



# Wasp Venom Peptide (Polybia MP-1) Shows Antimicrobial Activity Against Multi Drug Resistant Bacteria Isolated from Mastitic Cow Milk

Pallavi Shah<sup>1</sup> · Sameer Shrivastava<sup>1</sup> · Purnima Gogoi<sup>1</sup> · Sonal Saxena<sup>1</sup> · Saumya Srivastava<sup>1</sup> · Rajkumar James Singh<sup>1</sup> · Basant Godara<sup>1</sup> · Naveen Kumar<sup>2</sup> · Gyanendra Kumar Gaur<sup>3</sup>

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## Abstract

Increase in the incidence of Antimicrobial Resistance (AMR) in pathogens is a major concern for the human and animal health sector alike. AMR has been widely reported in bacteria that cause mastitis in dairy cattle. Antimicrobial peptides (AMPs) offer a lucrative and effective alternative to antibiotics for the treatment of mastitis. The Polybia MP-1 is a 14 amino acid long AMP obtained from the venom of the wasp *Polybia paulista*. In the present study, antimicrobial activity of Polybia MP-1 was evaluated against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from mastitic milk samples. In all, 20 *S. aureus*, 21 *E. coli* and 14 *K. pneumoniae* isolates were obtained from 40 mastitic milk samples. Antibiotic susceptibility of the isolates was determined by antibiotic disc diffusion assay and 19 *S. aureus*, 17 *E. coli* and 10 *K. pneumoniae* isolates were found to be multiple drug resistant (MDR) isolates. The synthetic Polybia MP-1 peptide effectively inhibited 32% (6/19) *S. aureus*, 47% (8/17) *E. coli* and 70% (7/10) *K. pneumoniae* MDR isolates. The peptide disrupted both the outer and inner bacterial membrane and at the same time showed very low haemolytic activity for mice and rabbit erythrocytes even up to 200 µM concentration. Moreover, it had no cytotoxic effect in Vero cells up to 160 µM concentration as assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay. The peptide did not result in mortality of the *Galleria mellonella* larvae up to a dose of 40 mg per kg bodyweight. These findings indicate that the Polybia MP-1 peptide is safe and can be effectively tried as an alternative to antibiotic for management of mastitis in cattle.

**Keywords** Polybia MP-1 · *Staphylococcus aureus* · *Escherichia coli* · *Klebsiella pneumoniae* · Antibiotic resistance · AMP · MDR

Pallavi Shah and Sameer Shrivastava have contributed equally for this work.

✉ Sameer Shrivastava  
sameer\_vet@rediffmail.com; sameer.shrivastava@icar.gov.in

<sup>1</sup> Facility for Research & Training on Bioassays and Biosensors, Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute (Deemed University), Izatnagar 243 122, India

<sup>2</sup> Division of Veterinary Surgery, ICAR-Indian Veterinary Research Institute, (Deemed University), Izatnagar 243 122, India

<sup>3</sup> Livestock Production & Management Section, ICAR-Indian Veterinary Research Institute, (Deemed University), Izatnagar 243 122, India

## Introduction

Inflammation of mammary glands of dairy cattle is defined as mastitis, often caused by bacterial infection and is associated with reduction in milk production and changes in milk composition (Boireau et al. 2018). Mastitis causes economic losses in dairy farms by affecting dairy cattle all over the world. India being the largest milk-producing country in the world suffers great losses due to udder infections estimated to be Rs. 6053.21 crores. 70 to 80 per cent of this loss has been attributed to sub-clinical mastitis. The causative pathogens of mastitis can be divided broadly into two groups namely, contagious pathogens and environmental pathogens. The major contagious pathogens are *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycoplasma* spp. which gain entry into mammary gland through teat canal-with exception of some mycoplasmal

infections whereas, other important bacteria are environmental coliforms like *Escherichia coli* and *Klebsiella* species (Gomes and Henriques 2016). In Canada, a recently established Mastitis Pathogen Culture Collection (MPCC) containing 16,000 mastitic isolates showed predominance of *S. aureus* (26%), coagulase negative *Staphylococcus* spp. (42.4%) and *Corynebacterium bovis* (8.3%) in the category of contagious pathogens whereas environmental pathogens included *E. coli* (3%), *Streptococcus dysgalactiae* (3%) and *Klebsiella* spp. (1.2%) (Dufour et al. 2019). Antibiotic treatment is a major part of treatment regimen for bacterial mastitis. Several cases of antimicrobial resistance (AMR) have been reported in pathogens isolated from mastitic samples such as methicillin resistant *Staphylococcus aureus* (MRSA) (Gogoi et al. 2021), Vancomycin-Resistant *Staphylococcus aureus* (VRSA) (Bhattacharyya et al. 2016), extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* (Ali et al. 2016), MDR *Pseudomonas aeruginosa* (Shah et al. 2021). AMR is a major threat to human and animal health and present times need alternative therapeutic interventions for treatment of multiple drug resistant (MDR) bacteria.

In the present study also, a number of *S. aureus* isolates (i.e., 20) were obtained from 40 milk samples with a significant high number of MDR isolates especially MRSA. The *S. aureus* is a gram positive highly contagious and notorious pathogen responsible for infections in humans and animals alike. It is responsible for one third of the clinical and sub clinical mastitis in cattle (Li et al. 2017a, b) and hence was selected for the present study. In the category of environmental pathogens *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Shah et al. 2021) were also isolated from mastitic milk samples, although the number of *Pseudomonas* isolates was quite less. We have therefore restricted our work to MDR isolates of *E. coli* and *K. pneumoniae* in the present study.

Antimicrobial Peptides (AMPs) offer a great alternative to present day antimicrobials for treatment of MDR bacteria. AMPs are short peptides that are widely present in nature as an important part of the innate immune system of different organisms and often termed as host defence peptides. These include peptides with low molecular weight and broad-spectrum antimicrobial and immunomodulatory activities against both Gram positive and Gram-negative infectious bacteria, fungi and viruses. AMPs are short peptides  $\leq 50$  amino acids approximately, with amphipathic cationic properties. Targeting of membrane is the most prominent mode of action against microbes reported for AMPs. Aside from targeting the membrane, the AMPs can enter the cells and act by inhibiting intracellular processes like protein synthesis and post translational modifications, nucleic acid synthesis, protease activity or by hampering cell division (Le et al. 2017). Multiple modes of action of AMPs make it difficult for the bacteria to develop resistance against them

(Mahlapuu et al. 2016). AMP sequences are obtained from natural sources, as well as, newer computer aided design methods and advanced artificial intelligence-based algorithms can also be used for screening and identification of synthetic AMPs (Sharma et al. 2021a, 2021b, 2021c, 2021d; Singh et al. 2021). Present day research aims at designing stable, efficient and specific AMPs that can be used therapeutically for treatment of MDR microbes.

Natural sources like wasp venom have been utilized for isolation and development of bioactive peptides especially AMPs. Mastoparans are a major category of naturally occurring bioactive peptides obtained from the social wasp venoms which possess mast cell degranulating and histamine releasing principles (Palma 2013). The mastoparans are mostly 14 amino acids long, found abundant in hydrophobic residues such as isoleucine, leucine, alanine and valine. They are amphipathic and attain an  $\alpha$  helical structure on interaction with phospholipids which imparts them various biological activities like interactions with the plasma membrane, mast cell degranulation and histamine release, and hemolysis (Konno et al. 2019) Polybia MP-1 (NH<sub>2</sub>-IDWKKLLDAAKQIL-CONH<sub>2</sub>) is a mastoparan peptide obtained from the venom of the vespid wasp *Polybia paulista*. It possesses immense antimicrobial activity as confirmed by numerous previous studies (Souza et al. 2005; Dos Santos Cabrera et al. 2008; Luong et al. 2017; Shah et al. 2021). In the present study, the antimicrobial efficacy of Polybia MP-1 against MDR *S. aureus*, *K. pneumoniae* and *E. coli* isolates obtained from mastitic milk was evaluated. Safety evaluation for the peptide was carried out by assessing haemolysis in different mammalian RBCs, in vitro cytotoxicity was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye reduction assay in Vero cells and in vivo toxicity was determined in *Galleria mellonella* larvae. Antibiotic susceptibility was determined for the isolates from milk samples by disc diffusion assay for standard antibiotics and based on resistance to  $\geq 3$  classes of antimicrobials they were classified as MDR.

