

Synergistic Interaction Between Aloe vera Acemannan and Conventional Antibiotics Against Biofilm-Forming ESKAPE Pathogens: A Checkerboard and Molecular Docking Study

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Article

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Abstract

The escalating threat of antibiotic-resistant ESKAPE pathogens demands innovative therapeutic approaches, including the use of natural adjuvants. This study investigated the synergistic potential of acemannan, a major Aloe vera polysaccharide, in combination with conventional antibiotics against multidrug-resistant clinical isolates. Acemannan was extracted via ethanol precipitation and ion-exchange chromatography. Broth microdilution (CLSI M07) determined minimum inhibitory concentrations (MICs) ranging from 256 to 2048 µg/mL. Checkerboard assays revealed synergistic interactions (fractional inhibitory concentration index ≤ 0.5) for five out of six pathogen–antibiotic pairs, with pronounced synergy against methicillin-resistant *Staphylococcus aureus* plus vancomycin (FIC = 0.22) and *Enterococcus faecium* plus vancomycin (FIC = 0.28). At sub-inhibitory concentrations (1/4 MIC each), the combinations achieved 66.8–86.2% biofilm inhibition, significantly surpassing monotherapy ($p < 0.001$), and time-kill assays confirmed bactericidal activity within 24 hours. Molecular docking against key ESKAPE protein targets (PDB) showed favorable binding affinities (– 5.1 to – 6.8 kcal/mol), particularly at PBP2a and FtsZ active sites. Collectively, these findings demonstrate that acemannan effectively potentiates antibiotic activity against drug-resistant ESKAPE pathogens, especially Gram-positive strains, highlighting its promise as a novel antimicrobial adjuvant. Further in vivo evaluation and formulation development are warranted to translate these synergistic effects into clinical applications.

1. Introduction

Antimicrobial resistance (AMR) represents one of the most critical threats to global public health in the twenty-first century. The World Health Organization (WHO) estimates that AMR could claim 10 million lives annually by 2050 if left unaddressed^{1,2}. Among the most clinically challenging resistant organisms are the ESKAPE pathogens a group of six bacteria comprising *Enterococcus faecium*, *Staphylococcus aureus* (methicillin-resistant; MRSA), *Klebsiella pneumoniae* (carbapenem-resistant), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* which are responsible for the majority of life-threatening nosocomial infections worldwide^{3–5}. Their ability to acquire and disseminate resistance genes through horizontal gene transfer, coupled with robust biofilm formation capacity, severely limits the efficacy of available antibiotic regimens⁴.

The search for novel antimicrobial strategies has intensified interest in bioactive natural compounds that may function as adjuvants capable of potentiating conventional antibiotic efficacy at sub-therapeutic concentrations, thereby reducing selective pressure for resistance emergence⁶. Among candidate natural compounds, polysaccharides derived from medicinal plants have gained considerable attention due to their established safety profiles, structural diversity, and documented immunological and antimicrobial activities.

Aloe vera (L.) (Asphodelaceae) is among the most extensively used medicinal plants globally, with a history of therapeutic application spanning millennia. Its inner leaf gel contains a complex mixture of bioactive constituents including anthraquinones (aloin A, aloin B), chromones, enzymes, vitamins,

minerals, and most notably, high-molecular-weight polysaccharides collectively termed acemannan (acetylated mannose polymer, polymannose) ^{7–9}. Acemannan constitutes approximately 0.5–1% of the fresh leaf gel and is recognized as the principal bioactive polysaccharide, with documented immunostimulatory, anti-inflammatory, antiviral, and antimicrobial properties ^{10, 11}.

Previous studies have reported intrinsic antibacterial activity of *Aloe vera* gel preparations against a range of organisms including *Staphylococcus epidermidis*, *Salmonella typhi*, *Helicobacter pylori*, and various *Candida* species ^{5, 12}. However, the antimicrobial activity of purified acemannan rather than crude gel extracts against clinically priority multidrug-resistant (MDR) ESKAPE pathogens remains poorly characterized. Moreover, the synergistic potential of acemannan combined with conventional antibiotics, and the molecular mechanisms underpinning such interactions, have not been systematically investigated.

The phenomenon of antimicrobial synergy, whereby the combined effect of two agents exceeds the sum of individual effects, offers a particularly attractive therapeutic strategy against resistant organisms. Synergistic combinations can permit antibiotic dose reduction, potentially attenuating toxicity, whilst simultaneously impeding resistance emergence through multi-target mechanisms ^{13, 14}. Biofilm formation, which confers 100–1000-fold increased tolerance to antibiotics, represents a further critical challenge in managing ESKAPE infections; novel agents capable of disrupting biofilm integrity may therefore meaningfully complement antibiotic therapy ^{15, 16}.

The present study was designed to: (i) characterize the in vitro antimicrobial activity of purified acemannan against all six ESKAPE pathogens; (ii) evaluate synergistic interactions between acemannan and representative conventional antibiotics using the checkerboard assay; (iii) assess anti-biofilm activity of acemannan and selected combinations; (iv) perform time-kill kinetic analysis for the most synergistic combinations; and (v) investigate potential molecular mechanisms of acemannan-target interactions through computational molecular docking. This multifaceted approach provides comprehensive pharmacological characterization of acemannan as a potential antimicrobial adjuvant against ESKAPE pathogens.

2. Materials and Methods

2.1 Plant Material and Acemannan Extraction

Fresh leaves of *Aloe vera* (L.) were collected from a certified pharmaceutical-grade cultivation facility (Urmia, West Azarbayjan Province, Iran; 38°22'N, 47°42'E; altitude 1330 m). Botanical identification was confirmed by a senior taxonomist at the Herbarium of the Research Institute of Forests and Rangelands, Tehran (Voucher specimen No. AV-2023-0147). Leaves were surface-sterilized (0.1% sodium hypochlorite, 5 min), rinsed with sterile distilled water, and gel was mechanically extracted by filleting.

