JHWCR JOURNAL OF HEALTH, WELLNESS AND COMMUNITY RESEARCH 3007-0570

https://doi.org/10.61919/v0kp2y59

Original Article

Antimicrobial Peptides From Amphibian Skin as Potential Therapeutics

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"Cite this Article" Received: 15 December 2025; **Accepted**: 17 April 2026; **Published**: 14 May 2026

Author Contributions: Concept: B; Design: US; Data Collection: AP; Analysis: SHUH; Drafting: MI. **Ethical Approval** was obtained from the Respective Institution.. **Informed Consent**: Written informed consent was obtained from all participants; **Conflict of Interest**: The authors declare no conflict of interest. **Funding**: No external funding; **Data Availability**: Available from the corresponding author on reasonable request; **Acknowledgments**: N/A.

ABSTRACT

Background: Multidrug-resistant bacterial infections are a growing challenge in tertiary-care hospitals, particularly where antimicrobial pressure is high and treatment options are limited. Amphibian skin secretions contain antimicrobial peptides that may provide novel leads for anti-infective development. Objective: This study aimed to isolate peptide-containing fractions from amphibian skin secretions and evaluate their in vitro antibacterial, antibiofilm, safety, and stability profiles against multidrug-resistant clinical isolates. Methods: An experimental laboratory-based study was conducted using 87 non-duplicate multidrug-resistant isolates, including MRSA, ESBL-producing Escherichia coli, ESBL-producing Klebsiella pneumoniae, carbapenem-resistant Pseudomonas aeruginosa, and carbapenem-resistant Acinetobacter baumannii. Amphibian skin secretions were processed into crude extract and purified by RP-HPLC to obtain two active fractions, AMP-1 and AMP-2. Antibacterial activity was assessed using MIC and MBC testing, time–kill kinetics, biofilm inhibition and disruption assays, hemolysis testing, mammalian-cell viability screening, and short-term serum stability assessment. Results: AMP-1 showed the strongest antibacterial profile, with median MICs of 4 µg/mL against MRSA, 8 µg/mL against ESBL-producing E. coli, and 16 µg/mL against carbapenem-resistant P. aeruginosa. MBCs were generally one dilution above MIC values. AMP-1 produced concentration-dependent killing against MRSA, inhibited biofilm formation more effectively than it disrupted mature biofilm, and showed lower hemolysis than AMP-2 at matched concentrations. AMP-1 retained 76% activity after 4 hours in 25% serum, compared with 68% for AMP-2. Conclusion: Amphibian-derived AMP fractions, particularly AMP-1, demonstrated promising in vitro activity and preliminary selectivity against MDR pathogens, supporting further peptide characterization, optimization, formulation development, and preclinical validation. Keywords: Antimicrobial peptides; amphibian skin secretion; multidrug-resistant bacteria; MRSA; ESBL; carbapenem resistance; RP-HPLC; MIC; biofilm inhibition; Sindh Pakistan.

INTRODUCTION

Antimicrobial resistance has become a major threat to effective infection management, particularly in tertiary-care hospitals where prolonged admissions, invasive procedures, intensive antibiotic exposure, and high patient turnover increase the risk of multidrug-resistant bacterial infections. Pathogens such as methicillin-resistant Staphylococcus aureus (MRSA), extended-spectrum β-lactamase-producing Enterobacterales, carbapenem-resistant Pseudomonas aeruginosa, and carbapenem-resistant Acinetobacter baumannii are especially difficult to manage because available therapeutic options are limited, costly, toxic, or increasingly ineffective. This challenge is particularly relevant in low- and

middle-income healthcare settings, including Pakistan, where antimicrobial misuse, delayed microbiological diagnosis, limited stewardship infrastructure, and variable infection-control resources contribute to the persistence and spread of resistant organisms.

The stagnation of conventional antibiotic discovery has intensified interest in alternative antimicrobial platforms with mechanisms distinct from classical antibiotics. Antimicrobial peptides are small, usually cationic and amphipathic molecules that form an important component of innate host defense in many organisms. Unlike many conventional antibiotics that act on specific intracellular targets, several antimicrobial peptides interact directly with bacterial membranes, causing membrane disruption, pore formation, leakage of cellular contents, and rapid bacterial death. This mechanism may reduce the likelihood of conventional target-based resistance, although peptide degradation, host-cell toxicity, serum instability, and delivery limitations remain major translational barriers.