## Materials and Methods

### Chemicals and Reagents

All the chemicals for peptide synthesis like Rink amide MBHA resin (loading efficiency 0.78 mmol/ g), N, N'-Diisopropylcarbodiimide/ Hydroxybenzotriazole (DIPC/HOBt), Hydroxybenzotriazole-2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), Diisopropylethylamine (DIEA), Fmoc protected amino acid derivatives, piperidine, Trifluoroacetic acid (TFA), Triisopropylsilane (TIPS), Thioanisole, 1,2-Ethanedithiol (EDT), Phenol and HPLC grade water

were purchased from Novabiochem (Merck, Germany) and Merck, Germany. 1-N-phenyl-naphthylamine (NPN), HEPES buffer and Triton X-100 were procured from Sigma Aldrich, USA whereas SYTOX™ Green Nucleic Acid Stain was purchased from Invitrogen, USA. Bacterial culture media like Mueller–Hinton Agar (MHA), Mueller–Hinton Broth (MHB), Brain Heart Infusion broth (BHI), Mannitol Slat Agar (MSA), Eosin Methylene Blue (EMB) and HiCrome™ Klebsiella Selective Agar Base were all purchased from HiMedia, India. Animal cell culture Earl's Minimum Essential Medium (EMEM) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were procured from Sigma Aldrich, USA. Foetal Bovine Serum (FBS) was purchased from Gibco, Thermo Fisher Scientific, USA.

### Bacterial Isolates

The standard ATCC isolates of *Staphylococcus aureus* (ATCC 29,213), *Escherichia coli* (ATCC 25,922) and *Klebsiella pneumoniae* (ATCC 13,883) were purchased from HiMedia, India. The bacterial isolates of *E. coli*, *S. aureus* and *K. pneumoniae* used in the study were isolated from mastitic cow milk samples.

### Peptide Synthesis and Purification

Synthesis of Polybia MP-1 was carried out on an automated peptide synthesizer (CSBio 336X) following Solid Phase

$$\% \text{ Growth Inhibition} = \frac{(\text{OD}_{600} \text{ of untreated cell control} - \text{OD}_{600} \text{ of treated sample}) \times 100}{(\text{OD}_{600} \text{ of untreated cell control})}$$

Peptide Synthesis (SPPS) methodology using standard 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry (Merri-field, 1963). The successive addition of the amino acids was carried out by using HOBT-DIPC or HBTU-HOBT-DIEA as activating agents on Rink amide MBHA resin (loading efficiency of 0.78 mmol/g). 20% piperidine was used for the removal of Fmoc protecting group from consecutive amino acids of the growing peptide chain. On completion of the peptide sequence the cleavage was done by using K reagent (85% TFA, 1% TIPS, 5% Thioanisole, 2.5% EDT, 1.5% Phenol and 5% HPLC grade water). Precipitation of the cleaved peptide was carried out by adding chilled diethyl ether. The precipitated peptide was dried under vacuum and was reconstituted in HPLC grade water. It was purified by reverse phase high pressure liquid chromatography (RP-HPLC) (Shimadzu, Japan) on a C18 column (Shim-pack GIST C18 5 μm, 250 × 14 mm) using a linear acetonitrile:

water gradient (95 to 5%) in presence of 0.05% TFA at a flow rate of 1.5 mL/min. The molecular weight of the peptide was predicted by the software PepCalc (<https://pepcalc.com/> (Innovagen)-Peptide property calculator) which was further confirmed by Mass Spectrometry.

### Minimum Inhibitory (MIC) and Minimum Bactericidal Concentrations (MBC)

The MIC of Polybia MP-1 for *Staphylococcus aureus* (ATCC 29,213), *Escherichia coli* (ATCC 29,522) and *Klebsiella pneumoniae* (ATCC 13,883) was determined by the standard broth microdilution assay performed in a 96 well micro-titer plate as per the method of Wiegand et al. 2008. The bacterial cultures were grown overnight at 37 °C in MHB till mid log phase (OD<sub>600</sub> 0.5 approximately equivalent to 4 × 10<sup>8</sup> cells/mL) and used for the assay. These cells were further diluted to a final concentration of 10<sup>6</sup> cells/mL in fresh MHB for seeding in wells. Two-fold serial dilutions of the peptide ranging from 5 μM to 50 μM were prepared in 50 μL of sterile water. To these dilutions 50 μL of 10<sup>6</sup> cells/mL in MHB were added. The final concentration of cells was 5 × 10<sup>5</sup> cells/mL (in 100 μL). Cells with no treatment were used as the normal control. The plates were incubated at 37 °C for 24 h in a humidified chamber and OD<sub>600</sub> was taken in a microplate reader (Multiskan Go, Thermo Fischer Scientific, USA). MIC was defined as the minimum concentration of the peptide which inhibited more than 99% visible growth of bacterial cells after incubation of 24 h at 37 °C. The percentage of growth inhibition was calculated by the formula as given where OD<sub>600</sub> stands for optical density at 600 nm

MBC was determined by microdilution assay similar to MIC determination, set up at 6 different concentrations of the peptide i.e., half of MIC, MIC, 2 × MIC, 4 × MIC, 8 × MIC and 16 × MIC. After incubating the plate for 24 h at 37 °C, 10 μL was taken from each well and diluted 10,000 times in sterile PBS. This dilution was then spread plated on MHA plates and plates were incubated overnight at 37 °C. MBC was defined as the minimum concentration of the peptide which completely (>99.9%) eliminated bacterial growth on MHA plates.

### Complete Elimination Concentration (CE values) determination by Drop Plate Method

CE values of Polybia MP-1 were determined for *S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522) based on the method described by Farkas et al. (2018) with some modifications. The bacterial cultures i.e.,

*S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522) were grown overnight at 37 °C in MHB till mid log phase ( $OD_{600}$  0.5 approximately equivalent to  $4 \times 10^8$  cells/mL) and were further diluted to  $10^6$  cells/mL in fresh MHB. To 50 µL of these cultures, 50 µL of different two-fold peptide dilutions (0.5, 1, 2, 4 times of MIC for respective ATCC isolate), Streptomycin (10 µg/mL) and Ampicillin (10 µg/mL) made in sterile water, were added. The final concentration of cells in 100 µL corresponded to  $5 \times 10^5$  cells/mL. These mixtures were incubated at 37 °C and 2 µL drops from them were placed on MHA plates after 0, 15, 30, 45, 60, 120 and 180 min of incubation. The drops were allowed to dry and the plates were incubated in inverted position in 37 °C overnight. This experiment was carried out in triplicate.