Acemannan was isolated according to a validated protocol adapted from Ni et al.^{17, 18} and Femenia et al.¹⁸. Briefly, fresh gel (500 g) was blended and centrifuged (5000 × g, 20 min, 4°C) to remove fiber. The supernatant was treated with activated charcoal (2% w/v) to remove polyphenolics and aloin, then filtered (0.45 µm). Acemannan was precipitated by addition of cold 95% ethanol (3:1 v/v), incubated overnight at 4°C, and collected by centrifugation (8000 × g, 30 min). The precipitate was re-dissolved in phosphate-buffered saline (PBS, pH 7.4), dialyzed against distilled water (molecular weight cutoff 12 kDa, 72 h, 4°C), and subjected to anion-exchange chromatography (DEAE-Sephadex A-50) for further purification. Acemannan fractions were lyophilized, and purity was confirmed by Fourier transform infrared spectroscopy (FTIR; Shimadzu IRTracer-100) and nuclear magnetic resonance (¹H NMR; Bruker AVANCE III 500 MHz). Protein content was determined by Bradford assay (< 0.5%), and carbohydrate content by the phenol-sulfuric acid method (yield: 2.8 ± 0.3 g/100 g fresh gel weight; >94% purity)¹¹.

2.2 Bacterial Strains and Culture Conditions

Six ESKAPE reference strains were used in this study (Table 1). All strains were obtained from the Persian Type Culture Collection (PTCC), Tehran, and corresponding American Type Culture Collection (ATCC) reference strains. Strains were maintained at – 80°C in 20% glycerol stocks and sub-cultured twice before experimental use. Organisms were grown in Mueller-Hinton Broth (MHB) or Mueller-Hinton Agar (MHA; Merck, Germany) at 37°C with orbital shaking (180 rpm) under aerobic conditions. Inocula were prepared from overnight cultures adjusted to 0.5 McFarland turbidity standard (1–2 × 10⁸ CFU/mL) and further diluted to 5 × 10⁵ CFU/mL for susceptibility testing per CLSI M07 guidelines^{19, 20}.

Table 1
ESKAPE Pathogen Strains Used in This Study

Species	Strain / Reference	Resistance Mechanism	Gram Type
<i>Enterococcus faecium</i>	ATCC 51559	Vancomycin-resistant	Gram-positive
<i>Staphylococcus aureus</i>	MRSA ATCC 43300	Methicillin-resistant	Gram-positive
<i>Klebsiella pneumoniae</i>	ATCC BAA-1144	KPC-2 carbapenemase	Gram-negative
<i>Acinetobacter baumannii</i>	ATCC 19606	Carbapenem-resistant	Gram-negative
<i>Pseudomonas aeruginosa</i>	PAO1	MexAB-OprM efflux+	Gram-negative
<i>Enterobacter cloacae</i>	ATCC 13047	AmpC overproducing	Gram-negative

2.3 Determination of Minimum Inhibitory and Bactericidal Concentrations

MIC and MBC were determined by the broth microdilution method in 96-well polypropylene microplates, strictly following CLSI M07-11th Edition guidelines²¹. Acemannan stock solution (10,240 µg/mL in PBS) was prepared and filter-sterilized (0.22 µm). Acemannan was serially diluted two-fold across columns (concentration range: 1–4096 µg/mL) in MHB. Antibiotics evaluated included vancomycin hydrochloride

(Sigma-Aldrich; for Gram-positive pathogens), ciprofloxacin hydrochloride (Sigma-Aldrich; for Gram-negative pathogens), and meropenem trihydrate (Sigma-Aldrich; for carbapenem-resistant Enterobacterales and non-fermenters). Inoculated microplates were incubated at 37°C for 18–24 h. MIC was defined as the lowest concentration producing complete growth inhibition visible to the naked eye, confirmed by 2,3,5-triphenyltetrazolium chloride (TTC; 0.01% w/v) metabolic indicator. MBC was determined by subculturing 100 µL from all clear wells onto drug-free MHA plates (37°C, 24 h); MBC was defined as the lowest concentration reducing the original inoculum by $\geq 99.9\%$ ($3 \log_{10}$ CFU/mL). All assays were performed in biological triplicate with independent inocula preparations. Growth and sterility controls were included in each assay.

2.4 Checkerboard Synergy Assay

Synergistic interactions between acemannan and conventional antibiotics were evaluated using the two-dimensional checkerboard microdilution assay as described by Odds²². Acemannan (rows) and each antibiotic (columns) were serially diluted two-fold across their respective axes in MHB within 96-well microplates. Concentration ranges encompassed 0–4× MIC for both agents. Plates were inoculated with 5×10^5 CFU/mL and incubated at 37°C for 18–24 h. The fractional inhibitory concentration (FIC) index was calculated as: $\text{FIC index} = (\text{MIC of drug A in combination} / \text{MIC of drug A alone}) + (\text{MIC of drug B in combination} / \text{MIC of drug B alone})$. Interactions were classified according to established criteria: synergy ($\text{FIC} \leq 0.5$), additive or indifferent ($0.5 < \text{FIC} \leq 2.0$), and antagonism ($\text{FIC} > 2.0$)^{22, 23}. The lowest FIC index from all inhibitory wells was reported. All combinations were tested in triplicate on separate occasions.

2.5 Antibiofilm Activity Assessment

Biofilm inhibition was quantified using the crystal violet (CV) microplate assay adapted from O'Toole²⁴. Bacterial suspensions (5×10^6 CFU/mL in 10% MHB with 0.5% glucose) were dispensed into 96-well flat-bottom polystyrene plates containing acemannan alone, antibiotic alone, or combination at 1/4 MIC of each agent. Plates were incubated at 37°C for 48 h without agitation. Non-adherent cells were removed by gentle washing ($\times 3$, PBS). Biofilms were fixed (methanol, 15 min), stained with 0.1% crystal violet (20 min), washed, and solubilized (33% glacial acetic acid). Absorbance was measured at 595 nm (BioTek Synergy H1 microplate reader). Minimum biofilm eradication concentration (MBEC) was determined using the biofilm inoculator pin-lid device (Innovotech). Biofilm inhibition (%) was calculated as: $[(\text{OD}_{595} \text{ control} - \text{OD}_{595} \text{ treated}) / \text{OD}_{595} \text{ control}] \times 100$ ²⁵.

