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A 6.7 kDa Cationic antimicrobial peptide from *Moringa oleifera* seeds: Purification and characterisation

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Antimicrobial cationic peptides are innate host-defence molecules found across diverse species. The broad-spectrum bioactivities of these small basic peptides could be effectively employed against the growing number of drug-resistant microbes. The high stability of plant-derived antimicrobial peptides brings in a promising template for designing novel antibiotics. *Moringa oleifera*, a member of the Moringaceae family, is widely cultivated around the world due to its exceptional nutritional profile. Various plant parts have demonstrated diverse biological activities, underscoring the species' longstanding importance in traditional medicinal practices. While numerous bioactive constituents from *M. oleifera* have been identified, the identities of several compounds that may contribute to its therapeutic potential are still elusive. The present investigation was targeted to isolate and characterise cationic antimicrobial peptide(s) from *M. oleifera* seeds. The peptide components extracted under acidic conditions were fractionated by ammonium sulfate precipitation and subsequently purified by size exclusion chromatography. One purified basic peptide of approximately 6.7 kDa exhibited broad range antimicrobial activity against *S. typhimurium*, *S. aureus* and *C. albicans*, with minimum inhibitory concentration (MIC) values of 16, 32, and 32 µg/mL, respectively. The activity was stable up to 70°C and at pH values of 5–8. Treatment with pronase, proteinase K, and trypsin fully inactivated the purified peptide, abolishing its activity against all three tested pathogens. Divalent (Ca^{2+}, Mg^{2+}) and trivalent (Fe^{3+}) cations had a weak inhibitory effect on the activity. This study hence brings to the fore characteristics of an unreported, highly stable, low molecular weight cationic peptide that significantly contributes to the antimicrobial properties of *M. oleifera* seeds.

Keywords: AMPs, Antimicrobials, Cationic peptides, *Moringa oleifera*, Seed peptide, Thermostable peptides

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Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved defence molecules distributed across all species of life from microbes to higher multicellular organisms, where they function as a crucial component of the innate immune system[1,2]. These peptides constitute one of the earliest natural defence mechanisms against invading pathogens and play a critical role in protecting organisms from microbial infections. The majority of AMPs identified in plants are amphipathic, cysteine-rich, cationic peptides of molecular mass less than 10 kDa, and are effective against diverse phyto-pathogens as well as human pathogens[3]. The presence of cysteine residues and multiple disulfide linkages (usually 2-6) endows them with remarkable structural stability even under exceedingly intense physicochemical conditions such as extremes of temperature or pH, and enzymatic degradation[3-5]. Their distinct structural features, broad antimicrobial spectrum, and lower propensity to induce resistance development have attracted significant interest in recent years. Consequently, plant-derived AMPs are increasingly being explored as prospective candidates for the development of novel peptide-based antibiotics and alternative antimicrobial therapeutics[3,5].

Moringa oleifera (*aka* drumstick tree or horseradish tree), a best-known and most widely cultivated species of the Moringaceae family has earned the title of 'Miracle tree' due to its enormous health benefits and medicinal properties[6]. The importance of *M. oleifera* extracts in traditional herbal medicine has been well accepted worldwide for centuries. Leaves, flowers, and pods of this plant are rich in vitamins, proteins, minerals, and many other healthful compounds, making them valuable as dietary supplements and functional foods[6,7]. Many

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scientific studies have demonstrated a wide range of pharmacological applications of *M. oleifera,* including antibacterial, antifungal, antioxidant, antiulcer, antipyretic, antitumor, diuretic, antidiabetic, antineoplastic, hepatoprotective, cardioprotective, and anti-inflammatory activities[7–13]. Such diverse bioactivities have attracted considerable interest in the plant as a promising source of therapeutic compounds for modern medicine.

The antimicrobial activity of *M. oleifera* extracts has been extensively studied, and several bioactive compounds such as alkaloids, flavonoids, triterpenes, saponins, tannins, polyphenols, anthraquinones, sterols, and coumarins, as identified from aqueous, ethanol, and methanol extracts of *M. oleifera* leaves, bark, and fruits, with activity against bacterial, fungal, and viral pathogens have been reported[9-11]. The seeds of *M. oleifera* have drawn significant attention for their application as natural agents to inhibit microbial growth and improve water quality through purification processes. Antimicrobial effects of *M. oleifera* seed extracts have so far been mainly ascribed to a variety of glycosides, glucosinolates (glucomoringin or 4-(α-L-rhamnopyranosyloxy benzyl glucosinolate), isothiocyanates (4-α-L-rhamnopyranosyloxy benzyl isothiocyanate) and naturally occurring flocculating agents[11-13].