### Safety profile of Polybia MP-1

#### Haemolysis Assay

The haemolytic activity of Polybia MP-1 was assessed by measuring the amount of haemoglobin released on erythrocyte lysis. Polybia MP-1 was assessed for haemolytic activity against erythrocytes from different species viz., rabbit and mice as per the procedure described by Bhagavathula et al. 2017 and Tan et al. 2017 with some modifications. Blood samples from healthy animals was collected in EDTA vials and erythrocytes were collected by centrifugation (1000 g for 5 min at 4 °C). The collected erythrocytes were washed in Alsever's solution (pH 6.1) and stored overnight at 4 °C for ageing. Overnight-aged erythrocytes were washed with fresh sterile Alsever's solution (pH 6.1), then resuspended to 4% in the same buffer. 80 µL of 4% RBC solution was incubated with 20 µL buffer containing various amounts of Polybia MP-1 i.e., 2.5, 5, 10, 25, 50, 75, 100, 150 and 200 µM in 0.6 mL micro-centrifuge tubes. After incubation at 37 °C for 1 h, the mixtures were centrifuged (1000 g for 5 min at 4 °C) and the supernatants were transferred to a 96-well plate. The amount of haemoglobin released was determined by measuring absorbance at 570 nm using a micro plate reader. 4% erythrocyte suspension with buffer alone ( $A_{\text{blank}}$ ) and 20 µL of 0.1% Triton X-100 ( $A_{\text{Triton}}$ ) were used as control samples for 0% and 100% hemolysis respectively. Percentage of Haemolysis was determined by

$$\% \text{ Haemolysis} = \left[ \frac{(A_{\text{Sample}} - A_{\text{Blank}})}{(A_{\text{Triton}} - A_{\text{Blank}})} \right] \times 100$$

#### In Vitro Cytotoxicity Determination by MTT

##### [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Dye Reduction Assay in Vero Cell Line

MTT dye reduction assay was performed as per the protocol of Freshney (2011). Vero cells were grown in EMEM

medium in 96 well plates for 24 h at a concentration of  $5 \times 10^4$  cells per ml ( $10 \times 10^3$  cells/well, 100 µl/well) at 37 °C in 5% CO<sub>2</sub>. Cultured medium was replaced with medium containing various concentrations of Polybia MP-1 ranging from 5 µM and 160 µM and cells were incubated further for 24 h. After incubation, the medium was removed, and the cells were washed with DPBS to remove any residue of different compounds used. All the wells were fed with 100 µl of fresh serum free medium and 20 µl of MTT (5 mg/ml in PBS). The plate was wrapped with aluminium foil and incubated for 4 h in a humidified atmosphere at 37 °C. After this, the medium containing MTT was removed and all the wells were fed with 200 µl of DMSO and incubated for 15 min. The plate was then read in a plate reader to record the absorbance at 570 nm immediately.

#### Peptide Toxicity in *Galleria mellonella* (Greater Wax Moth) Larvae

This study was carried out by the method of Ignasiak and Maxwell (2017). Larvae ranging from 200–300 mg weight were selected from the fresh stock maintained in the laboratory for experimental purpose. Eight groups were made of five larvae each based on similar average weights. The larvae were injected with a 10 µl Hamilton syringe in the second rear proleg with different concentrations of Polybia MP-1 (5, 20, 40, 80, 160, 240, 320 mg per kg body weight). The control group larvae were injected with PBS (buffer control) and prick group had larvae only pricked with the needle. The negative control larvae were injected with 50% Triton X-100. After injecting the larvae were maintained at 37 °C and survival was monitored for 72 h by prodding for movement and lack of melanisation.

#### Mechanism of Action on Bacterial Membrane

The effect of the peptide Polybia MP-1 on bacterial cell membrane was assessed by determining its activity on both outer and inner (cytoplasmic) membrane permeability based on fluorescent dye-based assays.

#### Action on Outer Membrane

Permeation of bacterial outer membrane by Polybia MP-1 was measured by utilizing the hydrophobic fluorescent dye, 1-N-phenyl-naphthylamine (NPN) as described by Yan



et al. 2013 and Rahaman et al. 1998. Overnight grown *E. coli* (ATCC 29,522) cells with an OD<sub>600</sub> 0.5 approximately equivalent to  $4 \times 10^8$  cells/mL were centrifuged at 3000 g for 10 min and resuspended in half volume of 5 mM HEPES buffer (pH 7.2). 100 µL of this suspended bacterial culture and 80 µL of Polybia MP-1 were mixed with 20 µL of NPN (500 µM of NPN stock was prepared in acetone and was used at a final concentration of 10 µM). The final concentrations for the peptide corresponded to MIC i.e., 10 µM. Excitation and emission wavelengths for NPN were set at 350 and 420 nm, respectively. A positive control was set up with 0.5% Triton X-100 whereas HEPES buffer was used in buffer control. An increase in fluorescence units due to permeabilization of NPN into outer membrane was observed as a function of time until no further increase in the intensity was detected. Data is represented as normalized relative fluorescence intensity (RFU) with respect to the initial 0 min reading.

### Action on Inner (Cytoplasmic) Membrane

To assess cytoplasmic membrane depolarization, SYTOX green assay was performed as described by Yasir et al. 2019 with few modifications. Overnight grown *E. coli* (ATCC 29,522) cells with an OD<sub>600</sub> 0.5 approximately equivalent to  $4 \times 10^8$  cells/mL, were centrifuged at 3000 g for 10 min and resuspended in PBS to get a dilution of  $2 \times 10^7$  cells / ml based on OD 600. 100 µL of these bacterial cells were dispensed into wells of 96-well plates along with 30 µl of 500 nM SYTOX green (Invitrogen, USA). Plates were incubated for 15 min in the dark at room temperature and then 20 µL of Polybia MP-1 was added so as to achieve a concentration equivalent to MIC i.e., 10 µM. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 522 nm, every 3 min until no further increase in fluorescence was detected. Triton X-100 at a concentration of 0.5% (Sigma Aldrich, St Louis, MO, USA) in PBS was used as positive control to disrupt the cytoplasmic membrane of bacteria. Data is represented as normalized relative fluorescence intensity (RFU) with respect to the initial 0 min reading.

### Isolation, Identification and Antibiogram of Bacteria from Milk Samples

Mastitic milk samples were collected from different dairy farms. The milk samples were aseptically collected in sterile 15 ml centrifuge tubes from infected quarters of the cow. Before collection the udder was disinfected with 5% potassium permanganate and collected milk samples were transported to the laboratory in an icebox. 1 mL of each sample was homogenized with 9 mL of BHI broth and grown overnight (16 h approx.) at 180 rpm and 37 °C for enrichment. These samples were then used for streaking on MSA plates, EMB plates and Klebsiella Selective Agar medium plates. Streaked plates were further incubated overnight in inverted position at 37 °C. *S. aureus* colonies appeared as yellow medium sized colonies on MSA plates whereas *E. coli* colonies gave distinctive green metallic sheen on EMB agar plates. Purple coloured colonies were obtained for *K. pneumoniae* on Klebsiella selective agar plates. Single, yellow medium sized *S. aureus* colonies were picked from MSA plates and added to fresh BHI broth and grown overnight at 37 °C till OD reached 0.5 ( $4 \times 10^8$  cells/mL) and used for DNA isolation by CTAB method (Minas et al. 2011). Similarly, single *E. coli* colonies with green metallic sheen on EMB plates and purple-coloured single colonies from Klebsiella selective agar plates were picked and grown in BHI broth overnight at 37 °C till OD reached 0.5 ( $4 \times 10^8$  cells/mL) and used for DNA isolation. All the isolates were confirmed by PCR and details of the primers used are given in Table 1.