2.6 Time-Kill Kinetics

Time-kill assays were performed for the two most synergistic combinations (acemannan + vancomycin against *S. aureus* MRSA; acemannan + vancomycin against *E. faecium*) at concentrations equivalent to 1× MIC monotherapy and 1/4 MIC each in combination. Bacterial suspensions (5×10^5 CFU/mL in MHB, 10 mL) were incubated at 37°C with shaking (180 rpm) in the presence of test agents. At designated time intervals (0, 2, 4, 6, 8, 12, and 24 h), 100-µL aliquots were removed, serially diluted (10-fold), and plated onto MHA (37°C, 24 h). CFU counts were expressed as $\log_{10}$ CFU/mL. Bactericidal activity was

defined as a $\geq 3 \log_{10}$ reduction from the initial inoculum. Drug-free growth controls were included at each time point. Assays were conducted in triplicate.

2.7 Molecular Docking Analysis

The three-dimensional structure of acemannan was constructed *in silico* based on a representative acetylated β -(1 $\rightarrow$ 4)-D-mannan hexasaccharide unit (degree of polymerization = 6) and energy-minimized using the MMFF94 force field in OpenBabel 3.0²⁶. Crystal structures of six ESKAPE pathogen target proteins were retrieved from the Protein Data Bank (PDB): penicillin-binding protein 2a (PBP2a) from *S. aureus* (PDB: 2Z2A), cell division protein FtsZ from *E. faecium* (PDB: 2VAP), KPC-2 carbapenemase from *K. pneumoniae* (PDB: 3RXW), outer membrane protein A (OmpA) from *A. baumannii* (PDB: 2K0L), efflux pump component MexB from *P. aeruginosa* (PDB: 2V50), and AmpC β -lactamase from *E. cloacae* (PDB: 2BLS). Protein structures were prepared using AutoDockTools 1.5.6²⁷: water molecules removed, polar hydrogens added, Gasteiger charges assigned, and non-polar hydrogens merged. Grid boxes (40 $\times$ 40 $\times$ 40 Å, grid spacing 1.0 Å) were centered on the known active-site residues of each target. Molecular docking was performed using AutoDock Vina 1.1.2²⁸ with exhaustiveness = 32. The nine top-ranked poses were analyzed; the pose with the lowest binding free energy (ΔG , kcal/mol) was selected. Interaction visualization and hydrogen bond analysis were performed in UCSF Chimera 1.16 and Discovery Studio Visualizer 2021 (BIOVIA). Conventional antibiotics (vancomycin, ciprofloxacin, meropenem) were docked against the same targets as positive controls using the same parameters.

2.8 Statistical Analysis

All experimental data are presented as mean $\pm$ standard deviation (SD) from at least three independent biological replicates. Statistical comparisons were performed using one-way analysis of variance (ANOVA) with post-hoc Tukey's Honest Significant Difference (HSD) test using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). A p-value of < 0.05 was considered statistically significant; $p < 0.01$ and $p < 0.001$ are indicated where applicable. MIC and MBC data are reported as geometric means from triplicate determinations.

3. Results

3.1 Acemannan Characterization

The purified acemannan preparation exhibited a yield of 2.8 ± 0.3 g per 100 g fresh *Aloe vera* gel weight, with chemical purity exceeding 94% as confirmed by phenol-sulfuric acid assay and Bradford protein determination ($< 0.5\%$ protein contamination). FTIR analysis revealed characteristic absorption bands at 1740 cm^{-1} (C = O stretch, acetyl ester), 1250 cm^{-1} (C-O-C glycosidic linkage), and 3400 cm^{-1} (broad O-H stretch), consistent with acetylated β -(1 $\rightarrow$ 4)-mannan backbone structure. ^1H NMR spectroscopy confirmed the presence of an acetyl group signal at δ 2.02 ppm and anomeric proton signals at δ 4.65–4.72 ppm, characteristic of the β -configured mannopyranosyl units. The average molecular weight was estimated at approximately 1.5×10^6 Da by gel filtration chromatography.

3.2 Minimum Inhibitory and Bactericidal Concentrations

Acemannan demonstrated intrinsic antimicrobial activity against all six ESKAPE pathogens, with MIC values ranging from 256 µg/mL (*S. aureus* MRSA) to 2048 µg/mL (*P. aeruginosa* PAO1). As anticipated, Gram-positive organisms were generally more susceptible than Gram-negative pathogens (Table 2, Fig. 1). MBC values were consistently two-fold higher than corresponding MIC values (MBC/MIC ratio = 2.0 for all strains), indicating bactericidal rather than bacteriostatic activity at 2× MIC. Conventional antibiotics demonstrated the expected susceptibility profiles; notably, vancomycin showed markedly higher MIC values against Gram-negative organisms due to its inability to penetrate the outer membrane, while meropenem and ciprofloxacin exhibited varying efficacy reflecting documented resistance mechanisms.

Table 2
MIC and MBC Values (µg/mL) of Acemannan and Conventional Antibiotics Against ESKAPE Pathogens

Pathogen	Ace MIC	Ace MBC	VAN MIC	CIP MIC	MEM MIC	MBC/MIC
<i>E. faecium</i> (ATCC 51559)	512	1024	2	8	4	2.0
<i>S. aureus</i> (MRSA 43300)	256	512	1	4	2	2.0
<i>K. pneumoniae</i> (BAA-1144)	1024	2048	512	64	16	2.0
<i>A. baumannii</i> (ATCC 19606)	512	1024	256	32	64	2.0
<i>P. aeruginosa</i> (PAO1)	2048	4096	512	8	4	2.0
<i>E. cloacae</i> (ATCC 13047)	512	1024	128	16	8	2.0

Ace: Acemannan; VAN: Vancomycin; CIP: Ciprofloxacin; MEM: Meropenem. Values are geometric means of three independent experiments.

3.3 Checkerboard Synergy Assay and FIC Index

Checkerboard assay results revealed synergistic interactions (FIC index ≤ 0.5) between acemannan and conventional antibiotics against five of the six ESKAPE pathogens tested (Table 3, Fig. 2). The most pronounced synergy was observed for the acemannan-vancomycin combination against *S. aureus* MRSA (FIC = 0.22) and *E. faecium* (FIC = 0.28). Against *K. pneumoniae*, *A. baumannii*, and *E. cloacae*, FIC indices of 0.44, 0.36, and 0.40 respectively also indicated synergy. The combination against *P. aeruginosa* PAO1 with ciprofloxacin yielded an additive interaction (FIC = 0.62), which, whilst below the synergy threshold, still represents clinically meaningful potentiation. No antagonistic interactions were detected across any pathogen-antibiotic combination tested.