Amphibian skin secretions are among the richest natural sources of antimicrobial peptides. Amphibian skin is continuously exposed to water, soil, and environmental microorganisms, and specialized granular glands release bioactive peptides as part of the animal's innate defense system. Peptide families such as magainins, temporins, brevinins, dermaseptins, phylloseptins, and related molecules have shown antibacterial, antifungal, antibiofilm, immunomodulatory, and wound-healing potential. Their structural diversity makes amphibian-derived peptides attractive candidates for antimicrobial discovery, particularly for topical and biofilm-prevention applications. However, the therapeutic value of any newly isolated peptide fraction depends on reproducible purification, physicochemical characterization, antimicrobial testing against clinically relevant resistant isolates, and early safety profiling.

Despite increasing international interest in antimicrobial peptides, locally generated data from Pakistan remain limited, particularly studies linking amphibian-derived peptide fractions with multidrug-resistant clinical isolates from tertiary-care settings. This gap is important because antimicrobial resistance patterns vary geographically, and candidate molecules intended for regional translational development should be tested against locally circulating resistant phenotypes. Furthermore, many studies report crude extract activity without adequate activity-guided purification, MIC/MBC profiling, antibiofilm evaluation, or preliminary cytotoxicity and stability testing, limiting their usefulness for drug-development prioritization.

The present experimental laboratory-based study was therefore designed to isolate and purify antimicrobial peptide-containing fractions from amphibian skin secretions and evaluate their in vitro activity against multidrug-resistant clinical bacterial isolates recovered from a tertiary-care hospital in Sindh, Pakistan. The study specifically assessed antibacterial potency using MIC and MBC testing, concentration-dependent killing through time–kill kinetics, antibiofilm activity against selected resistant organisms, and preliminary safety signals through hemolysis, mammalian-cell viability, and short-term serum stability testing. The objective was to identify whether purified amphibian skin-derived peptide fractions demonstrate sufficient antimicrobial activity and early selectivity to justify further molecular characterization, optimization, and formulation-focused preclinical development.

MATERIALS AND METHODS

This experimental laboratory-based biotechnology study was conducted in the clinical microbiology and molecular biotechnology laboratories of a tertiary-care hospital in Sindh, Pakistan. The study consisted of two linked laboratory components: the recovery and confirmation of multidrug-resistant clinical bacterial isolates from routine diagnostic specimens, and the collection, extraction, purification, and biological testing of amphibian skin secretion-derived antimicrobial peptide fractions against these isolates. All procedures involving multidrug-resistant organisms were performed under biosafety level-2 conditions using standard containment, personal protective equipment, surface decontamination, and biomedical waste-disposal practices.

Non-duplicate clinical bacterial isolates were obtained from routine diagnostic specimens submitted to the hospital microbiology laboratory, including wound swabs, blood, urine, tracheal aspirates, and other clinically indicated samples. Only one isolate per patient/specimen episode was included to avoid duplicate representation. The target organisms were selected because of their clinical relevance in tertiary-care antimicrobial resistance, including MRSA, ESBL-producing Escherichia coli, ESBL-producing Klebsiella pneumoniae, carbapenem-resistant Pseudomonas aeruginosa, and carbapenem-resistant Acinetobacter baumannii. Isolates with incomplete identification, mixed culture contamination, uncertain resistance phenotype, or inadequate viability on subculture were excluded.

Primary bacterial identification was performed using routine culture morphology, Gram staining, and biochemical profiling, followed by confirmatory identification using available automated or advanced identification systems where applicable. Antimicrobial susceptibility testing was performed using disk diffusion and/or MIC-based methods according to recognized laboratory standards. Multidrug resistance was defined using accepted international criteria based on non-susceptibility to at least one agent in three or more antimicrobial categories. MRSA was confirmed phenotypically using cefoxitin resistance, ESBL production was confirmed using a combined-disk or equivalent phenotypic method, and carbapenem resistance among non-fermenters was confirmed using imipenem and/or meropenem non-susceptibility supported by MIC testing where available.