M. oleifera seeds are rich in edible oils and contain a high proportion (>40%) of water-soluble proteins[14]. A substantial share of the proteins characterised to date from *M. oleifera* seeds were identified as lectins with strong coagulation or flocculating properties, making them quite effective in water treatment applications, particularly in primary clarification, where they help remove suspended particles and contaminants[14,15]. Despite a growing interest in *Moringa*-derived antimicrobials, significant research gaps persist regarding low molecular weight cationic peptides, particularly those with direct antimicrobial activity[15-17]. Most prior studies have focused instead on hydrolysate-derived, predominantly hydrophobic or anionic peptides, such as MOp2 and MOp3 (<1 kDa), which have shown inhibitory effects against *Staphylococcus aureus*[16,17]. Relatively few investigations have targeted distinct, water-soluble cationic peptides with potent antimicrobial properties, and comprehensive data on their purification, physicochemical stability, and molecular and functional characteristics remain scarce. This gap restricts the exploitation of *Moringa* seeds beyond conventional applications such as in water clarification, limiting their potential role in addressing antimicrobial resistance or developing natural food preservation strategies[14-17].

The present study was therefore undertaken to isolate and characterise cationic antimicrobial peptide(s) specifically from *M. oleifera* seeds. The study centres on the identification of a stable, low-molecular-weight peptide with broad-spectrum antimicrobial activity and on evaluating the physicochemical properties that influence its biological function. The findings are expected to contribute to the growing knowledge of plant-derived antimicrobial peptides and support the development of novel peptide-based antimicrobial agents.

Materials and Methods

Extraction and purification of *M. oleifera* seed peptide

Fresh seeds of *M. oleifera* plants (identification done by Dr. S. Krishnan, Sr. Professor of Botany, Goa University) found in Zuarinagar (15.3775ºN, 73.8810ºE; Goa, India) were collected during July and August. They were cleaned and blended to a 10% homogenate with 10% (v/v) acetic acid solution (pH 2.9), left for 24 h at 4°C, and filtered through a muslin cloth[18]. The resulting filtrate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was fractionated with ammonium sulfate; the precipitates obtained at different ammonium sulfate saturations were redispersed in 10 mM sodium acetate buffer (pH 5.6) and screened for antimicrobial activity[18,19]. Chromatographic fractionation of the samples was performed using a pre-equilibrated Sephadex G-50 column (1.2 x 50 cm). Sodium acetate was used as elution buffer at a flow rate of 14 mL/h, and the absorbance of eluted fractions was read at 280 as well as 220 nm. Fractions were also tested for antimicrobial activity. The active peak fractions were pooled together, lyophilised to a powder form, and stored at 4°C.

Gel electrophoretic analysis

The purified samples were subjected to native polyacrylamide gel electrophoresis (Native-PAGE) under non-reducing conditions on a 15% resolving gel and SDS (sodium dodecyl sulfate)-PAGE on an 18% resolving gel under reducing conditions[20]. The cationic or basic nature of the sample was analysed by Acid-Urea PAGE (AU-PAGE) using a 15% resolving gel with 6 M urea and 5% acetic acid[21]. After electrophoresis, the protein/peptide bands were detected by Colloidal Coomassie staining[22].

Mass Spectral analysis

Mass spectral analysis was carried out using an Agilent 6460 Triple quadrupole LC/ESI-MS (Liquid Chromatography/ Electrospray Ionization-Mass Spectrometry) and Bruker-Ultraflextreme MALDI-TOF/TOF MS (Matrix-Assisted Laser Desorption/ Ionization-Time-of-flight Mass Spectrometry) system. For LC/ESI-MS analysis, 10 µL of purified peptide sample reconstituted in deionised water with 0.01% (v/v) formic acid was injected into a High-Performance Liquid Chromatography (HPLC; C18) column, and the fractions were eluted in 20% (v/v) acetonitrile/water. The MS fragmentor voltage was adjusted to 150V. The analyte ion detection was carried out in positive ion mode based on the mass-to-charge (m/z) ratio, and the deconvolution of m/z spectra was performed using Agilent MassHunter BioConfirm software. For MALDI-TOF analysis, mass spectra were acquired in linear-positive ionisation mode across a 5–20 kDa mass range, using an ultraflex TOF/TOF instrument (Brucker Daltonics).