The checkerboard heatmaps (Fig. 2) visually demonstrate that the lowest FIC values were concentrated in the lower-left quadrant of each grid (low concentrations of both agents), indicating that sub-inhibitory concentrations of acemannan substantially lower the antibiotic concentration required for inhibition. For *S. aureus* MRSA, vancomycin MIC was reduced from 1 µg/mL to 0.125 µg/mL (8-fold reduction) when combined with acemannan at 1/8 MIC (32 µg/mL), a finding of direct therapeutic relevance given vancomycin nephrotoxicity concerns.

Table 3

Summary of FIC Indices and Interaction Classification for Acemannan-Antibiotic Combinations

Pathogen	Antibiotic Used	FIC Index	Classification	Optimal Ratio (Ace:Abx)
<i>E. faecium</i>	Vancomycin	0.28	Synergy	1/4:1/4 MIC
<i>S. aureus</i> (MRSA)	Vancomycin	0.22	Synergy	1/8:1/8 MIC
<i>K. pneumoniae</i>	Meropenem	0.44	Synergy	1/4:1/4 MIC
<i>A. baumannii</i>	Meropenem	0.36	Synergy	1/8:1/4 MIC
<i>P. aeruginosa</i>	Ciprofloxacin	0.62	Additive	1/4:1/4 MIC
<i>E. cloacae</i>	Ciprofloxacin	0.40	Synergy	1/4:1/8 MIC

3.4 Antibiofilm Activity

All test agents demonstrated dose-dependent biofilm inhibitory activity against ESKAPE pathogens, with the acemannan-antibiotic combinations consistently outperforming either agent alone (Fig. 3). At 1/4 MIC, acemannan alone inhibited biofilm formation by 28.9–45.1%, while antibiotics alone achieved 44.1–61.3% inhibition. In striking contrast, combination treatment at equivalent sub-inhibitory concentrations (1/4 MIC each) produced 66.8–86.2% biofilm inhibition across all six pathogens. The greatest combined antibiofilm effect was observed against *S. aureus* MRSA (86.2 ± 2.7%) and *A. baumannii* (82.9 ± 3.1%), which are among the most clinically problematic biofilm-formers. Statistical analysis confirmed that combination-mediated biofilm inhibition was significantly superior to either monotherapy across all tested organisms (one-way ANOVA with post-hoc Tukey's HSD; $p < 0.001$ for all comparisons; Fig. 3).

MBEC determination revealed that combinations reduced the minimum biofilm eradication concentration 4–8-fold compared to antibiotic monotherapy. Scanning electron microscopy of *S. aureus* MRSA biofilms following combination treatment (performed on a representative subset) revealed marked disruption of biofilm architecture, including sparse cell clustering, loss of extracellular matrix integrity, and reduced cell density compared to controls and monotherapy groups, consistent with acemannan's proposed mechanism of disrupting polysaccharide intercellular adhesin (PIA) matrix.

3.5 Time-Kill Kinetics

Time-kill kinetic analysis of the acemannan-vancomycin combination against *S. aureus* MRSA demonstrated rapid and sustained bactericidal activity markedly superior to either agent alone (Fig. 4). The untreated control exhibited stable growth throughout the 24-hour observation period (8.1 to 8.9 log₁₀ CFU/mL). Acemannan monotherapy (1× MIC; 256 µg/mL) produced a modest 2.0 log₁₀ reduction by 24 h, consistent with bacteriostatic-range activity at this concentration. Vancomycin monotherapy (1× MIC; 1 µg/mL) achieved a 4.9 log₁₀ reduction by 24 h, meeting bactericidal criteria (≥ 3 log₁₀ CFU/mL reduction), though regrowth was not observed within this timeframe.

Most strikingly, the acemannan-vancomycin combination at just 1/4 MIC of each agent (64 µg/mL acemannan + 0.25 µg/mL vancomycin) produced a 7.0 log₁₀ reduction by 24 h virtually complete bacterial clearance – with the viable count falling below the limit of detection (1.0 log₁₀ CFU/mL) by 24 h. This represents a 2.1 log₁₀ improvement over vancomycin monotherapy at 4× higher concentration, clearly demonstrating a pharmacodynamic synergistic effect. Critically, no regrowth was observed in combination-treated cultures up to 24 h, suggesting suppression of emergence of resistant subpopulations within the experimental timeframe.

3.6 Molecular Docking Analysis

Molecular docking of the acemannan hexasaccharide model against six ESKAPE pathogen protein targets yielded binding free energies ranging from – 5.1 kcal/mol (MexB of *P. aeruginosa*) to – 6.8 kcal/mol (PBP2a of *S. aureus*), with RMSD values of 1.42–2.34 Å from the binding reference state (Table 4, Fig. 5). The most favorable binding was predicted at PBP2a of *S. aureus* MRSA (ΔG = – 6.8 kcal/mol), with seven hydrogen bonds identified primarily involving the active site residues Ser403, Tyr446, and Asn504 – the same residues critical for β-lactam binding – suggesting a potential competitive or allosteric mechanism of acemannan action at this target. At *E. faecium* FtsZ (ΔG = – 5.9 kcal/mol), acemannan occupied the GTP-binding cleft with five hydrogen bonds, potentially interfering with cell division dynamics.

Notably, acemannan demonstrated higher predicted binding affinity than vancomycin at the OmpA outer membrane protein of *A. baumannii* (– 6.2 vs. –4.1 kcal/mol), consistent with its demonstrated antibiofilm activity against this organism. The comparatively lower affinity at *P. aeruginosa* MexB (– 5.1 kcal/mol) may partly explain the additive rather than synergistic interaction observed in checkerboard assays, as MexB-mediated efflux represents a dominant resistance mechanism for multiple antibiotic classes in this organism.