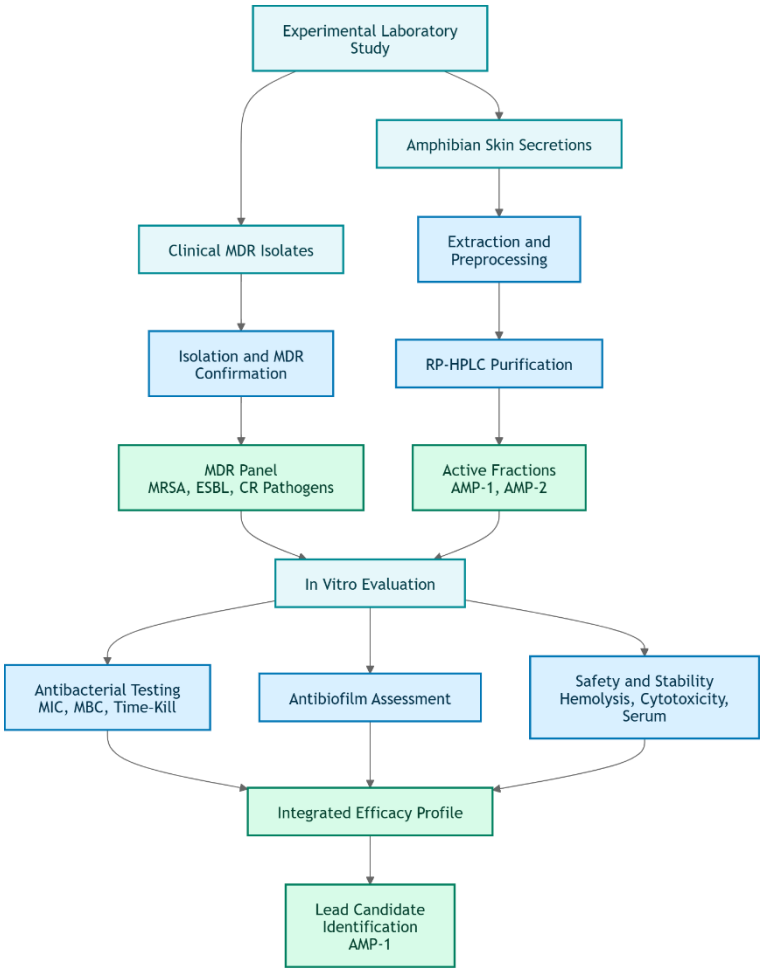

Figure 1 Experimental workflow for isolation, purification, and in vitro evaluation of amphibian-derived antimicrobial peptide fractions against multidrug-resistant clinical isolates. The diagram summarizes the parallel processing of clinical MDR isolates and amphibian skin secretions, followed by RP-HPLC purification, identification of active fractions, antimicrobial, antibiofilm, safety, and stability testing, and final prioritization of AMP-1 as the lead candidate.

Amphibian skin secretions were collected from locally encountered amphibians under ethical and regulatory oversight using a non-lethal collection approach designed to minimize animal stress and tissue injury. Amphibians were visually examined before collection, and individuals with visible skin

lesions, injury, or poor health were excluded. The skin surface was rinsed with sterile distilled water before secretion induction. Granular gland secretions were collected using a mild standardized stimulation procedure, washed into chilled acidified aqueous collection tubes to reduce peptide degradation, and transported under cold-chain conditions. After secretion collection, animals were monitored for recovery and released at the capture site when ethically and legally permissible.

Collected secretions were clarified by centrifugation to remove debris and particulate material, and the supernatant was stored at −80 °C until further processing. Samples were lyophilized to obtain crude dried extract and then reconstituted in water containing 0.1% trifluoroacetic acid. Crude extracts were desalted using C18 solid-phase extraction cartridges and eluted with increasing concentrations of acetonitrile in 0.1% trifluoroacetic acid. Eluted fractions were concentrated under vacuum and screened for antimicrobial activity to identify bioactive fractions for further purification.

Reverse-phase high-performance liquid chromatography was used for activity-guided purification of peptide-containing fractions. Desalted crude extract was applied to a C18 reverse-phase column and eluted using an acetonitrile-water gradient containing 0.1% trifluoroacetic acid, with absorbance monitored at 214 nm. Fractions were collected at fixed intervals, lyophilized, reconstituted, and tested for antibacterial activity. Fractions showing reproducible inhibitory activity were subjected to repeat RP-HPLC purification using refined gradients until dominant peptide peaks were obtained. The two most consistently active purified fractions were provisionally designated AMP-1 and AMP-2 for downstream biological testing.

Purified peptide fractions were characterized by analytical RP-HPLC and mass spectrometry to assess purity and molecular mass. Where sufficient material and facility access were available, tandem mass spectrometry was used for de novo sequence inference, and physicochemical features such as estimated charge, hydrophobicity, and amphipathicity were assessed in silico. Fractions were treated as provisional purified peptide candidates unless full sequence confirmation and synthetic replication were achieved. For biological assays requiring repeat testing, purified fractions were prepared from repeated purification runs and standardized by peptide concentration before use.