The antibiotic susceptibility of the isolates was evaluated by Kirby-Bauer disk diffusion method (Bauer et al. 1966) as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2019). *S. aureus* isolates were tested for susceptibility against 10 antibiotics namely chloramphenicol 30 µg (C 30), tetracycline 30 µg (TET 30), erythromycin 15 µg (E 15), enrofloxacin 5 µg (EX 5), co-trimoxazole (Sulpha/ Trimethoprim) 25 µg (COT 25), vancomycin 5 µg (VA 5), linezolid 30 µg (LZ 30), penicillin-G 10 units (P 10), oxacillin 1 µg (OX 1) and cefoxitin 30 µg (CX 30). *K. pneumoniae* and *E. coli* isolates were tested against 9

**Table 1** List of primers for genes used for identification of *S. aureus*, *E. coli* and *K. pneumoniae*

Bacteria	Genes	Encodes for	Primers	Amplicon Size
<i>S. aureus</i>	<i>nuc</i>	Nuclease	F- GCGATTGATGGTGATACGGTT R- AGCCAAGCCTTGACGAACATAAAGC	278
	<i>23 s rRNA</i>	23 s rRNA	F-AGCGAGTCTGAATAGGGCGTTT R-CCCATCACAGCTCAGCCTTAAC	894
<i>K. pneumoniae</i>	<i>tyr B</i>	Tyrosine Amino transferase	F- GGCTGTACTACAACGATGAC R- TTGAGCTAATCCACTTTG	931
<i>E. coli</i>	<i>uidA</i>	β-D Glucuronidase	F- TACCGACGAAAACGGCAAGA R- CGGTGATATCGTCCACCCAG	119

antibiotics viz., tetracycline 30 µg (TET 30), gentamycin 10 µg (GEN10), cefotaxime 30 µg (CTX 30), ciprofloxacin 5 µg (CIP 5), amikacin 30 µg(AK30), chloramphenicol 30 µg (C30), amoxicillin 30 µg (AMX 30), ampicillin 10 µg (AMP 10) and ceftriaxone 30 µg (CTR 30). An isolate resistant to three or more than three antimicrobial classes is defined as Multiple Drug Resistant (MDR) (Magiorakos et al. 2012). Selected antibiotics for the study covered different classes such as tetracycline and chloramphenicol both inhibit protein synthesis, erythromycin belongs to the macrolide class of antibiotics; enrofloxacin and ciprofloxacin are fluoroquinolones; penicillin, oxacillin, amoxicillin and ampicillin belong to penicillin class of antibiotics; gentamycin and amikacin are aminoglycosides; ceftriaxone, cefotaxime and cefoxitin are cephalosporins; linezolid belongs to oxazolidinones; vancomycin is a glycopeptide and co-trimoxazole is a sulfonamide.

### Antimicrobial Activity Screening of Polybia MP-1 Against Bacterial Isolates from Milk

The antimicrobial activity of Polybia MP-1 was assessed against *S. aureus*, *K. pneumoniae* and *E. coli* isolates by broth microdilution assay as mentioned for MIC determination at a concentration of MIC and 2×MIC (MICs used for the respective *S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522)). All isolates whose ≥ 90% growth was inhibited by the peptide were considered susceptible to the AMP.

### Statistical Analysis

Total variation present among groups was determined by two-way analysis of variance (ANOVA). Student's *t* test and Tukey's multiple comparison test was used for determining significance. All values represent mean ± SEM.

## Results

### Synthesis of Polybia MP-1

The peptide was obtained with a free N terminal and a C terminal amide group once cleaved from Rink amide MBHA resin. After HPLC purification, the peptide had > 90% purity. The HPLC chromatogram is given in Fig. 1A, which shows the peak for Polybia MP-1 at a retention time of 22.3 min. The predicted mass of the peptide by in silico analysis was 1654.01 g/mol and the same was observed by MS analysis as well (Fig. 1B).

### Determination of MIC and MBC

After screening for a range of concentrations, the MIC of Polybia MP-1 for *S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522) were found to be 15 µM (24.81 µg/mL), 45 µM (74.43 µg/mL) and 10 µM (16.54 µg/mL), respectively (Table 2). The MBC for Polybia MP-1 was found to be equal to MIC for all the three bacteria i.e., 15 µM, 45 µM and 10 µM for *S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522) respectively. At this concentration the bacteria were completely eliminated and no growth was observed on MHA plates after overnight incubation at 37 °C.

### Determination of CE Values

4×MIC of Polybia MP-1 was highly effective against *S. aureus* (ATCC 29,213) just after 15 min of incubation followed by 2×MIC which was effective after 60 min of incubation. MIC took 180 min of incubation to eliminate the bacteria whereas no inhibition of bacterial growth was observed for half of MIC even after 180 min of incubation (Fig. 2A).

2×MIC and 4×MIC of Polybia MP-1 was effective against *K. pneumoniae* (ATCC 13,883) after 15 min of incubation whereas MIC was effective after 30 min of incubation. Half of the MIC and 10 µg/mL of Ampicillin were found ineffective even after 180 min of incubation (Fig. 2B).

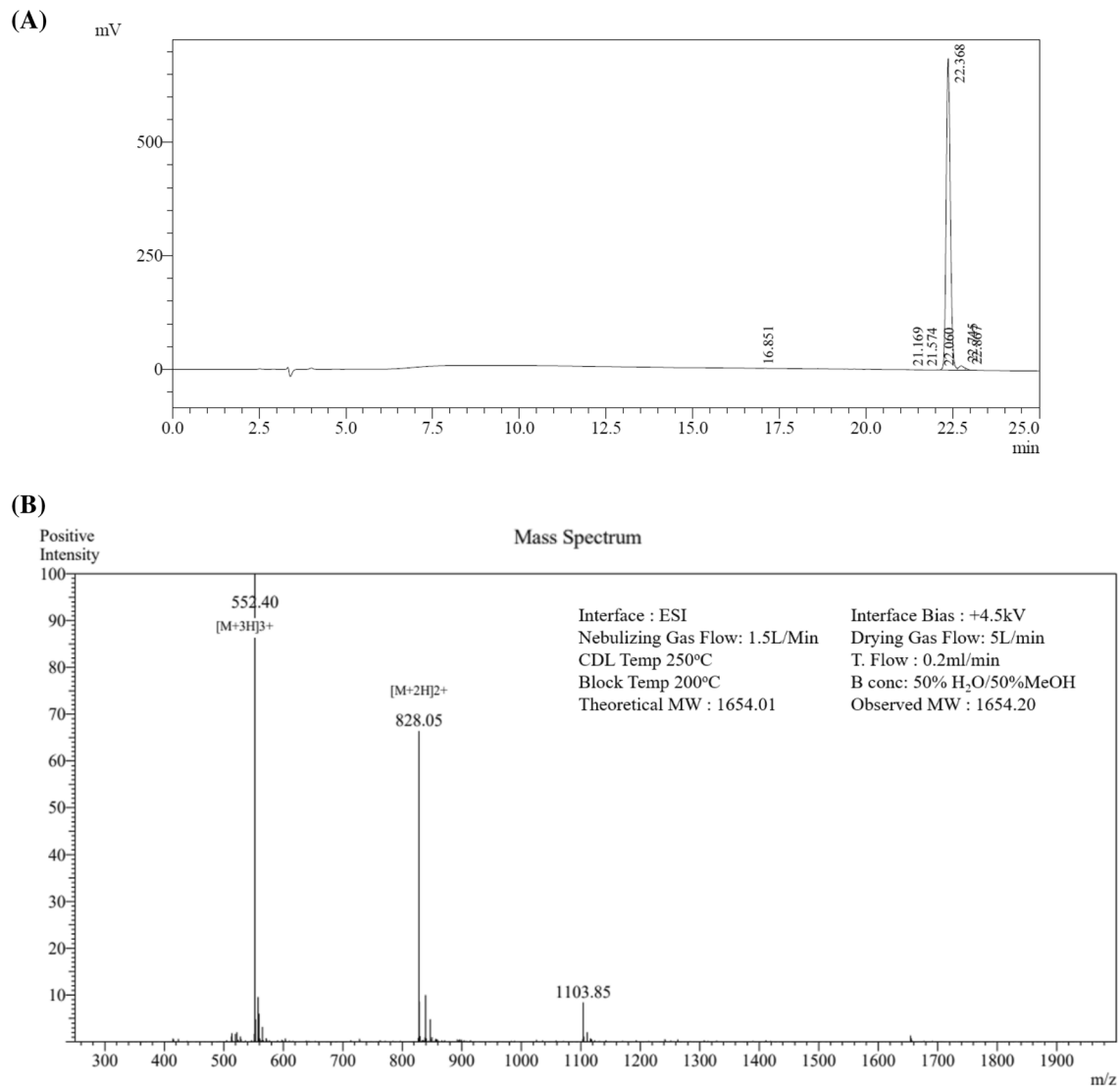
4×MIC of Polybia MP-1 was effective against *E. coli* (ATCC 29,522) after 120 min of incubation whereas half of MIC, MIC and 2×MIC were effective after 180 min of incubation (Fig. 2C).