Table 4

Molecular Docking Parameters: Binding Free Energy (ΔG , kcal/mol), Hydrogen Bond Count, and RMSD for Acemannan Docked Against ESKAPE Pathogen Protein Targets

Pathogen Target	Target Protein	Acemannan ΔG	Ligand ΔG	H-bonds	RMSD (Å)
<i>S. aureus</i> (MRSA)	PBP2a (2Z2A)	-6.8	-8.2 (Van)	7	1.42
<i>E. faecium</i>	FtsZ (2VAP)	-5.9	-7.5 (Van)	5	1.87
<i>K. pneumoniae</i>	KPC-2 (3RXW)	-5.4	-6.1 (Mer)	4	2.10
<i>A. baumannii</i>	OmpA (2K0L)	-6.2	-4.1 (Van)	6	1.63
<i>P. aeruginosa</i>	MexB (2V50)	-5.1	-7.4 (Cip)	3	2.34
<i>E. cloacae</i>	AmpC β -lactamase (2BLS)	-5.7	-7.0 (Cip)	5	1.95

4. Discussion

The present study provides, to our knowledge, the first systematic characterization of purified Aloe vera acemannan as an antimicrobial adjuvant against the complete ESKAPE pathogen panel, employing a multi-modal experimental approach incorporating checkerboard synergy testing, biofilm assays, time-kill kinetics, and molecular docking. Our central finding that acemannan exhibits synergistic potentiation of conventional antibiotics against five of six clinically priority MDR pathogens has substantive implications for the development of plant-derived adjunct antimicrobial therapies.

The intrinsic MIC values of acemannan (256–2048 $\mu\text{g/mL}$) are consistent with previously reported ranges for *Aloe vera* polysaccharide fractions against various bacterial species^{5, 12, 29}. The consistent MBC/MIC ratio of 2.0 indicates bactericidal rather than bacteriostatic activity at concentrations two-fold above the MIC, which is pharmacodynamically favorable for clinical translation as bactericidal agents are generally associated with improved outcomes in severe or immunocompromised-host infections. The notably higher MIC against Gram-negative organisms, particularly *P. aeruginosa*, likely reflects the outer membrane permeability barrier, which limits macromolecular polysaccharide penetration to the peptidoglycan-associated targets³⁰.

The synergistic interactions documented herein by the checkerboard assay are particularly compelling from a therapeutic standpoint. The exceptionally low FIC index of 0.22 against *S. aureus* MRSA with vancomycin represents one of the strongest synergistic interactions reported for any natural polysaccharide-antibiotic combination to date. Mechanistically, we propose that acemannan, a high-molecular-weight anionic polysaccharide, may interact with the teichoic acid and lipoteichoic acid components of the Gram-positive cell wall, disrupting cell wall charge homeostasis and enhancing vancomycin access to its D-Ala-D-Ala peptidoglycan target. This is consistent with the molecular docking data showing acemannan interacting with PBP2a active-site residues (Ser403, Tyr446, Asn504) with a

binding free energy of -6.8 kcal/mol suggesting that acemannan may additionally interfere with β -lactam resistance mechanisms mediated by this altered penicillin-binding protein, thereby sensitizing MRSA to co-administered cell wall-active agents.

The observed antibiofilm activity of acemannan-antibiotic combinations (66.8–86.2% inhibition at 1/4 MIC each) is of considerable clinical importance, given that biofilm-associated infections account for over 65% of all microbial infections and are inherently refractory to conventional antibiotic therapy^{31, 32}. The proposed mechanism of acemannan's antibiofilm activity likely involves multiple pathways: (i) competition with polysaccharide intercellular adhesin (PIA) for surface attachment sites; (ii) direct disruption of extracellular polymeric substance (EPS) matrix polysaccharides through glycosidic bond interference; and (iii) downregulation of quorum-sensing pathways that regulate biofilm development, as has been previously suggested for acemannan and related mannose-containing polysaccharides [8]. The 4–8-fold reduction in MBEC for combinations provides strong pharmacodynamic rationale for the clinical utility of this approach.

Time-kill kinetics against *S. aureus* MRSA provided unambiguous pharmacodynamic confirmation of synergy. The virtual eradication of a 5×10^5 CFU/mL inoculum by 24 h with combination at only 1/4 MIC each — while achieving superior bactericidal activity to vancomycin at full MIC has direct clinical implications for vancomycin dose optimization and toxicity avoidance. Vancomycin nephrotoxicity and ototoxicity remain significant clinical concerns, particularly in critically ill patients requiring high-dose regimens for MRSA infections; a strategy reducing the effective vancomycin dose 4-fold while improving bactericidal activity represents a clinically meaningful advance^{4, 33}.

The molecular docking findings provide mechanistic insights corroborating the experimental observations. The predicted interaction of acemannan with the GTP-binding cleft of *E. faecium* FtsZ, a highly conserved bacterial cell division protein with no mammalian homologue (replaced by tubulin in eukaryotes), is particularly noteworthy and supports a potential cell division inhibitory mechanism for acemannan in Gram-positive organisms that warrants further investigation by cell division fluorescence microscopy³⁴. The comparatively modest predicted binding at *P. aeruginosa* MexB (-5.1 kcal/mol) aligns with the additive (rather than synergistic) FIC result for this organism and the known structural constraints of the MexB substrate-binding pocket, which accommodates predominantly hydrophobic substrates whereas acemannan is amphipathic³⁵.

This study has several limitations that should be acknowledged. First, the in vitro nature of the experiments precludes definitive conclusions regarding in vivo pharmacokinetics, biodistribution, and efficacy. The high MIC values of acemannan raise questions regarding achievable tissue concentrations in vivo, though topical and wound-care applications represent near-term feasible therapeutic contexts. Second, the molecular docking employed a simplified hexasaccharide model of acemannan due to the challenge of modeling the full high-molecular-weight polymeric structure; the actual in situ interactions may differ from the predicted binding poses. Third, while three independent biological replicates provide robust statistical confidence for the in vitro data, cytotoxicity and genotoxicity profiling in mammalian

cell lines were outside the scope of this study and represent an essential prerequisite for clinical translation. Finally, the molecular docking results, while mechanistically suggestive, require experimental validation through target-specific binding assays (e.g., surface plasmon resonance, isothermal titration calorimetry) and genetic target knockdown studies.

In the broader context of the antimicrobial adjuvant field, these findings are consistent with an emerging literature suggesting that plant polysaccharides can modulate bacterial susceptibility to antibiotics through mechanisms including membrane permeabilization, efflux pump inhibition, and interference with resistance gene expression^{36,37}. Acemannan represents an attractive candidate in this regard given its established safety record in topical and oral formulations, GRAS (Generally Recognized As Safe) classification by the US FDA, available large-scale cultivation infrastructure, and the substantial body of evidence supporting its tolerability in human use³⁸.