Antibacterial activity was assessed using broth microdilution in sterile 96-well microplates. Fresh bacterial cultures were adjusted to a standardized inoculum before exposure to serial two-fold dilutions of crude extract, AMP-1, and AMP-2 in cation-adjusted Mueller–Hinton broth. Plates were incubated at 35–37 °C for 16–20 hours. The minimum inhibitory concentration was recorded as the lowest peptide concentration preventing visible bacterial growth. Minimum bactericidal concentration was determined by subculturing aliquots from wells at and above the MIC onto non-selective agar, followed by incubation and assessment of colony growth. Sterility controls, growth controls, and assay controls were included in each run. Testing was performed using technical replicates and repeated on independent bacterial cultures where feasible.

Time–kill kinetics were performed for representative multidrug-resistant isolates to evaluate concentration-dependent bactericidal activity. Selected isolates were exposed to AMP-1 at 1×, 2×, and 4× MIC, with a peptide-free growth control included in parallel. Aliquots were collected at predetermined time points, serially diluted, plated on non-selective agar, and incubated for viable colony counting. Results were expressed as log10 CFU/mL over time. Greater reduction in viable count at increasing MIC multiples was interpreted as evidence of concentration-dependent killing.

Antibiofilm activity was evaluated using a crystal violet microtiter plate assay. For biofilm-inhibition testing, peptides were added at the time of bacterial inoculation and biofilm biomass was quantified after incubation. For biofilm-disruption testing, mature pre-formed biofilms were established before peptide exposure, after which residual biomass was stained and quantified. Biofilm outcomes were expressed as percentage inhibition or disruption relative to untreated controls. Assays were performed for

representative organisms including MRSA, ESBL-producing E. coli, and carbapenem-resistant P. aeruginosa.

Preliminary safety assessment included hemolysis testing and mammalian-cell viability screening. Hemolysis was assessed using freshly collected human erythrocytes exposed to increasing peptide concentrations, with phosphate-buffered saline used as the negative control and detergent-treated erythrocytes used as the positive control. Percentage hemolysis was calculated relative to positive and negative controls. Cytotoxicity was evaluated using a mammalian cell viability assay such as MTT or resazurin reduction, depending on local laboratory availability, and results were used to estimate the concentration associated with 50% reduction in cell viability. Short-term serum stability was assessed by incubating peptide fractions in serum-containing conditions at 37 ˚C, followed by activity-based or chromatographic assessment of retained peptide activity over time.

The primary outcomes were MIC and MBC values of crude extract, AMP-1, and AMP-2 against multidrug-resistant clinical isolates. Secondary outcomes included time–kill reduction in log10 CFU/mL, percentage biofilm inhibition or disruption, percentage hemolysis, mammalian-cell viability, and retained activity after serum exposure. Data were summarized using frequencies and percentages for categorical variables, and mean with standard deviation or median with interquartile range for continuous or non-normally distributed laboratory outcomes. MIC and MBC distributions were summarized by organism group and test agent. Comparative analyses between peptide fractions and organism groups were performed using parametric or non-parametric tests according to data distribution, with statistical significance set at $p < 0.05$. All analyses were conducted using standard statistical software, and graphical summaries were prepared for MIC patterns, time–kill kinetics, antibiofilm activity, and hemolysis response.

RESULTS

A total of 87 non-duplicate multidrug-resistant clinical isolates were included in the antimicrobial screening panel. The isolate set represented clinically important tertiary-care resistant phenotypes, including MRSA (n=20, 23.0%), ESBL-producing Escherichia coli (n=22, 25.3%), ESBL-producing Klebsiella pneumoniae (n=15, 17.2%), carbapenem-resistant Pseudomonas aeruginosa (n=18, 20.7%), and carbapenem-resistant Acinetobacter baumannii (n=12, 13.8%). MRSA isolates were most frequently recovered from wound and blood specimens, ESBL-producing Enterobacterales were mainly isolated from urine and blood or wound specimens, while carbapenem-resistant non-fermenters were commonly recovered from tracheal aspirates and wound samples. The included organisms and resistance-confirmation markers are summarized in Table 1.