### Haemolytic Activity

The haemolytic activity of Polybia MP-1 was assessed on rabbit and mice erythrocytes. As observed in Fig. 3, the HC<sub>50</sub> (the peptide concentration that resulted in 50% haemolysis of erythrocytes) for Polybia MP-1 exceeded 200 µM for RBCs of both mice and rabbit. Polybia MP-1 showed maximum haemolysis of 4.5% for mice erythrocytes at 200 µM.

### In Vitro Cytotoxicity Determination in Vero Cells

The peptide showed no cytotoxicity at a range of concentrations up till 160 µM as shown in Fig. 4.



**Fig. 1** **A** HPLC chromatogram of Polybia MP-1 showing major peak at a retention time of 22.3 min **B** Mass spectrum of Polybia MP-1 using ESI-MS. The theoretical molecular weight of peptide was 1654.01 and the observed MW was 1654.20

### Peptide Toxicity in *Galleria mellonella* (Greater Wax Moth) Larvae

The viability of larvae in different treatment groups was monitored after every 12 h by prodding for movement and checking for melanisation (Fig. 5 inset). The larvae in buffer control and prick control showed no mortality even after 72 h. Similar results were obtained for 5, 20 and 40 mg per kg body weight treatment groups of Polybia MP-1. 80% survival was obtained for 160 mg/kg bodyweight and 60% survival was obtained for 320 mg/kg bodyweight treatment with Polybia MP-1 after 60 h. The negative control group showed 20% survival after 12 h of treatment with Triton X-100 whereas 0% survival was observed after just 24 h (Fig. 5).

### Membrane Disruption by Polybia MP-1

The damage to bacterial outer membrane by Polybia MP-1 was measured by 1-N-phenyl naphthylamine (NPN) uptake assay. The fluorescence intensity of NPN increased significantly ( $p < 0.05$ ) in a time dependent manner for *E. coli* (ATCC 29,552) cells treated with 1 MIC of Polybia MP-1 similar to the positive control i.e., Triton X 100 (0.5% v/v) (Fig. 6A). However, the overall increase in the fluorescent intensity for Polybia MP-1 was less than Triton X 100.

The SYTOX Green fluorescence was measured for determining the damage to inner cytoplasmic membrane of *E. coli* (ATCC 29,552) cells by Polybia MP-1 (1 MIC) and Triton X 100 (0.5% v/v). The membrane permeabilization increased significantly ( $p < 0.05$ ) for Polybia MP-1 and

**Table 2** Minimum Inhibitory Concentration (MIC) for Polybia MP-1 for *S. aureus* (ATCC 29,213), *K. pneumoniae* (ATCC 13,883) and *E. coli* (ATCC 29,522) (highlighted in red)

Peptide ( $\mu\text{M}$ )	% Inhibition of growth for bacterial isolates		
	<i>S. aureus</i> (ATCC 29,213)	<i>K. pneumoniae</i> (ATCC 13,883)	<i>E. coli</i> (ATCC 29,522)
50	100 $\pm$ 0.00	100 $\pm$ 0.00	100 $\pm$ 0.00
45	100 $\pm$ 0.00	99.9 $\pm$ 0.06	99.9 $\pm$ 0.01
40	99.9 $\pm$ 0.03	94.9 $\pm$ 0.23	99.9 $\pm$ 0.07
35	99.8 $\pm$ 0.14	82.8 $\pm$ 0.21	99.8 $\pm$ 0.19
30	99.9 $\pm$ 0.14	61.7 $\pm$ 0.40	99.9 $\pm$ 0.07
25	99.8 $\pm$ 0.13	50.4 $\pm$ 0.56	99.8 $\pm$ 0.05
20	99.5 $\pm$ 0.69	32.9 $\pm$ 0.01	99.8 $\pm$ 0.05
15	99.9 $\pm$ 0.06	29.5 $\pm$ 0.70	99.7 $\pm$ 0.25
10	64.9 $\pm$ 0.03	18.4 $\pm$ 0.72	99.8 $\pm$ 0.08
5	51.8 $\pm$ 0.17	5.4 $\pm$ 0.18	52.3 $\pm$ 0.79
Control	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00

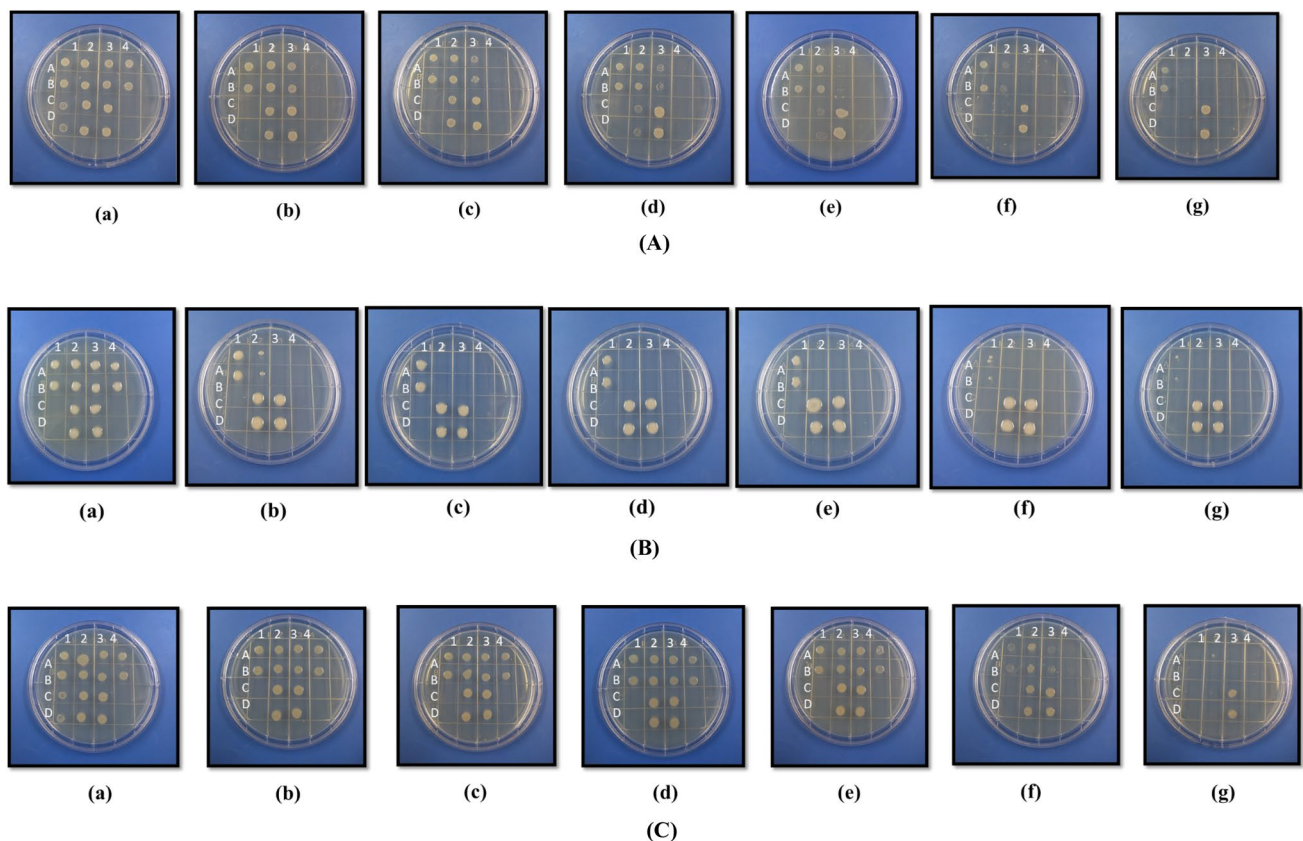
All values represent Mean  $\pm$  SEM (n=2)

Triton X 100 with increasing time of incubation (Fig. 6B). Polybia MP-1 showed significant increase in internal membrane permeabilization even higher than the positive control i.e., Triton X 100.