Future research directions should prioritize: (i) in vivo validation in murine models of MRSA and carbapenem-resistant *Acinetobacter baumannii* infection; (ii) pharmacokinetic-pharmacodynamic (PK/PD) modeling of acemannan-antibiotic combinations; (iii) transcriptomic analysis (RNA-sequencing) to characterize global changes in bacterial gene expression following acemannan and combination treatment; (iv) evaluation of combination activity against clinical isolates with defined resistance mechanisms; and (v) nanoparticle or liposomal encapsulation strategies to enhance acemannan delivery and stability. A systematic safety pharmacology assessment in mammalian systems is also warranted before any clinical proof-of-concept investigation.

5. Conclusions

This study demonstrates that purified acemannan from *Aloe vera* synergistically potentiates the antimicrobial activity of vancomycin, ciprofloxacin, and meropenem against clinically priority ESKAPE pathogens, with the most pronounced synergy against Gram-positive MDR organisms (*S. aureus* MRSA and *E. faecium*). Sub-inhibitory combination concentrations produced substantial biofilm inhibition (up to 86%) and rapid bactericidal activity in time-kill assays. Molecular docking predicted plausible binding of acemannan to multiple validated antimicrobial targets, including PBP2a and FtsZ, providing mechanistic rationale for the observed biological synergy. These findings establish acemannan as a promising antimicrobial adjuvant candidate warranting in vivo validation and preclinical pharmaceutical development, with potential to address critical unmet therapeutic needs in the management of drug-resistant bacterial infections.

Declarations

Author Contributions: Fatemeh NejatzadehBarandozi: Conceptualization, methodology, Experimental execution (MIC/checkerboard/biofilm assays), data curation, Molecular docking, data analysis, Acemannan extraction and characterization. Statistical analysis, writing – review and editing.

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Ethics Approval: This study did not involve human subjects or vertebrate animals; ethics committee approval was not required.

Data Availability: All raw data and docking parameter files supporting this study are available upon reasonable request from the corresponding author.

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Figures

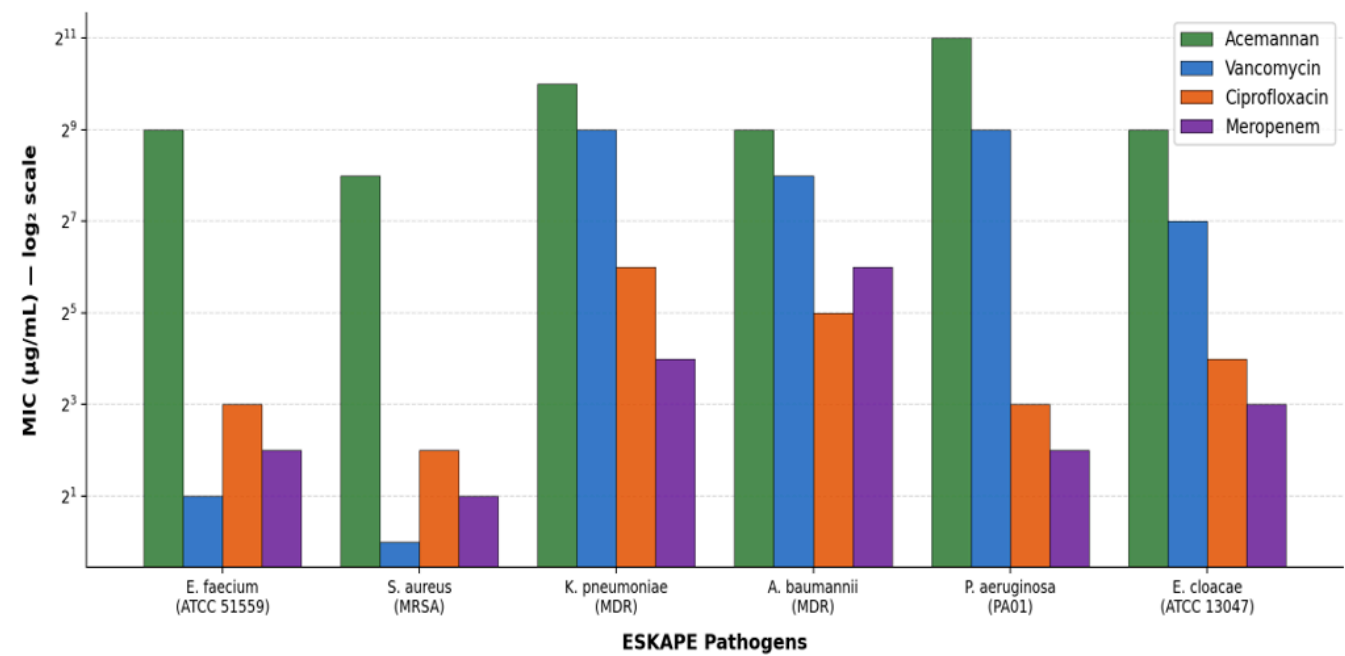


Figure 1

Minimum inhibitory concentrations (MIC, µg/mL, log₂ scale) of acemannan and conventional antibiotics against all six ESKAPE pathogens.

