The results are presented in Table 8.

Following the identification of mi-saponin B as the best-docked compound, its binding modes were further analyzed. The interaction patterns with the selected targets are illustrated in Figures 6 and 7.

For *S. aureus*, docking within UPPS and ClfA revealed stable binding configurations involving multiple hydrogen bonds and hydrophobic interactions (Figure 6).

For *K. pneumoniae*, similar interaction profiles were observed with LptDE and FimH, where mi-saponin B exhibited stronger binding compared to reference antibiotics (Figure 7).

Mi-saponin B formed multiple hydrogen bonds and hydrophobic interactions with all investigated targets in the docking simulations.

For *S. aureus* UPPS (PDB ID: 4H8E), the compound was predicted to form hydrogen bonds with residues Gln247, Arg249, Glu220, Tyr218, and Trp214, along with hydrophobic interactions involving Leu158 and Trp214.

In ClfA (PDB ID: 1N67), mi-saponin B was predicted to exhibit extensive hydrogen bonding with residues such as Glu453, Glu456, Pro452, Phe455, Ser290, Asp340, Asn284, Ile339, and Val450, in addition to hydrophobic interactions involving Ile488, Val288, Pro251, Tyr338, and Phe455.

For *K. pneumoniae* LptDE (PDB ID: 5IV9), the compound was predicted to form multiple hydrogen bonds with residues including Gly324, Arg748, Asp85, Ala305, Asn292, Asp306, Ser336, Arg302, Asn304, and Lys322.

In FimH (PDB ID: 9AT9), mi-saponin B was predicted to interact through hydrogen bonds with residues Thr40, Thr25, Asn33, Asn23, and Tyr21, along with additional interactions involving Phe43 and Ala6.

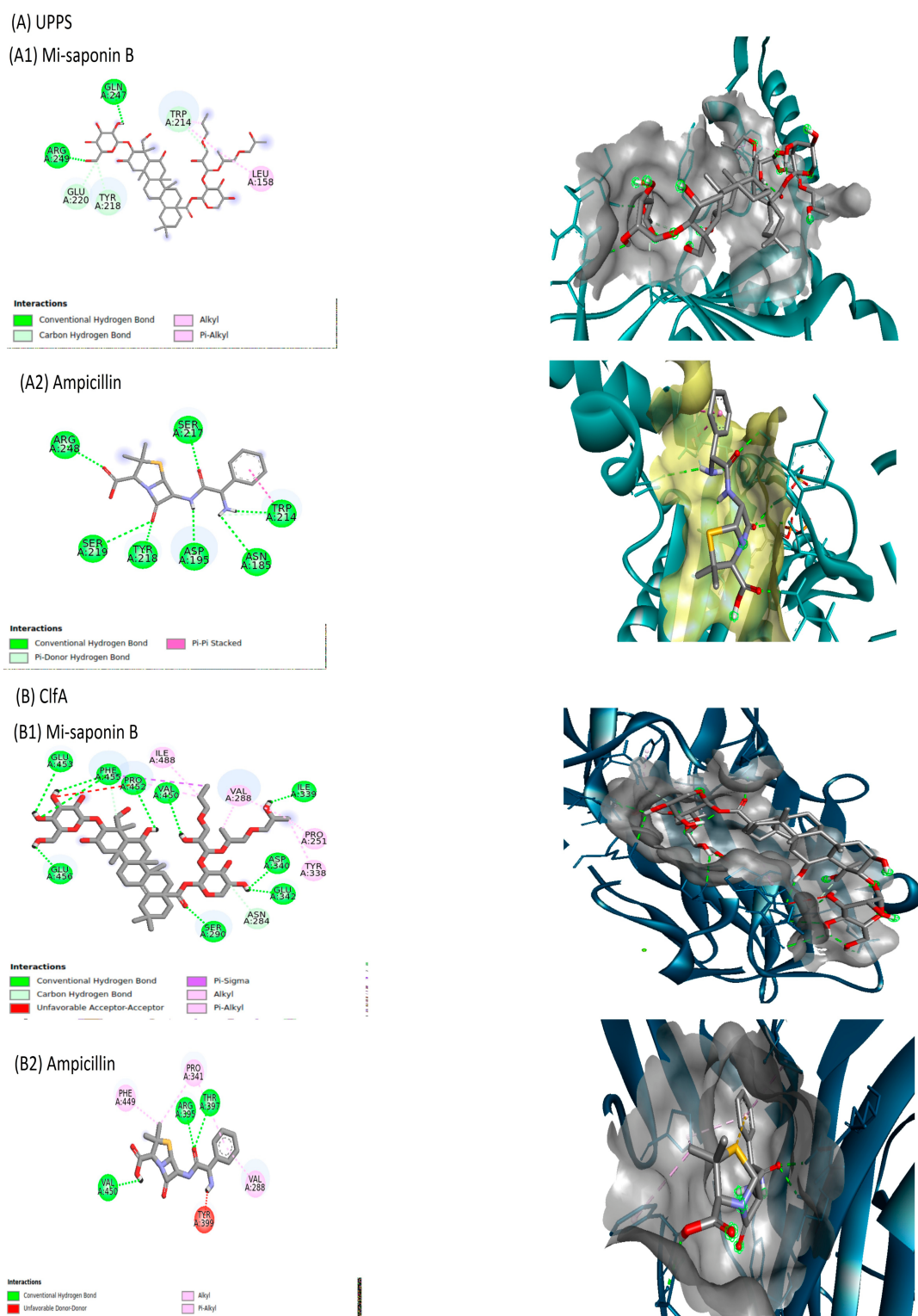
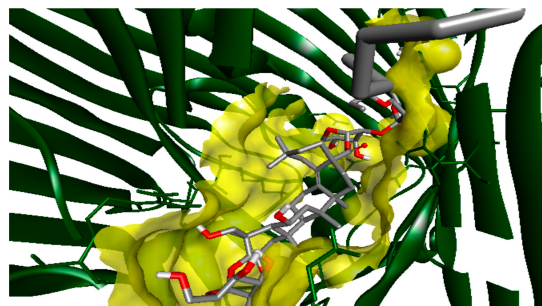
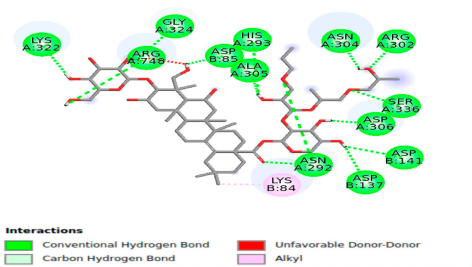