Antimicrobial activity assay and MIC determination

Antimicrobial activity of the *M. oleifera* seed peptide sample was tested by the agar well diffusion method[19], using six different pathogenic strains procured from the MTCC (Microbial Type Culture Collection and Gene Bank, India): *Staphylococcus aureus* MTCC 3160, *Salmonella typhimurium* MTCC 3231, *Aeromonas hydrophila* MTCC 1739, *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 741, and *Candida albicans* MTCC 227. Mueller-Hinton broth or Mueller-Hinton agar was used for microbial susceptibility tests[23]. Chloramphenicol, ampicillin, amphotericin, tetracycline, and gentamicin were maintained as appropriate antibiotic standards against various pathogenic strains used in this study. The Minimal Inhibitory Concentrations (MICs) were evaluated using the broth microdilution method according to CLSI (Clinical and Laboratory Standards Institute) guidelines[23]. In short, two-fold serial dilutions of the peptide solution (1-128 µg/mL) were carried out in a microwell plate, microbial inoculum prepared in Mueller–Hinton broth was added to each well at a final density of 3-5 x 10^5 CFU/mL, and the plates were incubated at 37°C for 24 h (48 h in the case of *C. albicans*). After incubation, the absorbance at 595 nm was recorded using an iMark Microplate Absorbance Reader (Bio-Rad). The minimum peptide concentration required to inhibit 99.9% of microbial growth was designated as the MIC[23]. All experiments were conducted at least three times, each time in triplicate, to ascertain high reproducibility.

Temperature and pH sensitivity assays

The peptide solution was pre-treated at temperatures ranging from 40–100°C for 1 h before antimicrobial activity tests[24,25]. The sample maintained at room temperature served as the control for these thermostability studies.

The pH sensitivity of the peptide was assessed by evaluating its antimicrobial activity after incubation in 50 mM citrate buffer (for pH 5 and 6), phosphate-buffered saline (for pH 7), and Tris–HCl buffer (for pH 8) for 1 h at room temperature. The peptide sample in deionised water served as a control[24,26].

Sensitivity to proteolytic degradation

Purified peptide samples incubated (3 h, 37°C) with different proteases such as pronase (Sigma), proteinase K (HiMedia), and trypsin (HiMedia) at a peptide to enzyme ratio of 100:1 (w/w), were subjected to antimicrobial activity testing by the agar well diffusion assay. Untreated peptide dissolved in buffer and the corresponding enzyme alone in buffer were included as controls[24,27].

Salt sensitivity assays

The salt sensitivity of the peptide was evaluated as previously described[28,29]. Microbial inocula were prepared in Mueller-Hinton broth at final salt concentrations of 150 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, or 4 µM $FeCl_3$. The broth microdilution method was used to evaluate antimicrobial activity, with peptide activity in the absence of salts serving as the control.

Results and Discussion

Extraction and purification of *M. oleifera* seed peptide

The crude extract of *M. oleifera* seeds prepared in acetic acid solution was partially purified by fractionation with ammonium sulfate. The precipitates pelleted down at various saturation concentrations were screened for inhibitory activity against six different pathogenic microbial strains, using the agar well diffusion assay. The sample obtained at 0–30% ammonium sulfate saturation showed inhibitory effect against *S. typhimurium*, *S. aureus*, and *C. albicans* (Table 1); no activity was detected against *P. aeruginosa*, *E. coli* or *A. hydrophila*. Since proteins that pelleted down at higher ammonium sulfate saturations were found inactive against all the tested pathogens, the 0–30% fraction was chosen for further procedures. This active preparation was then purified using Sephadex G-50 column chromatography. Column fractions scanned for absorbance at 280 and 220 nm showed perfect overlap of the profiles, but since the 220 nm data showed sharper peaks, these were plotted (Fig. 1). Fractions were then pooled based on the antimicrobial activity elution profile and concentrated for further analysis.

Gel electrophoresis and Mass Spectral analyses

Electrophoretic analysis of the purified *M. oleifera* seed sample by native-PAGE (Fig. 2a) yielded a single band, confirming the homogeneity of the preparation. The SDS-PAGE analysis indicated a molecular size of <7 kDa (Fig. 2b). During AU-PAGE on a 15% resolving gel, the *M. oleifera* seed peptide exhibited cathodal migration, reiterating its basic or cationic nature (Fig. 2c). The purified *M. oleifera* seed cationic peptide was further analysed by LC/ESI-MS, where the peptide elution pattern reflected as a single peak in the chromatogram (Fig. 3a); the deconvoluted mass spectrum revealed a dominant peak at 6708.16 Da (Fig. 3, b and c). The intact mass of *M. oleifera* seed peptide was detected as 6693.485 Da by MALDI-TOF/TOF MS analysis (Fig. 4). All of these results unequivocally established the purified antimicrobial activity as due to a single peptide of cationic nature and with a molecular mass of ~6.7 kDa.

Table 1 — Antimicrobial activity in ammonium sulfate fractions of *M. oleifera* seed extracts

	Zone of inhibition (mm)			
Test culture	0 – 30% fraction	30 – 60% fraction	60 – 90% fraction	Positive control[a]
S. typhimurium	21±1	9±0.5	0	16±0.6
S. aureus	18±0.5	8±1	0	15±0.5
C. albicans	17±0.5	0	0	19±1

[a] Chloramphenicol, gentamicin, and amphotericin served as positive controls against *S. typhimurium*, *S. aureus* and *C. albicans*, respectively. The 'zone of inhibition' value does not include the well diameter

Antimicrobial spectrum and MIC of the *M. oleifera* seed peptide

Antimicrobial activity of the purified *M. oleifera* seed peptide was examined by agar well diffusion assay (Fig. 5), and the MIC values were evaluated by the broth microdilution method. The pathogenic strains *S. typhimurium*, *S. aureus,* and *C. albicans* showed intermediate susceptibility to the *M. oleifera* seed peptide, with MIC values of 16, 32, and 32 µg/mL, respectively, while *P. aeruginosa*, *E. coli* and *A. hydrophila* remained unaffected.