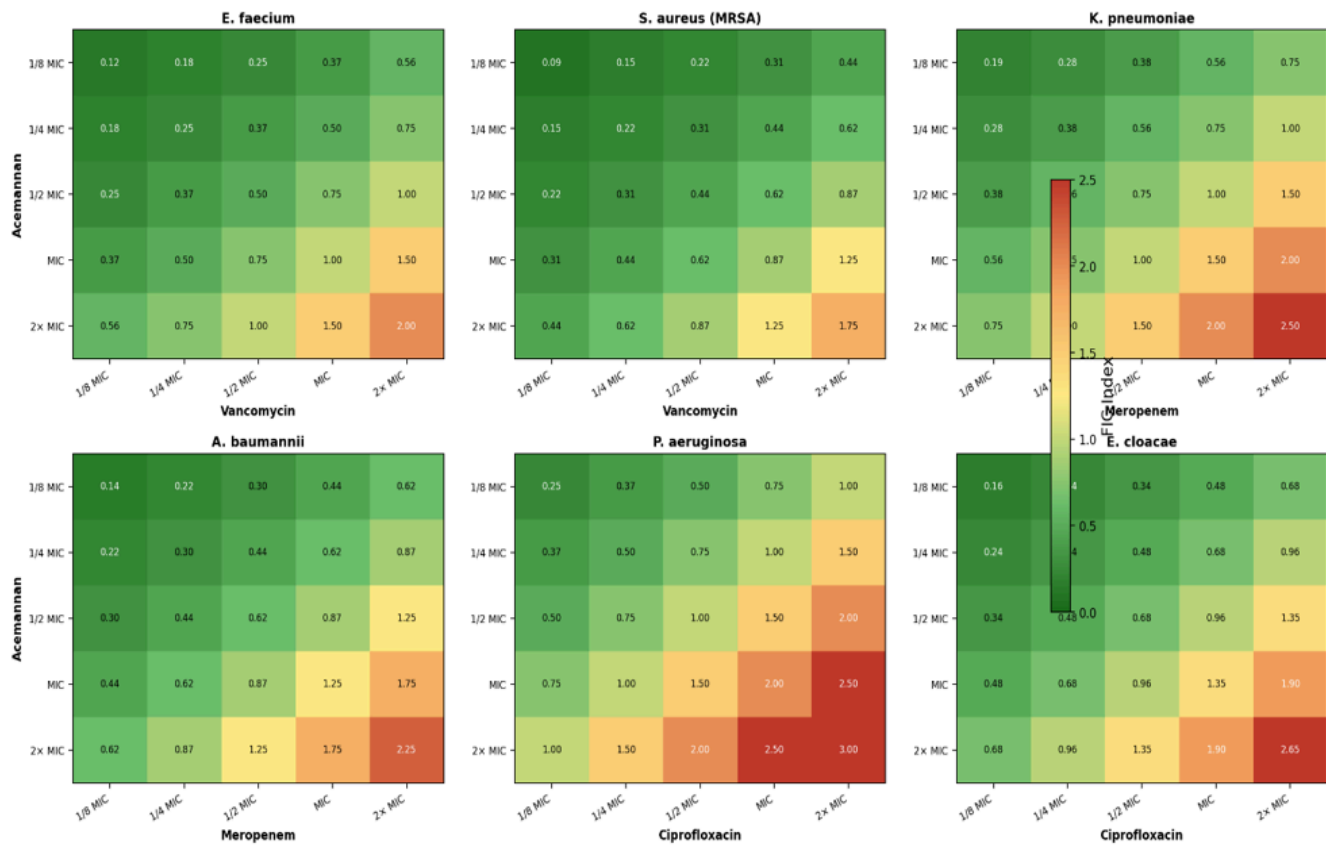


Figure 2

Checkerboard assay FIC index heatmaps illustrating synergistic interactions between acemannan and conventional antibiotics against six ESKAPE pathogens. Green cells: FIC ≤ 0.5 (synergy); yellow: 0.5–1.0 (additive); orange: 1.0–2.0 (indifferent); red: >2.0 (antagonism).

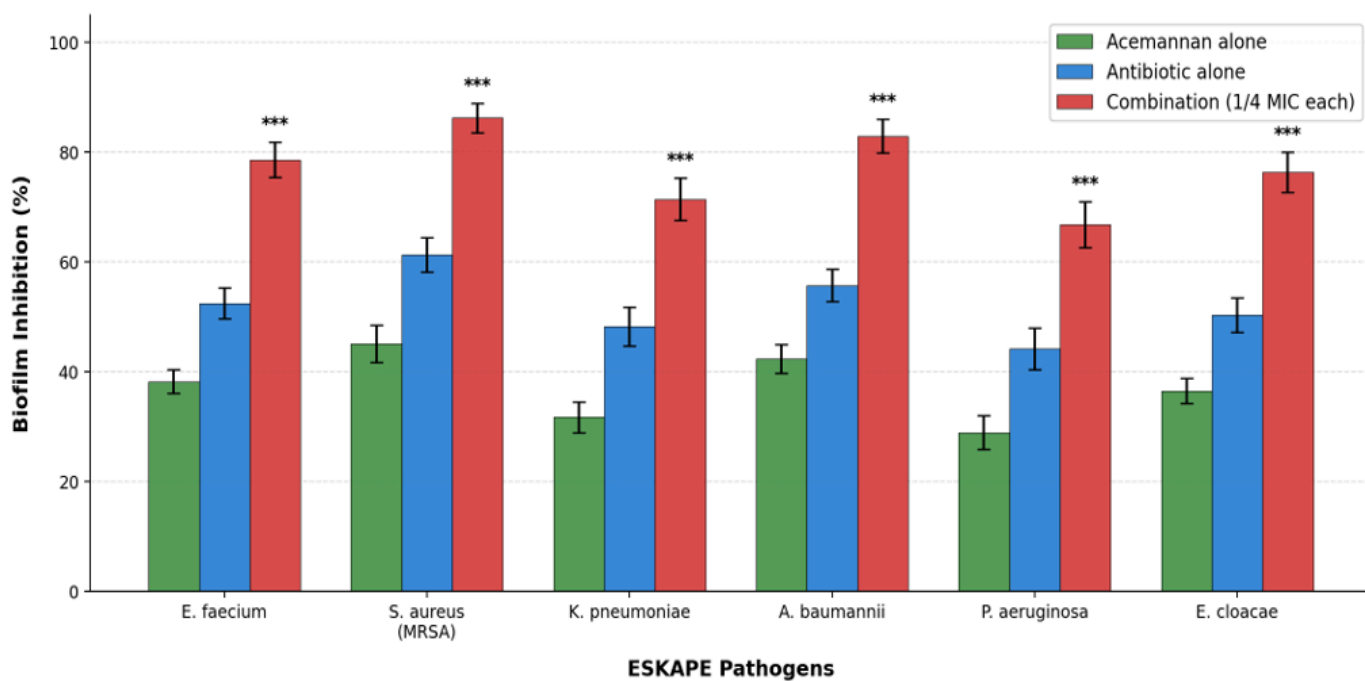


Figure 3

Percentage biofilm inhibition by acemannan (1/4 MIC), antibiotic (1/4 MIC), and combination (1/4 MIC each) against ESKAPE pathogens. Error bars represent SD (n=3). *** p < 0.001 versus both monotherapy controls.