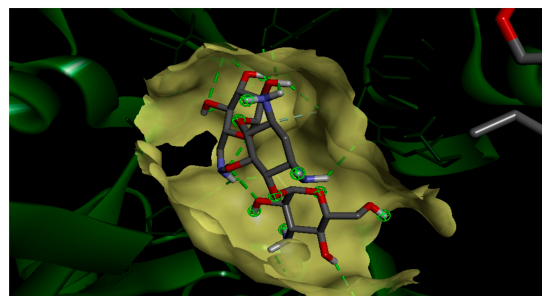
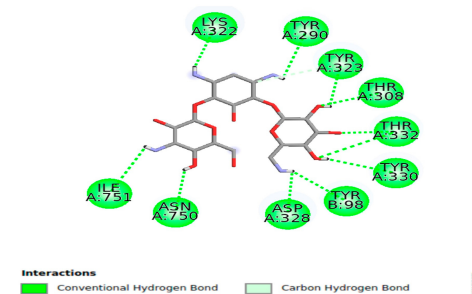
Figure 6. Two-dimensional (2D) and three-dimensional (3D) visualizations of Mi-saponin B and ampicillin docked into *Staphylococcus aureus* target proteins. (A) UPPS: (A1) Mi-saponin B, (A2) ampicillin. (B) ClfA: (B1) Mi-saponin B, (B2) ampicillin. UPPS, undecaprenyl pyrophosphate synthase; ClfA, clumping factor A.