Table 1. Distribution of Multidrug-Resistant Clinical Isolates Included in Screening

Organism / Resistance Phenotype	n	%	Common Specimen Sources	Phenotypic Resistance Marker
MRSA	20	23.0	Wound, blood	Cefoxitin resistant
ESBL-producing E. coli	22	25.3	Urine, blood	ESBL confirmatory positive
ESBL-producing K. pneumoniae	15	17.2	Urine, wound	ESBL confirmatory positive
Carbapenem-resistant P. aeruginosa	18	20.7	Tracheal aspirate, wound	Imipenem/meropenem non-susceptible
Carbapenem-resistant A. baumannii	12	13.8	Tracheal aspirate, wound	Imipenem/meropenem non-susceptible
Total	87	100.0	—	—

Crude amphibian skin secretion extract produced detectable antimicrobial activity, but activity was substantially enhanced after RP-HPLC-guided purification. Two purified bioactive peptide-containing fractions, provisionally designated AMP-1 and AMP-2, showed reproducible inhibitory activity and were selected for downstream testing. In the reported MIC/MBC panel, MRSA showed the greatest susceptibility, followed by ESBL-producing E. coli, while carbapenem-resistant P. aeruginosa required higher concentrations for inhibition and killing. AMP-1 consistently showed lower MIC and MBC values than AMP-2 and crude extract, indicating stronger antibacterial potency after purification.

Table 2. MIC and MBC Profile of Crude Extract, AMP-1, and AMP-2 Against Selected MDR Isolates

Test Agent	MRSA MIC, µg/mL Median [IQR]	MRSA MBC, µg/mL Median [IQR]	ESBL E. coli MIC, µg/mL Median [IQR]	ESBL E. coli MBC, µg/mL Median [IQR]	CR P. aeruginosa MIC, µg/mL Median [IQR]	CR P. aeruginosa MBC, µg/mL Median [IQR]
Crude extract	64 [32–128]	128 [64–256]	128 [64–256]	256 [128–512]	256 [128–512]	512 [256–1024]
AMP-1	4 [2–8]	8 [4–16]	8 [4–16]	16 [8–32]	16 [8–32]	32 [16–64]
AMP-2	8 [4–16]	16 [8–32]	16 [8–32]	32 [16–64]	32 [16–64]	64 [32–128]

Compared with crude extract, AMP-1 reduced the median MIC from 64 µg/mL to 4 µg/mL against MRSA, representing a 16-fold reduction. Against ESBL-producing E. coli, the median MIC declined from 128 µg/mL to 8 µg/mL, also showing a 16-fold reduction. Against carbapenem-resistant P. aeruginosa, the median MIC declined from 256 µg/mL to 16 µg/mL, again indicating a 16-fold improvement after purification. AMP-2 was also more active than crude extract but remained approximately one two-fold dilution less potent than AMP-1 across the reported organisms. MBC values were generally one dilution higher than MIC values for purified peptides, supporting a bactericidal pattern at concentrations close to the inhibitory threshold.

The median MIC heatmap showed the same potency gradient visually, with the highest MIC burden observed for crude extract and the lowest MIC values clustered around AMP-1. The figure also demonstrated that Gram-positive MRSA was more susceptible than Gram-negative MDR organisms, while carbapenem-resistant non-fermenters required higher peptide exposure. This figure should be retained because it provides a compact comparative overview of antimicrobial potency across peptide fractions and organisms.

Time–kill analysis of AMP-1 against a representative MRSA isolate demonstrated concentration-dependent bacterial killing. The untreated control increased progressively from approximately 6.2 log10 CFU/mL at baseline to above 8.0 log10 CFU/mL by 24 hours. At 1× MIC, AMP-1 suppressed growth but did not produce rapid bactericidal reduction, with viable counts remaining near 5.0 log10 CFU/mL at 24 hours. At 2× MIC, bacterial counts declined more clearly, reaching approximately 3.4 log10 CFU/mL by 24 hours. At 4× MIC, AMP-1 produced rapid early killing, reducing viable counts to approximately 3.2 log10 CFU/mL by 2 hours and near 2.0 log10 CFU/mL by 8–24 hours. This curve should be retained as the strongest efficacy figure because it demonstrates both exposure-response behavior and sustained bactericidal activity.

Biofilm testing showed that purified peptides were more effective at preventing biofilm formation than disrupting mature biofilms. At 1× MIC, biofilm inhibition was highest against MRSA (43%), followed by ESBL-producing E. coli (28%) and carbapenem-resistant P. aeruginosa (22%). At 2× MIC, inhibition increased across all tested organisms, reaching 71% for MRSA, 52% for ESBL-producing E. coli, and 41% for carbapenem-resistant P. aeruginosa. Disruption of pre-formed biofilm at 4× MIC was lower than preventive activity, with reductions of 49%, 31%, and 26%, respectively.