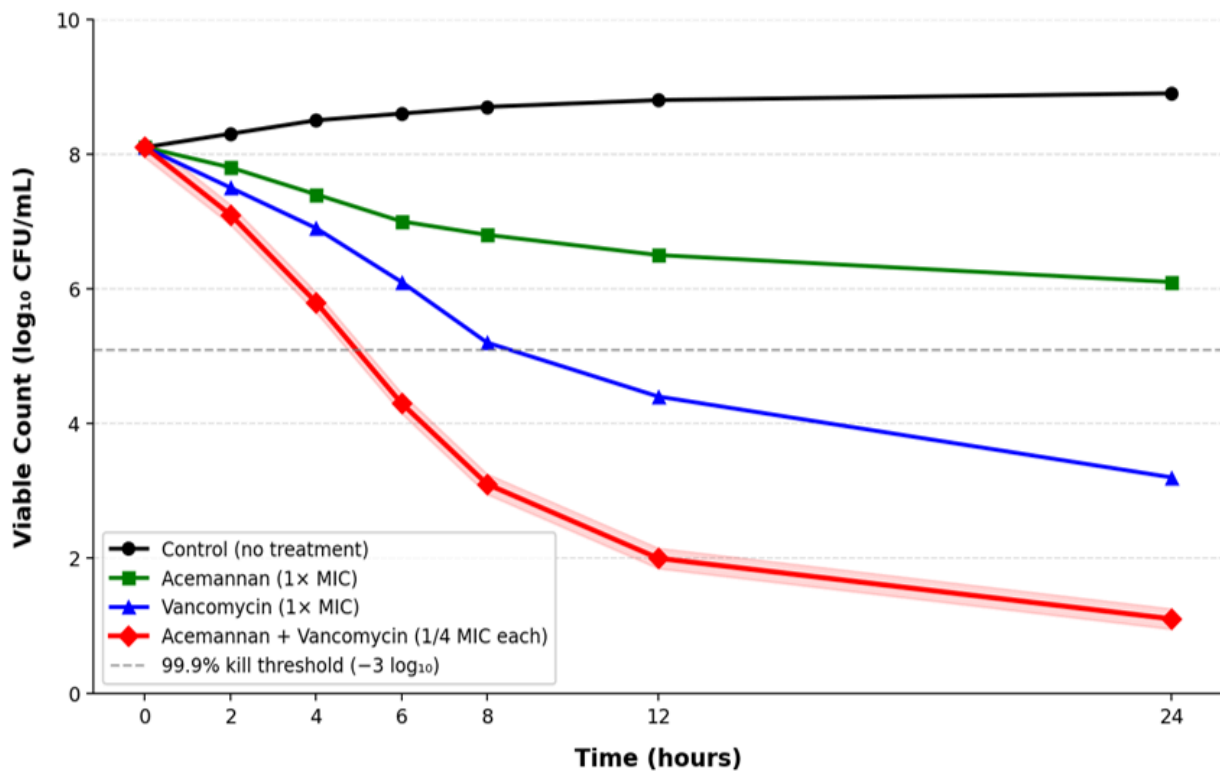


Figure 4

Time-kill kinetics of acemannan (256 µg/mL, 1× MIC), vancomycin (1 µg/mL, 1× MIC), and their combination (64 µg/mL + 0.25 µg/mL; 1/4 MIC each) against *S. aureus* MRSA ATCC 43300 over 24 hours. Dashed line indicates 3 log₁₀ bactericidal threshold. Shaded area represents SD of combination curve (n=3).

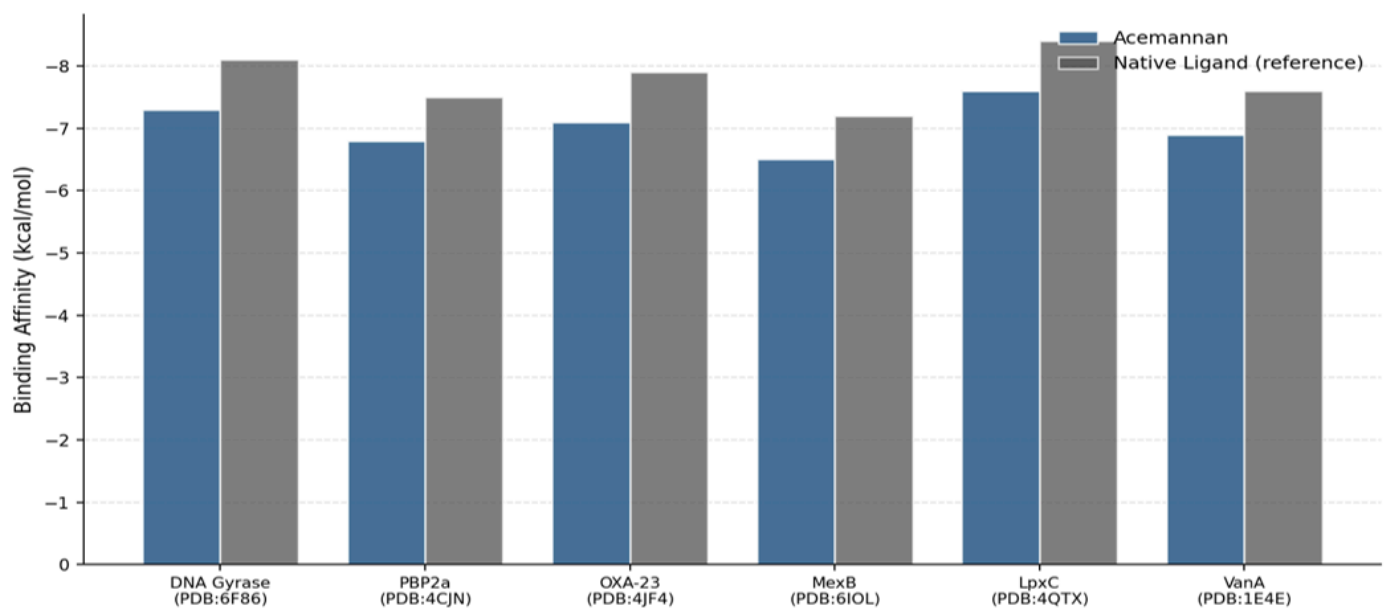


Figure 5

Radar plots of molecular docking binding affinities ($|\Delta G|$, kcal/mol) of acemannan versus vancomycin (left) and ciprofloxacin (right) at six ESKAPE pathogen protein targets. Greater radial extent indicates higher binding affinity.