(A) LptDE

(A1) Mi-saponin B

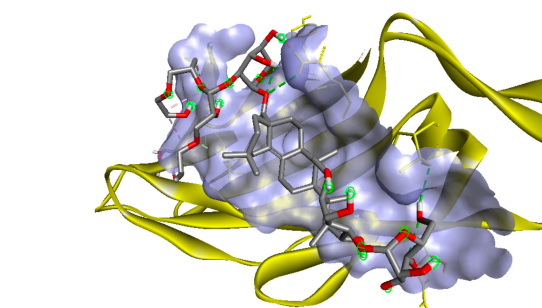
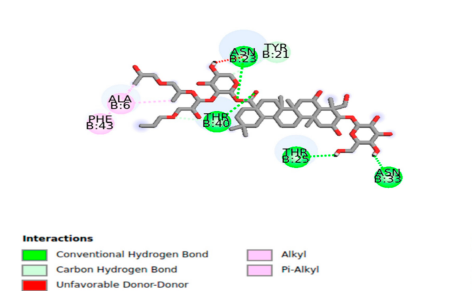


(A2) Kanamycin



(B) FimH

(B1) Mi-saponin B



(B2) Kanamycin

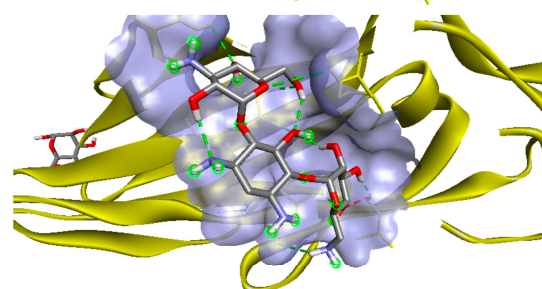
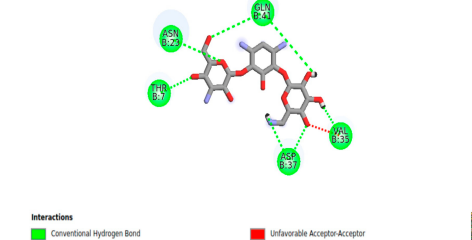


Figure 7. Two-dimensional (2D) interaction diagrams and three-dimensional (3D) binding poses of Mi-saponin B and kanamycin docked into *Klebsiella pneumoniae* target proteins. (A) LptDE: (A1) Mi-saponin B, (A2) kanamycin. (B) FimH: (B1) Mi-saponin B, (B2) kanamycin. LptDE, lipopolysaccharide transport protein D/E complex; FimH, type 1 fimbrial adhesin.

4. Discussion

Antibiotics have long been regarded as one of the most transformative achievements in modern medicine; however, their extensive and sometimes inappropriate use has resulted in unintended consequences. Over time, the widespread and repeated exposure of bacteria to these drugs has contributed to the emergence of multidrug-resistant strains, now recognized as a major global public health crisis [46]. In response to this growing threat and the diminishing effectiveness of conventional antibiotics, there is an urgent need to explore alternative therapeutic strategies. In this context, natural products have gained considerable

attention, particularly plant-derived extracts rich in bioactive metabolites. Recent evidence has shown that compounds such as saponins, flavonoids, tannins, and alkaloids exhibit measurable antibacterial properties, underscoring their relevance as promising reservoirs for new antimicrobial agents [47].

Argania spinosa (L.), renowned for its exceptional chemical diversity, emerges as a particularly promising candidate within this framework [48]. Its woody tissues, especially the trunk bark, contain substantial levels of triterpenoid saponins, accounting for approximately 5% of the dry weight. These compounds, primarily of the bayogenin and oleanane types, are widely associated with potent pharmacological and antimicrobial activities [18,49]. Within this context, the present study investigated how contrasting environmental conditions between two Algerian regions, coastal Stidia (ES) and Saharan Tindouf (ET), influence the saponin composition and antimicrobial efficacy of *A. spinosa* bark extracts, aiming to clarify the relationship between phytochemical variability and antibacterial performance.

To address this objective, extraction yields were first determined, followed by quantitative colorimetric assays and detailed HPLC-MS profiling to characterize metabolite diversity within saponin-enriched bark fractions. The obtained fractions showed comparable yields between the two regions, ES ($12.61 \pm 0.06\%$) and ET ($12.65 \pm 0.03\%$), reflecting the selective nature of the enrichment procedure. As previously reported [50], crude ethanolic extractions generally provide higher yields, whereas butanolic or precipitated fractions produce lower recoveries but are enriched in saponins. The fractions analyzed here follow this trend, representing complex bioactive matrices in which triterpenoid saponins coexist with phenolics, flavonoids, and tannins, whose combined effects likely contribute to biological activity [22].