### Identification and Antibiogram of Bacteria Isolated from Milk Samples

20 isolates of *S. aureus*, 21 isolates of *E. coli* and 14 isolates of *K. pneumoniae* were obtained from 40 milk samples as confirmed by PCR. Antibiogram for the isolates is given in Fig. 7A–C. 100% of *S. aureus* isolates were resistant to cefoxitin (cx 30) followed by 90% isolates showing resistance to penicillin (P 10). 80% of *S. aureus* isolates were resistant to linezolid (LZ 30), 75% isolates were resistant for oxacillin (OX 1) with minimum 40% isolates having resistance for chloramphenicol (C 30). 19 out of the 20 *S. aureus* isolates belonged to MDR category showing resistance for  $\geq 3$  classes of antimicrobials.

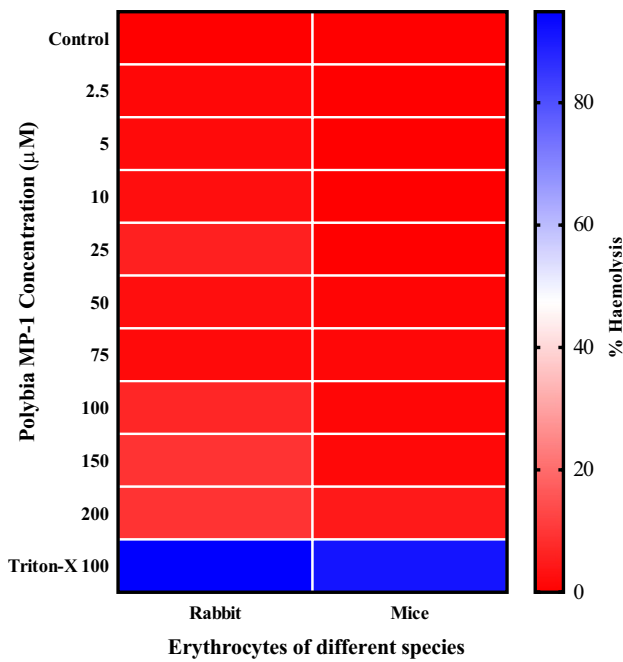
86% of *E. coli* isolates were found resistant for cefotaxime (Ctx 30) followed by 76% of resistance against ampicillin



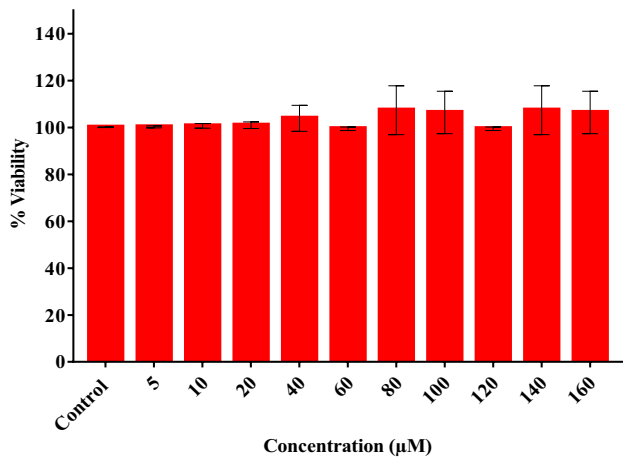
**Fig. 2** Complete Elimination study for Polybia MP-1 on **A** *S. aureus* (ATCC 29,213) **B** *K. pneumoniae* (ATCC 13,883) and **C** *E. coli* (ATCC 29,522). Different figures correspond to different incubation times i.e., (a) 0 min (b) 15 min (c) 30 min (d) 45 min (e) 60 min (f) 120 min (g) 180 min. 1A and 1B represent 0.5 MIC; 2A and 2B

represent MIC; 3A and 3B represent 2 $\times$ MIC; 4A and 4B represent 4 $\times$ MIC for Polybia MP-1; 1C and 1D represent Streptomycin at 10  $\mu\text{g}/\text{mL}$ ; 2C and 2D represent Ampicillin 10  $\mu\text{g}/\text{mL}$ ; 3C and 3D represent untreated cells and 4C and 4D represent blank (plain media)





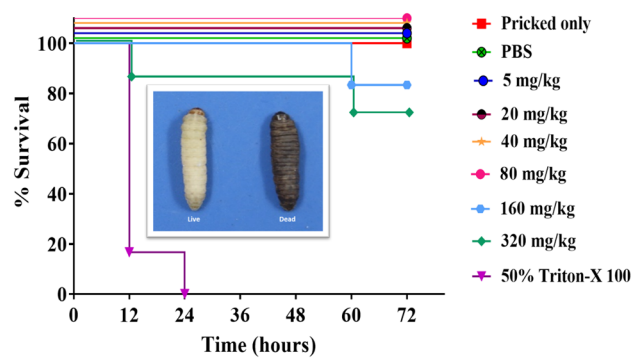
**Fig. 3** Heat map showing haemolytic activity of Polybia MP-1 on erythrocytes of rabbit and mice. All values represent % Haemolysis (Mean ± SEM, n=2) at different concentrations of Polybia MP-1 and 0.1% Triton X 100



**Fig. 4** MTT based cytotoxicity assay for Polybia MP-1. Vero cells were treated with increasing concentrations of the peptide for 24 h and cell cytotoxicity was determined by MTT assay. Results are expressed as % MTT absorption with respect to untreated control wells (n=6)

(Amp 10). Least resistance was observed for chloramphenicol (C 30) by 33% isolates. 17 out of 21 isolates were found to be MDR isolates with resistance for ≥ 3 classes of antimicrobials.

93% of *K. pneumoniae* isolates were resistant for Ampicillin (Amp 10) followed by 79% of isolates showing



**Fig. 5** Polybia MP-1 toxicity in *G. mellonella* larvae. Survival curves for larvae treated with Polybia MP-1 (5, 20, 40, 80, 160, 320 mg/kg bodyweight), PBS (buffer control), prick control and Triton X-100 (50% v/v). Each group had five larvae (n=5) and the injection volume was 10 µl. Figure in the inset shows representative image of a live and dead larva

resistance for amoxicillin (Amx 30). Least resistance in 36% isolates was observed for ciprofloxacin (Cip 5) and chloramphenicol (C 30). Out of 14, 10 isolates were found to be MDR with resistance for ≥ 3 classes of antimicrobials.