Antimicrobial activity due to several coagulant or flocculant proteins of *Moringa* seed aqueous

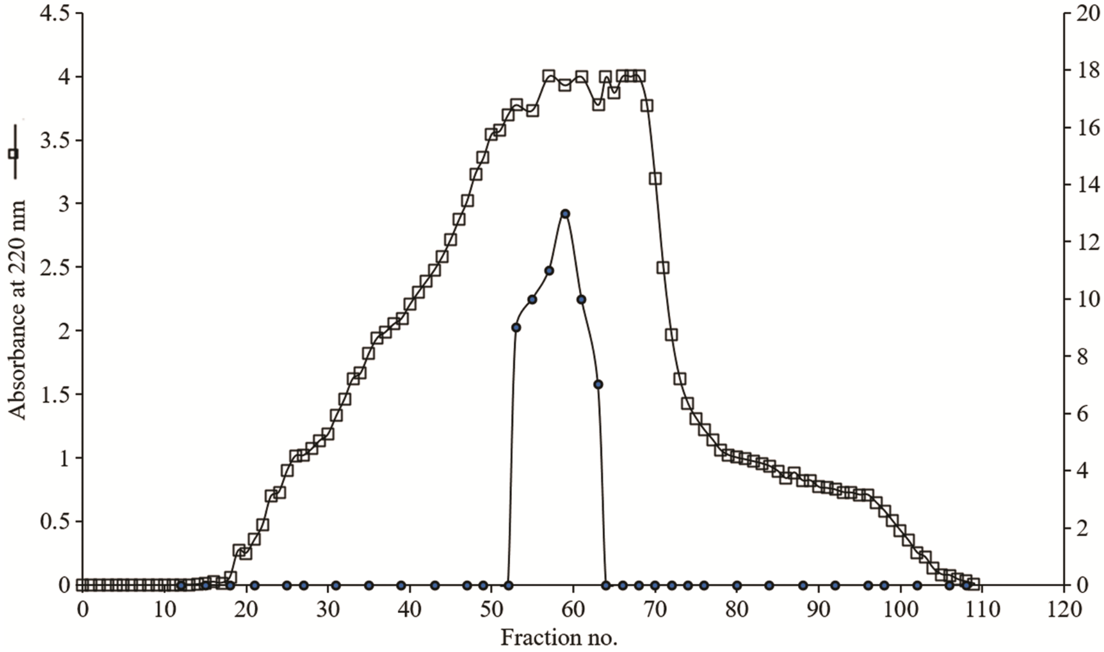

Fig. 1 — Gel filtration profile of the 0–30% ammonium sulfate fraction of *M. oleifera* seed extract on a Sephadex G-50 column matrix; zone of inhibition of eluted fractions was as tested against *S. typhimurium*.

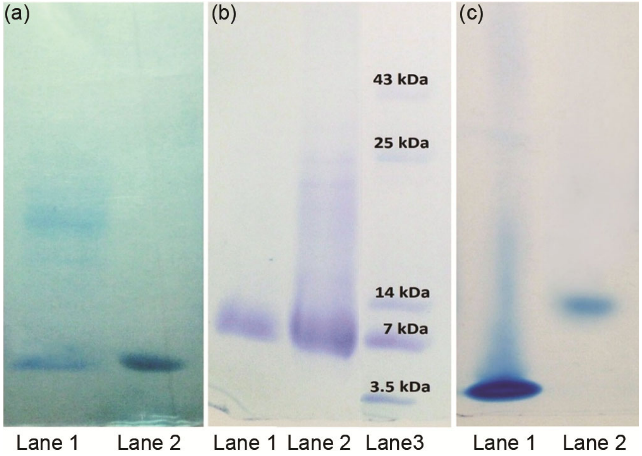

Fig. 2 — Electrophoretic analyses by (a) Native-PAGE: Lane 1 - 0–30% ammonium sulfate precipitate, Lane 2 – purified *M. oleifera* seed peptide, (b) SDS PAGE: Lane 1 - purified *M. oleifera* peptide, Lane 2 - 0–30% ammonium sulfate precipitate and Lane 3 - protein markers, and (c) AU-PAGE: Lane 1 – lysozyme as a 'basic protein' marker, Lane 2 - purified *M. oleifera* peptide.