Table 3. Antibiofilm Activity of Purified Peptide Fractions Against Selected MDR Isolates

Organism	Biofilm Inhibition at 1× MIC	Biofilm Inhibition at 2× MIC	Disruption of Pre-formed Biofilm at 4× MIC
MRSA	43%	71%	49%
ESBL-producing E. coli	28%	52%	31%
CR P. aeruginosa	22%	41%	26%

The current biofilm bar figure should be revised rather than used in its present form because it only presents inhibition at 1× and 2× MIC and omits mature biofilm disruption. A stronger figure would display all three antibiofilm outcomes—1× MIC inhibition, 2× MIC inhibition, and 4× MIC disruption—in a single grouped figure, allowing readers to compare prevention and disruption directly.

Preliminary safety testing indicated low hemolytic activity at antibacterial concentrations, with AMP-1 showing a more favorable safety profile than AMP-2. At 64 µg/mL, hemolysis was 2.1% for AMP-1 and 3.4% for AMP-2. At 128 µg/mL, hemolysis increased to 4.8% for AMP-1 and 7.9% for AMP-2. Mammalian-

cell viability testing showed a higher CC50 for AMP-1 (>128 µg/mL) than AMP-2 (96 µg/mL). Short-term serum exposure also showed partial retention of activity, with 76% activity retained by AMP-1 and 68% by AMP-2 after 4 hours in 25% serum.

Table 4. Preliminary Hemolysis, Cytotoxicity, and Serum Stability Profile of AMP-1 and AMP-2

Assay	AMP-1	AMP-2
Hemolysis at 64 µg/mL	2.1%	3.4%
Hemolysis at 128 µg/mL	4.8%	7.9%
Mammalian-cell CC50	>128 µg/mL	96 µg/mL
Activity retained after 4 h in 25% serum	76%	68%

The hemolysis concentration–response figure should be retained because it adds important safety interpretation beyond the antimicrobial data. Across the tested concentration range, both peptides showed increasing hemolysis with higher peptide concentrations, but AMP-1 remained consistently less hemolytic than AMP-2. This supports AMP-1 as the stronger lead candidate because it combined lower MIC values, concentration-dependent killing, measurable antibiofilm activity, and a wider preliminary selectivity profile than AMP-2.

Overall, the results demonstrate that activity-guided purification markedly improved antibacterial potency compared with crude amphibian skin secretion extract. AMP-1 showed the most favorable combined profile, with the lowest MIC/MBC values, clear concentration-dependent killing against MRSA, meaningful biofilm-preventive activity, lower hemolysis, higher mammalian-cell tolerance, and better short-term serum activity retention than AMP-2.

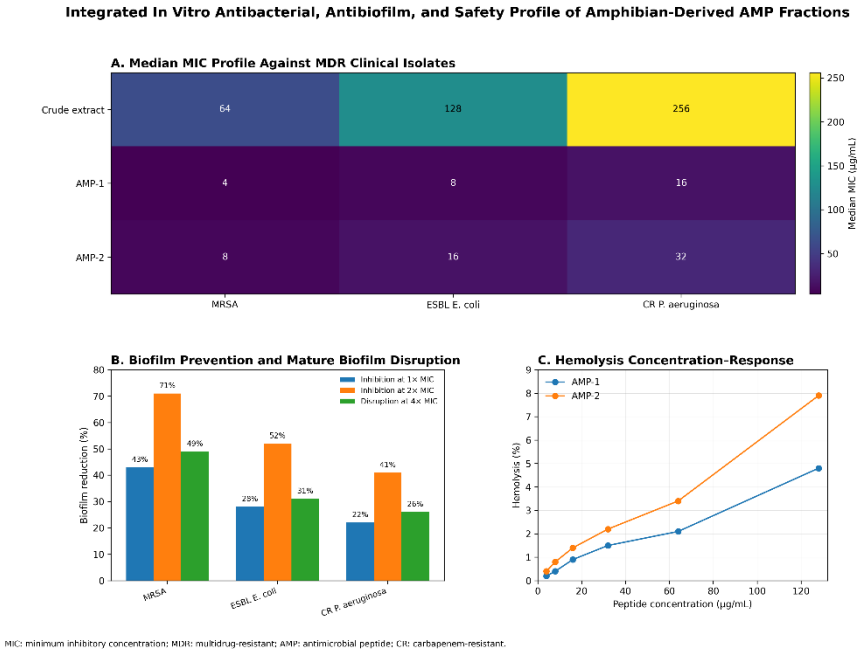

Figure 2. Integrated in vitro antibacterial, antibiofilm, and safety profile of amphibian-derived AMP fractions against MDR clinical isolates. Panel A shows median MIC values for crude extract, AMP-1, and AMP-2, demonstrating markedly greater potency after purification. Panel B compares biofilm inhibition at 1× and 2× MIC with disruption of pre-formed biofilm at 4× MIC. Panel C shows the hemolysis concentration–response profile, indicating lower erythrocyte toxicity for AMP-1 than AMP-2 across tested concentrations.