Colorimetric assays revealed region-dependent variations in phytochemical composition. While total phenolic contents were relatively comparable between extracts, the ES extract exhibited markedly higher flavonoid levels, whereas tannin contents remained similar. These differences likely reflect adaptive metabolic responses to local environmental conditions. In the humid and light-exposed environment of Stidia, bark tissues may favor the accumulation of antioxidant phenolics, as reported in *Salix* species [51]. Conversely, the arid conditions of Tindouf may promote the biosynthesis of stress-related metabolites, including triterpenoid saponins, potentially at the expense of certain phenolic subclasses [52]. This pattern is consistent with previous studies on *Argania spinosa*, which highlight variability in phenolic and flavonoid contents depending on plant organ and environmental constraints [50,53], and aligns with broader ecological adaptations observed in plants exposed to contrasting stresses [54]. This compositional variability is further supported by UHPLC-MS profiling, which revealed distinct differences in the distribution of secondary metabolites between the two extracts.

UHPLC-MS profiling confirmed a shared core of protobasic-type saponins in both extracts, including Arganine C, Arganine E, and Arganine J, commonly reported in kernels, press cake, and trunk tissues [48,55–57]. A particularly noteworthy result is the first detection of Mi-saponin B in *A. spinosa* bark, previously described only in *Madhuca longifolia* [58,59], and recently associated with anticancer and antimicrobial activity [60]. The two ecotypes also differed in flavonoid composition: both contained catechin and quercetin, while ES uniquely presented myricetin, whereas ET included several glycosylated derivatives such as quercetin-O-pentoside and hyperoside [61–63]. Such differences may reflect adaptive metabolic responses to environmental constraints, as flavonoid biosynthesis is known to be highly responsive to abiotic stress factors such as UV radiation, drought, and temperature variations [51,54].

Furthermore, ET exhibited higher levels of bayogenin-derived saponins, Arganine G and 3-O-bayogenin (Arganine L/R) [48,64], which may reflect enhanced accumulation of saponins under arid conditions. This trend is in agreement with previous studies showing that water deficit and environmental stress can stimulate the accumulation of triterpenoid saponins as part of plant defense and adaptation mechanisms [52,54]. Altogether, these observations highlight a strong environmental imprint on the chemical profile of *A. spinosa* bark. However, these interpretations should be considered with caution, as UHPLC–MS data provide only semi-quantitative information, and variations in signal intensity may arise from compound-dependent ionization efficiency rather than true differences in metabolite concentration.

Taken together, these results suggest that differences in chemical composition may contribute to the observed biological activity of the extracts, particularly their antimicrobial potential, which was therefore assessed.

The antimicrobial evaluation of *A. spinosa* bark fractions was designed to determine how these compositional differences translate into biological activity. Each analytical approach explored a complementary aspect of antimicrobial behavior, from diffusion in solid media to quantitative inhibition in liquid systems. In agar-well diffusion assays, both saponin-enriched fractions inhibited a broad spectrum of microorganisms, including *S. aureus* ATCC 25923, *L. innocua* ATCC 33090, *S. epidermidis*, *E. coli*, *P. mirabilis*, *S. marcescens*, *K. pneumoniae*, and *C. albicans*, with noticeable selectivity. The coastal extract (ES) generally produced larger inhibition zones against several Gram-positive bacteria and fungi, whereas ET showed relatively greater activity against some Gram-negative strains in the agar diffusion assay [65,66]. This distribution may partly reflect differences in chemical composition and polarity: ES is dominated by oleanane-type bidesmosidic saponins (Arganine C, E), while ET is richer in bayogenin derivatives (Arganine J, L, Mi-saponin B), which may interact with the outer LPS layer of Gram-negative bacteria [67].

These results extend previous observations [50], which reported limited activity in leaf and kernel extracts, particularly against *E. coli*. The influence of plant organ and solvent polarity appears decisive, as methanolic trunk-bark fractions preferentially extract saponin-rich compounds [68]. This interpretation is in agreement with the well-established tissue-specific distribution of plant metabolites [69].