extracts has been elaborated in previous studies[30–35]. Suarez *et al.*[30], had characterised a flocculating protein recovered from heat-stable fractions of *M. oleifera* seed extracts (Flo, of ~6.5 kDa), which exhibited bacteriostatic effect against *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli*, with MICs that ranged between 5–50 mg/mL. Detailed functional characterisations of recombinant Flo derivatives and synthetic Flo or Flo-derived peptides (<6.5 kDa) had demonstrated bacteriostatic or bactericidal actions against *S. pyogenes*, *S. aureus*, *S. pneumoniae*, *P. aeruginosa* and *E. coli*, at an MIC range of 2–10 mg/mL, but not against *C. albicans*[30,31]. Ghebremichael *et al.*[32] elucidated the coagulant and antimicrobial activity of a heat-resistant *M. oleifera* seed protein known as MOCP (MO Coagulant Protein) of less than 6.5 kDa, effective against *E. coli*, *P. aeruginosa,* and *B. thuringiensis*; the MS/MS analysis of MOCP revealed high sequence similarity to Flo. A coagulant *M. oleifera* Lectin or cMoL (~30 kDa), the first lectin identified from *Moringa* seeds, has demonstrated antimicrobial as well as insecticidal activities[33,35]. A Water-Soluble *M. oleifera* Lectin (WSMoL, ~60 kDa) isolated from *Moringa* seed extracts was categorised as an anionic, chitin-binding glycoprotein with bactericidal or bacteriostatic properties against several *Bacillus* sp., *Micrococcus* sp., *Pseudomonas* sp., *S. aureus, E. coli* and *Serratia marcescens*[34,36]. Different isoforms of Chitin-Binding Proteins from *M. oleifera* seeds (Mo-CBP3), usually of molecular mass >11.5 kDa, have been reported with *in vitro* antifungal activity against many phyto-pathogenic fungi[37]. Kini *et al.*[38] characterised two antifungal 8C-hevein-like peptides called morintides (mO1 and mO2, with a mass of 4.5 kDa) from aqueous extracts of *M. oleifera* leaves. In light of the literature reported to date, it is hence quite evident that the nature and antimicrobial properties of the 6.7 kDa peptide purified in the present study distinctly differed from those of previously reported molecules of similar molecular mass from seeds of *M. oleifera*.

Characteristics of the *M. oleifera* peptide activity

Stability to heat, pH, and proteolytic treatments

Physicochemical characterisation of the *M. oleifera* seed peptide was carried out by assessing the effect of temperature, pH, and proteolytic enzymes on its antimicrobial activity. Heat treatment of the peptide for up to 1 h at 60-70°C did not affect its activity against *S. typhimurium*, *S. aureus*, or *C. albicans* (Fig. 6). Still, incubation at temperatures ≥90°C for 1 h resulted in a drastic/complete loss of activity against all three pathogenic strains.

Thermostability appears to be a common characteristic of many flocculant or coagulant proteins previously isolated from *M. oleifera* seed extracts[35]. The Flo purified from heat-treated (80°C) *M. oleifera* seed extracts retained good flocculant and antibacterial potential[30]. The MOCP isolated by Ghebremichael *et al.*[32] exhibited high coagulation efficiency even after heating for 5 h at 95°C. Katre *et al.*[39] reported a thermostable hemagglutinin called *M. oleifera* Lectin (MoL, ~14 kDa) that remained active after treatment at 85°C for 30 minutes. Detailed structural characterisation of cMoL (~30 kDa), which exhibited stable antimicrobial activity after heat treatment at 80°C for 30 minutes, had revealed the presence of eight cysteine residues that were involved in disulfide linkages[33,35].

The pH sensitivity of our *M. oleifera* seed antimicrobial peptide activity was assayed by evaluating the residual activity after treatment at various pH values. The susceptibility of *S. typhimurium*, *S. aureus* and *C. albicans* to this peptide remained unchanged, and the MICs were observed as 16, 32 and 32 µg/mL, respectively, at pH values in the range of 5–8. The effect of proteases on the antimicrobial activity of the seed peptide was assessed by treating it with pronase, trypsin, or proteinase K at 37°C for 3 hours. None of the three