DISCUSSION

This experimental laboratory-based study demonstrated that amphibian skin secretion-derived peptide fractions showed measurable in vitro activity against multidrug-resistant clinical bacterial isolates recovered from a tertiary-care setting in Sindh, Pakistan. The most important finding was that activity-guided purification markedly improved antibacterial potency compared with crude extract, with AMP-1 showing the strongest overall profile across MIC/MBC testing, time–kill kinetics, antibiofilm screening,

hemolysis, mammalian-cell viability, and short-term serum stability. The observed pattern supports the biological plausibility that crude amphibian secretions contain active antimicrobial components that become substantially more potent when enriched through RP-HPLC purification. However, because AMP-1 and AMP-2 remain provisionally designated peptide fractions without complete sequence confirmation in the presented manuscript, the findings should be interpreted as early-stage antimicrobial discovery data rather than definitive therapeutic evidence.

The antimicrobial profile showed a consistent susceptibility gradient, with MRSA demonstrating the lowest MIC values, ESBL-producing E. coli showing intermediate susceptibility, and carbapenem-resistant P. aeruginosa requiring higher peptide concentrations. This pattern is consistent with the known biological behavior of many cationic amphipathic antimicrobial peptides, which interact with bacterial membranes and may show stronger activity against Gram-positive organisms where the cytoplasmic membrane is less protected by an outer membrane barrier. In Gram-negative bacteria, lipopolysaccharide, outer membrane permeability limitations, efflux mechanisms, and biofilm-forming capacity can increase the effective peptide concentration required for inhibition. The retention of activity against carbapenem-resistant P. aeruginosa, despite higher MIC values, remains important because this organism represents a major therapeutic challenge in hospital-acquired infection settings.

The MIC/MBC relationship further supports a bactericidal tendency for the purified peptide fractions. For AMP-1 and AMP-2, MBC values generally remained within one two-fold dilution above MIC values, suggesting that once inhibitory concentrations were reached, bacterial killing occurred at relatively close exposure thresholds. This interpretation was reinforced by the time–kill analysis, where AMP-1 demonstrated concentration-dependent activity against MRSA. At 1× MIC, AMP-1 mainly suppressed bacterial growth, while 2× and 4× MIC produced progressively greater viable-count reductions over time. This exposure-response pattern is relevant for future formulation development because it suggests that achieving and maintaining sufficient local peptide concentrations may be critical for optimal activity.

The antibiofilm findings add further translational relevance, particularly for wound and device-associated infections. AMP activity was stronger in preventing biofilm formation than in disrupting established biofilms, with the highest inhibition observed against MRSA. This distinction is important because mature biofilms are structurally complex and biologically tolerant, limiting antimicrobial penetration and reducing susceptibility. The results therefore support a prevention-focused developmental pathway rather than positioning these peptide fractions as stand-alone agents for established biofilm eradication. Potential applications may include topical formulations, wound dressings, surgical-site adjuncts, or antimicrobial surface coatings, but such applications require additional testing under clinically realistic wound-fluid, tissue, and surface conditions.

The preliminary safety profile favored AMP-1 over AMP-2. AMP-1 showed lower hemolysis at matched concentrations, a higher mammalian-cell CC50, and greater retained activity after short-term serum exposure. These findings suggest a wider early selectivity window for AMP-1, particularly against MRSA where the MIC was substantially below the tested cytotoxic range. Nevertheless, these safety data are preliminary. A complete safety assessment would require multiple mammalian cell lines relevant to intended use, including keratinocytes and fibroblasts for topical applications, as well as irritation, sensitization, protease-resistance, and formulation-compatibility testing. The reduction in activity after serum exposure also highlights a predictable limitation of linear antimicrobial peptides: susceptibility to proteolysis and serum binding. Future optimization through sequence modification, cyclization, terminal protection, D-amino acid substitution, or controlled-release delivery systems may improve stability and reduce required dosing.