To obtain quantitative insight, the broth microdilution method was applied, providing standardized MIC and MBC/MFC values [36]. ES exhibited MICs of 25–50 mg/mL and MBC/MFC values of 50–100 mg/mL, whereas ET required higher concentrations (50–100 mg/mL and 100–200 mg/mL, respectively). The higher efficacy of ES was evident against several tested microorganisms. Although ET showed measurable activity against Gram-negative bacteria, the MIC data do not clearly support a preferential activity toward Gram-negative strains. Similar findings were reported in [70], confirming that solvent polarity and triterpenoid enrichment influence antibacterial potency. Although the MIC values obtained in the present study were higher than those reported for some *Argania spinosa*-derived products, direct comparisons should be interpreted with caution because of differences in plant material, extraction procedures, phytochemical composition, and the microorganisms tested. Therefore, the observed activity may be considered moderate, while still demonstrating the antimicrobial potential of saponin-enriched bark fractions.

Mechanistically, differences between agar and broth assays can be explained by the structural properties of the media. In agar, polar metabolites diffuse radially, creating concentration gradients and larger inhibition zones [71]. In contrast, liquid media ensure uniform dispersion, which can dilute polar compounds and reduce their local activity, while favoring hydrophobic interactions with membranes [71]. Such methodological differences have been widely documented [72].

These complementary behaviors illustrate how structural diversity among triterpenoid saponins may contribute to differences in antimicrobial responses. Based on this polarity-driven model, the study further evaluated whether bark saponins could act as antibiotic adjuvants in combination with aminoglycosides using the checkerboard assay [73].

The results revealed distinct synergy patterns. ES enhanced the activity of gentamicin against *S. aureus* and *K. pneumoniae*, whereas ET showed no improvement with gentamicin against *S. aureus* but exhibited strong synergy with kanamycin against *K. pneumoniae*. These responses reflect selective compatibility between extract composition and bacterial envelope structure. Similar effects have been reported for ginsenosides and saponins from *Glycyrrhiza glabra* [20,74–76], supporting the role of triterpenoid saponins as membrane-active adjuvants [22].

To explore membrane involvement, crystal violet uptake assays were performed. Both extracts increased membrane permeability in *S. aureus* and *K. pneumoniae*, with ET inducing a stronger permeabilizing effect than ES. These findings are consistent with previous studies showing that glycosylated saponins can promote membrane perturbation [77] and modulate lipid organization at the molecular level [67]. However, despite inducing higher membrane permeability, ET did not exhibit greater antibacterial potency than ES, indicating that membrane permeabilization alone cannot fully account for the antimicrobial activity of the extracts. This observation suggests that ET may function primarily as a membrane sensitizer rather than as a direct antibacterial agent. By increasing membrane permeability, ET could facilitate the intracellular access of co-administered antimicrobials and thereby contribute to the synergistic interactions observed with aminoglycosides. Further studies are required to determine whether a concentration-dependent window for synergy exists, in which membrane permeabilization is enhanced without compromising bacterial viability.

AFM analysis further confirmed these effects by revealing nanoscale membrane alterations [75]. Treated cells displayed surface roughness, structural deformation, and membrane disruption, similar to observations reported in bacteria exposed to triterpenoid saponins [76,78–80].

Together, the permeability assays, AFM observations, and checkerboard results suggest that membrane perturbation may contribute to the antibacterial and antibiotic-potentiating effects of the extracts [22].

To further elucidate the molecular basis underlying these experimentally observed effects, *in silico* molecular docking was performed to explore the potential interactions between identified phytochemicals and key bacterial targets.

The present findings are consistent with the *in vitro* results, suggesting that phytochemicals derived from *Argania spinosa* contribute to the observed antibacterial activity. This activity may be largely associated with mi-saponin B and arganine derivatives (C, E, and J), which showed lower binding energy values toward the selected targets compared to flavonoids. This trend highlights the importance of the amphipathic nature and structural complexity of saponins in their interaction with bacterial proteins.