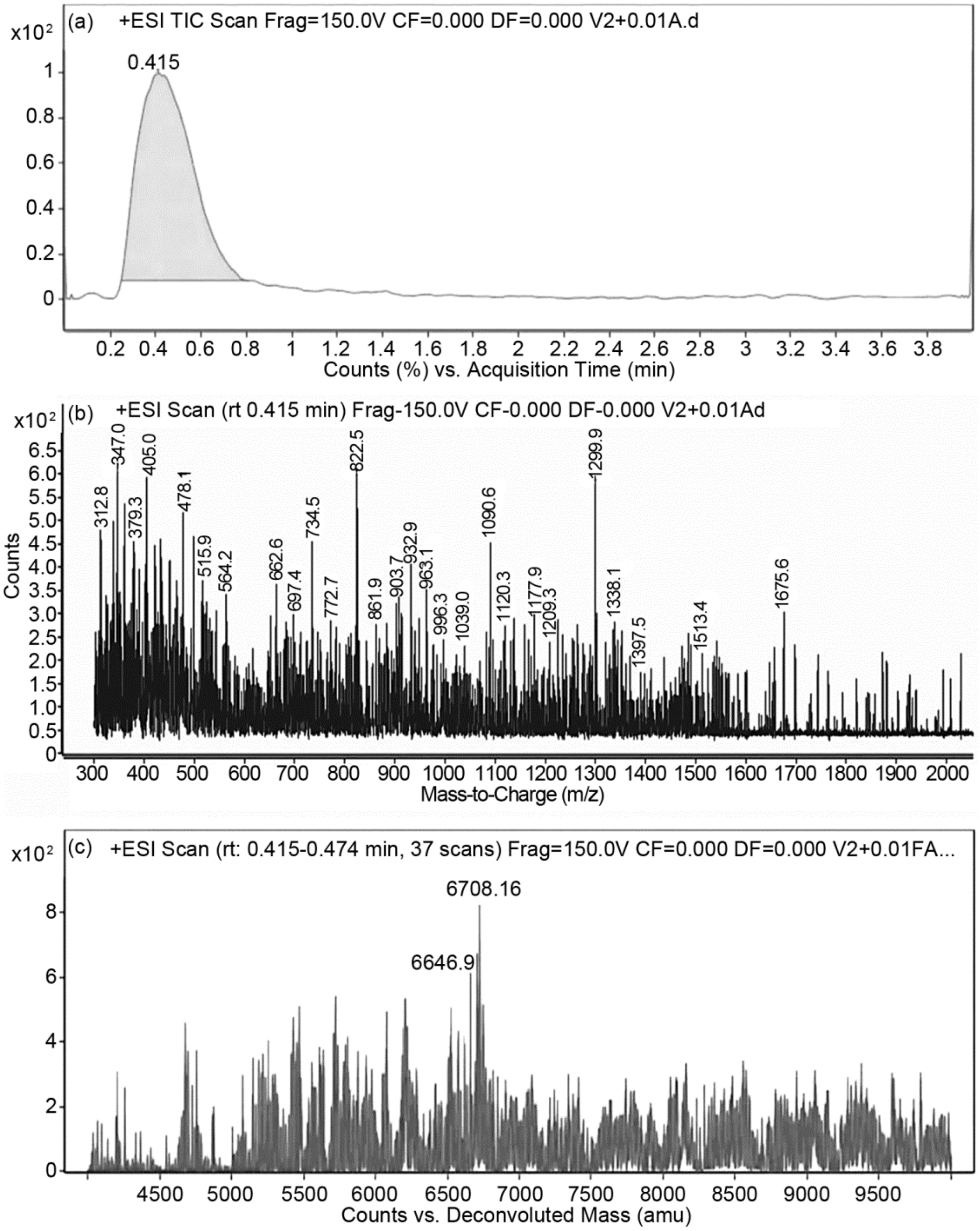

Fig. 3 — LC–ESI MS analysis of *M. oleifera* seed peptide: (a) LC elution profile, (b) ESI mass spectrum, and (c) Deconvoluted mass spectrum.

tested pathogens was affected by the protease-treated peptide. The effect on its activity against *S. typhimurium* in culture is captured in Fig. 7. The active molecule was thus sensitive to proteolytic degradation, confirming that intactness of the peptide structure was obligatory for its antimicrobial activity.

Salt sensitivity

Cationic peptides exert antimicrobial effect primarily by binding to phospholipid head groups of microbial membranes. Many studies have shown that the presence of high concentrations of salts or cations could interfere with the peptide binding to microbial membranes and thereby reduce the antimicrobial potential[40]. From this perspective, the effect of physiological concentration of salts on antimicrobial activity of *M. oleifera* peptide was examined by the broth microdilution method. While the pathogenic susceptibility to *M. oleifera* peptide did not vary notably in presence of the monovalent ions Na^+ and K^+, the divalent Mg^{2+}, Ca^{2+} and the trivalent Fe^{3+} ions exhibited some weak inhibitory effect on the peptide