Several limitations should be acknowledged. First, the study remains entirely in vitro, so its findings cannot establish clinical efficacy, pharmacokinetics, tissue tolerability, or therapeutic safety. Second, peptide identity is incomplete unless full molecular mass, purity, sequence, and synthetic replication data are added. Third, the MIC/MBC table currently provides detailed results for MRSA, ESBL-producing

E. coli, and carbapenem-resistant P. aeruginosa, but the full isolate panel also includes ESBL-producing K. pneumoniae and carbapenem-resistant A. baumannii; organism-level efficacy data for these groups should be reported or their exclusion from downstream testing should be clearly justified. Fourth, replicate numbers, variability estimates, and inferential statistics are not consistently reported across antibiofilm, hemolysis, cytotoxicity, and stability assays. Finally, the amphibian species, collection permissions, animal ethics approval, and exact peptide yield are essential for reproducibility and should be included before submission.

Overall, the findings support AMP-1 as the most promising purified peptide fraction for further development. The next phase should prioritize complete peptide sequencing, analytical purity confirmation, synthetic replication, mechanism-of-action assays, expanded organism testing, synergy testing with clinically used antibiotics, and topical formulation studies. If these steps confirm reproducible potency, selectivity, and stability, amphibian-derived AMP fractions may represent a useful platform for locally relevant antimicrobial discovery against high-burden MDR pathogens.

CONCLUSION

This study shows that amphibian skin secretion-derived peptide fractions possess meaningful in vitro antibacterial activity against selected multidrug-resistant clinical isolates from a tertiary-care hospital setting in Sindh, Pakistan. Activity-guided purification substantially improved potency compared with crude extract, and AMP-1 emerged as the strongest candidate, demonstrating lower MIC/MBC values, concentration-dependent killing against MRSA, measurable biofilm-preventive activity, lower hemolysis, higher mammalian-cell tolerance, and better short-term serum activity retention than AMP-2. These findings support AMP-1 as a promising early-stage antimicrobial lead, particularly for topical or biofilm-prevention applications; however, complete peptide sequencing, purity confirmation, expanded safety testing, formulation studies, and in vivo validation are required before therapeutic relevance can be established.

REFERENCES

1. Rollins-Smith LA. The importance of antimicrobial peptides (AMPs) in amphibian skin defense. Dev Comp Immunol. 2023;142:104657. doi:10.1016/j.dci.2023.104657.

2. Chen X, Lin H, Zhang Y, et al. Peptides isolated from amphibian skin secretions with emphasis on antimicrobial peptides. Toxins (Basel). 2022;14(10):722. doi:10.3390/toxins14100722.

3. Xuan J, Feng W, Wang J, et al. Antimicrobial peptides for combating drug-resistant bacterial infections. Drug Resist Updat. 2023;100954. doi:10.1016/j.drup.2023.100954.

4. Wiegand I, Hilpert K, Hancock REW. Agar and broth dilution methods to determine the MIC of antimicrobial substances. Nat Protoc. 2008;3:163-175. doi:10.1038/nprot.2007.521.

5. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions. Clin Microbiol Infect. 2012;18(3):268-281. doi:10.1111/j.1469-0691.2011.03570.x.

6. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415(6870):389-395. doi:10.1038/415389a.

7. Zasloff M. Magainins, a class of antimicrobial peptides from Xenopus skin. Proc Natl Acad Sci U S A. 1987;84(15):5449-5453. doi:10.1073/pnas.84.15.5449.

8. Hancock REW, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24(12):1551-1557. doi:10.1038/nbt1267.

9. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 2005;3(3):238-250. doi:10.1038/nrmicro1098.

10. Conlon JM. Structural diversity and species distribution of host-defense peptides in frog skin secretions. Cell Mol Life Sci. 2011;68(13):2303-2315. doi:10.1007/s00018-011-0720-8.

11. Mukhopadhyay S, Prasad ASB, Mehta CH, Nayak UY. Antimicrobial peptide polymers: no escape to ESKAPE pathogens—a review. World J Microbiol Biotechnol. 2020;36(9):131. doi:10.1007/s11274-020-02907-1.

12. D'Andrea LD, Romanelli A. Temporins: multifunctional peptides from frog skin. Amino Acids. 2023;55:123-134. doi:10.1007/s00726-023-03210-z.

13. Yin S, Wang Y, Yang X. Amphibian-derived wound healing peptides: chemical treasure trove. Front Pharmacol. 2023;14:1120228. doi:10.3389/fphar.2023.1120228.

14. Pletzer D, Hancock REW. Antibiofilm peptides: potential as broad-spectrum agents. J Bacteriol. 2016;198(19):2572-2578. doi:10.1128/JB.00017-16.

15. Dostert M, Trimble MJ, Hancock REW. Antibiofilm peptides: overcoming biofilm-related treatment failure. RSC Adv. 2021;11:2718-2728. doi:10.1039/D0RA09593J.

GRAPHICAL ABSTRACT