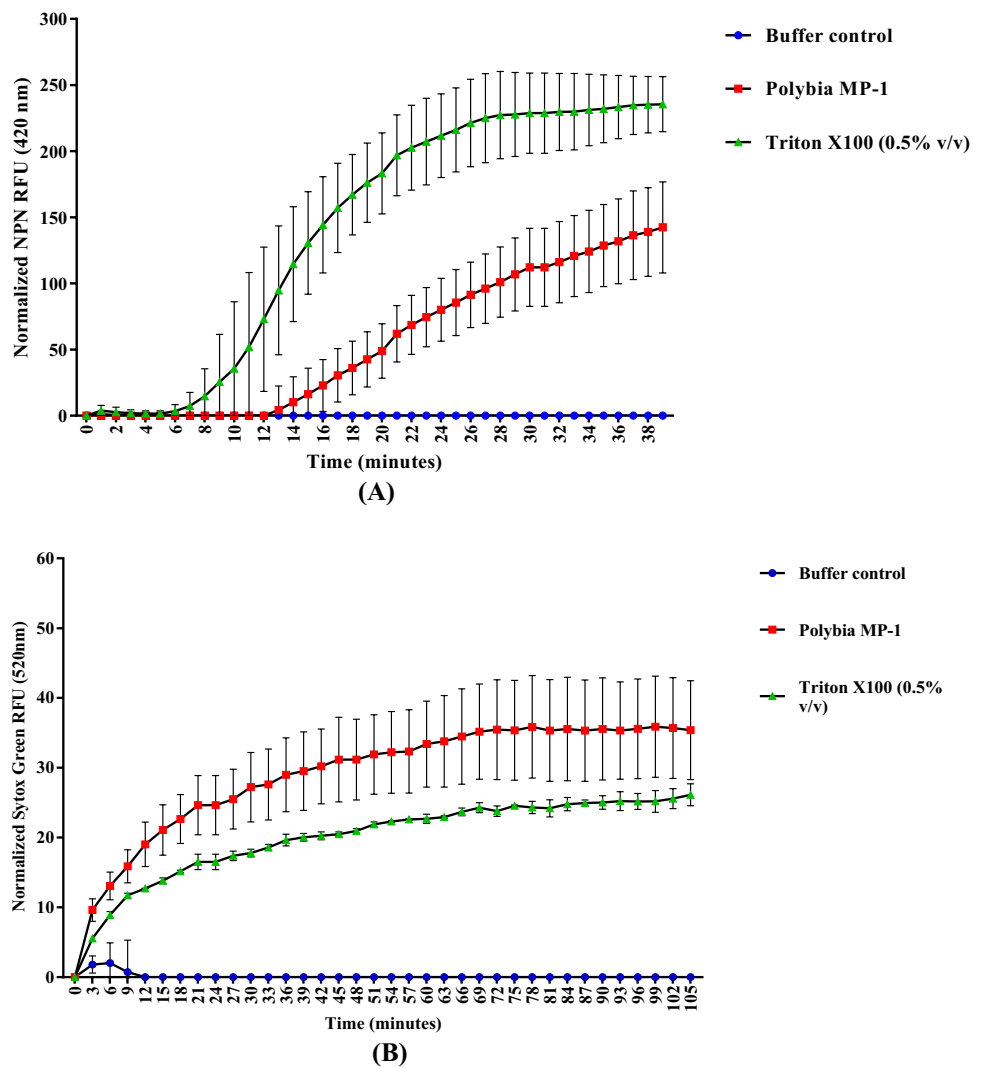
### Antimicrobial Activity of Polybia MP-1 Against Bacterial Isolates from Milk

The activity of Polybia MP-1 was evaluated against the 20 *S. aureus*, 21 *E. coli* and 14 *K. pneumoniae* isolates at MIC and 2 × MIC as determined for the standard *S. aureus* (ATCC 29,213), *E. coli* (ATCC 29,522) and *K. pneumoniae* (ATCC 13,883), respectively. Polybia MP-1 at 2 × MIC was able to effectively inhibit ≥ 90% growth of (IC<sub>90</sub> -Inhibitory Concentration at which 90% growth is inhibited) 32% (6/19) MDR isolates of *S. aureus*, 47% (8/17) MDR isolates of *E. coli* and 70% (7/10) MDR isolates of *K. pneumoniae* effectively. This elucidated the potent antimicrobial activity of the peptide against MDR isolates.

### Discussion

Mastoparan and similar peptides obtained from the venom of vespid wasps and hornets are an important class of naturally occurring antimicrobial peptides (Konno et al. 2019). Polybia MP-1 is a tetradecapeptide of this class with an α-helical structure and potent antimicrobial activity because of its interaction with the bacterial membrane (Alvares et al. 2016). Previous work done by Souza et al. (2005) showed that Polybia MP-1 had an MIC of 8, 8, 4 and 15 µg/mL for *Bacillus subtilis* (CCT 2576), *Escherichia coli* (ATCC 25,922), *Staphylococcus aureus* (ATCC 6538), and *Pseudomonas aeruginosa* (ATCC 15,422), respectively. Luong

**Fig. 6** Mechanism of action of Polybia MP-1 on bacterial membrane was determined for **A** Outer membrane by 1-N-phenyl naphthylamine (NPN) uptake assay by measuring its fluorescence at 420 nm (Excitation and emission wavelengths for NPN were set at 350 and 420 nm, respectively) **B** Inner Cytoplasmic membrane by measuring increase in fluorescence of SYTOX Green due to binding with DNA (Excitation and emission wavelengths for SYTOX green were set at 480 and 522 nm, respectively). Each value represents the mean  $\pm$  SEM (n = 2)