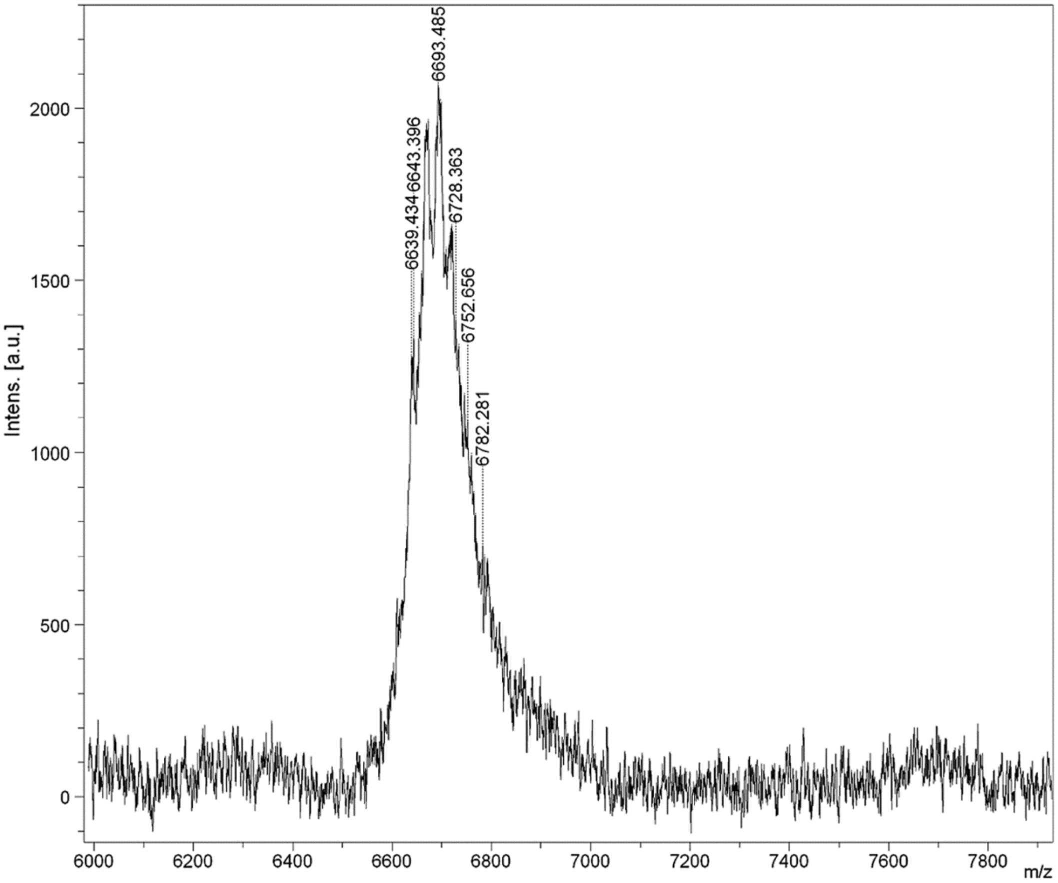

Fig. 4 — MALDI-TOF Mass spectrum of *M. oleifera* seed peptide.

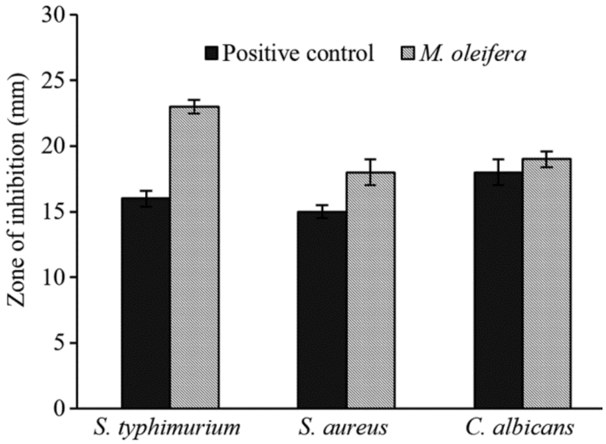

Fig. 5 — Antimicrobial susceptibility of purified *M. oleifera* peptide against *S. typhimurium*, *S. aureus* and *C. albicans* as determined by agar well diffusion assay (~25 µg peptide/well); Chloramphenicol, gentamicin and amphotericin at 25 µg/well served as positive controls against *S. typhimurium*, *S. aureus* and *C. albicans*, respectively.

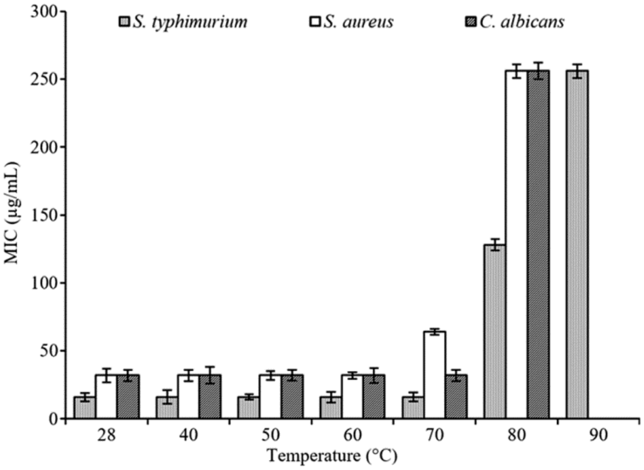

Fig. 6 — Effect of heat treatment on antimicrobial activity of *M. oleifera* peptide. The peptide treated at room temperature (28°C) was used as the control. The experiments were repeated at least thrice, in triplicate, to ensure reproducibility.

activity against *S. typhimurium*, *S. aureus*, and *C. albicans,* respectively (Fig. 8). In general, antimicrobial activity of the *M. oleifera* peptide was found tolerant to salts at physiological concentrations.