Molecular docking provides preliminary mechanistic insights, suggesting that the strong predicted binding of mi-saponin B may arise from the combined effect of hydrogen bonding mediated by its glycone moieties and hydrophobic interactions involving the aglycone core. These multivalent interactions may enhance ligand stability within target proteins, which may explain the lower binding energy values observed for saponins compared to flavonoids and reference antibiotics.

These data are in agreement with previous reports indicating that saponins interact with membrane-associated components and bacterial systems [67,81]. Similarly, studies [79,80], reported that saponins form stable interactions within bacterial environments, supporting their potential to interfere with targets such as UPPS, ClfA, LptDE, and FimH.

In addition, the surfactant-like properties of saponins may contribute to membrane destabilization and increased permeability [82]. These observations suggest that saponins may interact with both bacterial membranes and specific cellular targets; however, further experimental studies are required to confirm these mechanisms.

Although both saponins and flavonoids exhibit antibacterial activity, their combined effects remain poorly understood. A plausible explanation may involve a synergistic interaction, in which membrane perturbation induced by saponins may enhance the accessibility of bacterial cells to other bioactive compounds. This hypothesis remains tentative and requires further experimental validation but is supported by previous studies on quercetin-based complexes showing enhanced antibacterial activity [83].

Collectively, these findings suggest that saponins and arganine derivatives may target proteins involved in membrane integrity and bacterial colonization. Strong predicted binding to LptDE and FimH in *K. pneumoniae* suggests possible interference with outer membrane biogenesis and adhesion, while interactions with UPPS and ClfA in *S. aureus* indicate potential interactions with proteins involved in cell wall biosynthesis and colonization processes. However, these in silico findings remain predictive and require further experimental validation.

Despite these promising findings, several limitations should be acknowledged. While the in vitro assays support the antibacterial activity and membrane-disruptive effects of the extracts, the molecular docking results remain predictive and do not constitute direct experimental validation of specific protein–ligand interactions. Furthermore, although membrane perturbation was observed, the present study did not directly evaluate intracellular antibiotic uptake or accumulation. Therefore, any contribution of enhanced antibiotic penetration to the observed synergistic effects remains hypothetical and should be confirmed experimentally. In addition, the phytochemical characterization was based on semi-quantitative UHPLC–MS analysis, without absolute quantification of individual compounds. Furthermore, all experiments were conducted in vitro, and no toxicity or in vivo assessments were performed. Therefore, further studies, including quantitative analysis, mechanistic validation, and toxicity evaluation, are required to confirm the therapeutic potential of these extracts as antibiotic adjuvants.

Future research should focus on the isolation of major bioactive saponins, particularly Mi-saponin B and arganine derivatives, to clarify their individual contributions to antibacterial activity. Studies investigating antibiotic uptake, target-specific inhibition, and in vivo efficacy would further improve our understanding of the mechanisms underlying the antibacterial and synergistic effects of *A. spinosa* bark extracts.

5. Conclusions

Overall, this study highlights the significant antimicrobial and antibiotic-adjuvant potential of *Argania spinosa* bark saponins against clinically relevant multidrug-resistant bacteria. The comparative analysis of coastal (ES) and Saharan (ET) ecotypes revealed distinct chemotypes associated with differential biological responses, emphasizing the influence of environmental conditions on bioactivity. Importantly, the identification of mi-saponin B expands the phytochemical diversity of this species and provides new insight into its biological potential. These findings support the valorization of bark-derived saponins as promising multifunctional agents and natural adjuvants within a One Health framework.

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Abbreviations

The following abbreviations are used in this manuscript:

ATCC	American Type Culture Collection
CE	Catechin equivalents
ClfA	Clumping factor A
ES	Extract from Stidia (coastal Algeria)
ET	Extract from Tindouf (southern Algeria)
FimH	Type 1 fimbrial adhesion
GAE	Gallic acid equivalents
LptDE	Lipopolysaccharide transport protein D/E complex
MBC	Minimum bactericidal concentration
MDR	Multidrug-resistant
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
RT	Retention time
TFC	Total flavonoid content
TPC	Total phenolic content
TTC	Total tannin content
UHPLC–MS	Ultra-high-performance liquid chromatography–mass spectrometry
UPPS	Undecaprenyl pyrophosphate synthase

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