Analysing the data on the several *M. oleifera* seed antimicrobial molecules documented to date, there appear to be only two that bear similarity in molecular size to the 6.7 kDa cationic peptide being reported in the present study. Of these, one was the Flo molecule

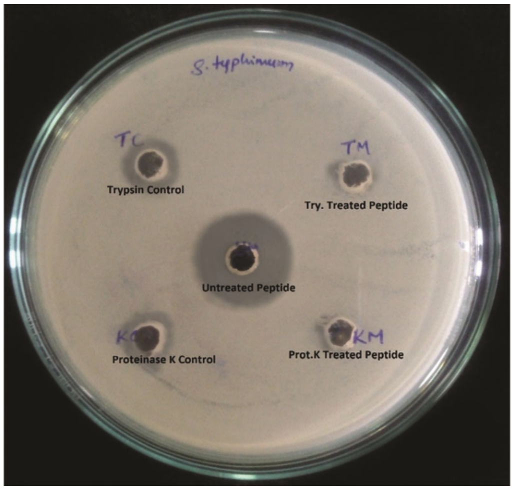

Fig. 7 — Effect of protease-treated *M. oleifera* peptide on *S. typhimurium* in culture. Untreated peptide in buffer and the respective enzyme in buffer served as controls.

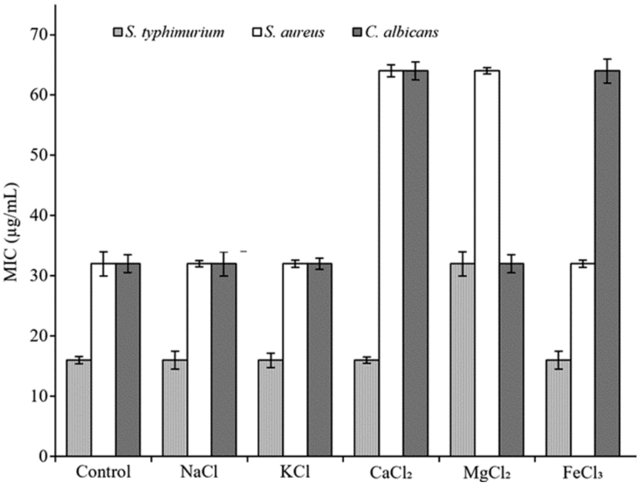

Fig. 8 — Effect of different salts on antimicrobial activity of *M. oleifera* peptide. Activity of peptide in the absence of salts was taken as the control. The experiments were repeated at least thrice, with samples in triplicate, to ensure maximum reproducibility.

of ~6.5 kDa, which demonstrated inhibitory action against *P. aeruginosa* and *E. coli* strains while *C. albicans* appeared to be resistant[30,31], in direct contrast to our results, wherein *P. aeruginosa* and *E. coli* strains showed resistance, while the growth of *C. albicans* was affected. They had also worked on recombinant or synthetic peptides modelled based on relevant Flo sequences. A comparative study on antibacterial properties of *M. oleifera* seed Flo and recombinant or synthetic Flo derivatives had described the bacteriostatic effect against *S. aureus*, with MIC ranges of 9–18 and 2–5 mg/mL, respectively[30], whereas the purified 6.7 kDa peptide in the present study exhibited inhibitory action against *S. aureus* at a significantly lower MIC of 32 μg/mL.

The other coagulant protein MOCP (<6.5 kDa), as described by Ghebremichael *et al.*, had high sequence similarity to Flo and was active against *P. aeruginosa* and *E. coli*[32]. While both Flo and MOCP showed high heat resistance and remained functional after heat treatment at 80–95°C[30,32], the thermostability pattern of our peptide depicted intense loss of activity when treated at 90°C and above. The acid-extracted cationic AMP emerging from the current study thus appears distinctly different from those reported earlier.

Conclusion

The study at hand highlights the antimicrobial potential and distinct physicochemical properties of what could be a novel low molecular weight cationic peptide purified from *M. oleifera* seeds. The major findings of this study not only underscore the value of *M. oleifera* seed-derived peptides as a promising antimicrobial agent but also pave the way for further exploration into their applications in pharmaceutical development and combating antimicrobial resistance. Detailed structural characterisation of this peptide figures in the ongoing research and will be reported in due course.

Conflict of interest

The authors declare that there are no conflicts of interest relevant to the content of this article.

AI use disclosure

No Artificial Intelligence tools were used in the preparation of the manuscript, data analysis, or interpretation.

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