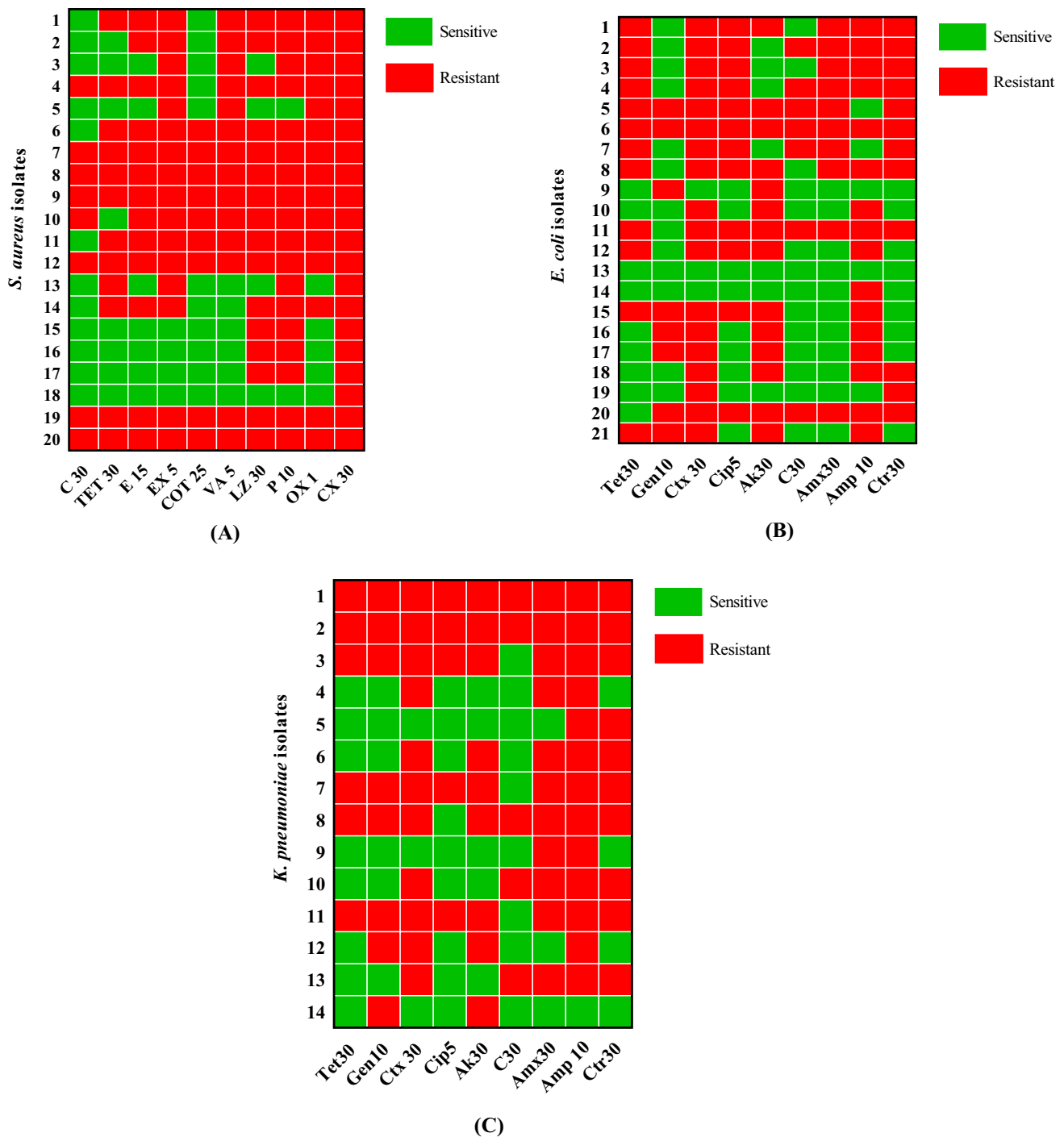


et al. (2017) reported an MIC greater than 100  $\mu\text{g}/\text{mL}$  for Polybia MP-1 against *P. aeruginosa*. Shah et al. (2021) determined the MIC of Polybia MP-1 against *P. aeruginosa* (ATCC 27,853) to be 75  $\mu\text{M}$  i.e., 127.5  $\mu\text{g}/\text{mL}$ . In the present study, the MIC for *S. aureus* (ATCC 29,213), *E. coli* (ATCC 29,522) and *K. pneumoniae* (ATCC 13,883) were determined to be 15  $\mu\text{M}$ , 10  $\mu\text{M}$  and 45  $\mu\text{M}$ , respectively (Table 1). Differences in MIC may have arisen due to difference in membrane bilayer composition which in turn would have influenced the interaction of the peptide with the bacterial membrane.

Polybia Mp-1 disrupts the bacterial membrane by forming pores as a result of its helical structure. The cationic residues of the peptide interact with the anionic bacterial surface and hydrophobic interactions in between hydrophobic helix residues and membrane's interior portion further aid the process. Lipid composition heavily influences the peptide and membrane interaction. A certain threshold amount of peptide concentration is thus required to interact

with the bacterial membrane and cause its disruption (Dos Santos Cabrera et al. 2008). This was well demonstrated by the time dependent complete elimination study performed by drop plate method in this study. Based on lipid composition of gram positive (*S. aureus* ATCC 29,213) and gram negative (*E. coli* ATCC 29,522 and *K. pneumoniae* ATCC 13,883) bacteria the peptide showed maximum and earliest disruption of the bacterial membrane at the highest  $4 \times \text{MIC}$  (Fig. 1).

Toxicity evaluation of an AMP is an important parameter to be considered for its further development into a therapeutic drug. Membrane active antimicrobial peptides can potentially affect mammalian cells. Hemolysis assay is a well-characterized test to study the ability of synthetic peptides to disrupt mammalian membranes. Previous reports have suggested little to no haemolytic activity by Polybia MP-1 even at a concentration of 100  $\mu\text{M}$  against rat RBCs (Souza et al. 2005). Our studies are in agreement with previous reports and show the selective nature of the peptide with



**Fig. 7** Antibiogram for **A** *S. aureus* isolates (n=20) **B** *E. coli* isolates (n=21) and **C** *K. pneumoniae* isolates (n=14)

high affinity for bacterial membranes and poor to no effect on mammalian RBCs. In vitro cytotoxicity of the peptide was assessed by MTT assay on Vero cell line. Positively charged compounds such as MTT readily penetrate viable cells. Viable cells with active metabolism are able to convert MTT into a purple-coloured formazan product by dehydrogenase activity in the mitochondria. This formazan when

dissolved in DMSO is measured at 570 nm. The peptide showed no cytotoxicity even at a higher concentration of 160 µM. These results are in agreement with the previous work done by Wang et al. (2008) where they demonstrated the low toxicity of Polybia MP-1 against NIH 3T3 mouse embryonic fibroblast cell line.

Toxicity testing is an extrapolation of antibiotic efficacy studies, and for this de novo *G. mellonella* larvae toxicity testing model have been popularly used. Apart from establishing a therapeutic dose of the antibiotic against the infecting bacteria, it is also important to determine an LD<sub>50</sub> dose (median lethal dose; a dose of the compound that is sufficient to kill 50% of a population of test larvae) for safety evaluation of the test compound. However, only recently *G. mellonella* larvae have been used in de novo toxicity testing (Ignasiak and Maxwell, 2017). *G. mellonella* larvae are generally considered to be an ethical alternative to studies in mammals (Titball, 2016). In the present study, no larvae mortality was observed till 40 mg/kg body weight of concentration even after 72 h. Recently, studies on the novel membrane-disruptive antimicrobial peptide from frog skin secretion, Japponicin-2LF was reported non-toxic for *G. mellonella* larvae up to the highest concentration (50 mg/kg body weight) of the peptide tested (Yuan et al., 2019).

To understand the mode of action of the peptide, damage to the outer bacterial membrane was assessed by NPN uptake assay. A dose dependent increase in fluorescence intensity of *E. coli* (ATCC 29,522) cells was observed after treatment with Polybia MP-1. The AMP likely targets the bacterial outer membrane leading to partitioning of NPN into the outer membrane and thus an increase in fluorescence. Similar dose dependent increase in fluorescence was reported by Mohamed et al. (2017) for the peptide analog D-RR4 when mixed with *P. aeruginosa* PAO1 and NPN. Damage of the bacterial inner (cytoplasmic) membrane by peptides was monitored by measuring the fluorescence intensity of *E. coli* (ATCC 29,522) cells combined with SYTOX green and then subsequently treated with Polybia MP-1. When the inner membrane is disrupted the impermeable SYTOX Green nucleic acid stain is able to enter the bacterial cells and bind with the DNA. A dose and time dependent increase in the fluorescence was observed confirming perturbations in the inner membrane of bacteria. Similar results were obtained by Mohamed et al. (2017) for the peptide analog D-RR4 on *P. aeruginosa* PAO1 cells with Propidium iodide (PI). In the present study, the peptide induced more damage to the inner membrane of *E. coli*. The interaction between the outer membrane of Gram negative bacteria and AMPs is poorly understood because of the complexity of lipopolysaccharide (LPS). Initially the AMP gets adsorbed on the outer membrane and the major interaction is brought about by the electrostatic interaction in between cationic AMP and anionic LPS molecules. Any changes in the electrostatic driving force may lead to poor interaction in between the two and loss of antimicrobial activity. Apart from electrostatic interaction the hydrophobic residues of AMP further interact with the lipid tails of LPS. These interactions disrupt the outer membrane and AMP gets diffused into the periplasmic space. It reaches the cytoplasmic membrane and gets

adsorbed on the surface. After reaching a certain threshold concentration the AMP induces severe cytoplasmic membrane disruptions followed by loss of membrane potential and ultimate death of the bacteria (Hollmann et al. 2018). Our findings also indicate that the peptide is active against both inner and outer membrane of bacteria and has the ability to disrupt both these membranes. The potent antibacterial activity of this AMP against the MDR bacteria also may be due to the disruption of the inner and the outer membrane of bacteria.

The antibiogram data revealed the presence of 19 MDR *S. aureus*, 17 MDR *E. coli* and 10 MDR *K. pneumoniae* isolates. These results signify the increase in the incidence of antimicrobial resistance (AMR) in dairy farms due to unmonitored and rampant use of antibiotics to treat mastitis. Development of AMPs as alternatives to antibiotics for treatment of mastitis and other infections will surely hold the key to success against AMR. In the present work, Polybia MP-1 was able to effectively inhibit the growth of MDR isolates which elucidated its immense potential for development as a therapeutic intervention against mastitis. Presently, a number of AMPs are being tested against bacteria causing mastitis. Islas-Rodríguez et al. (2009) reported the activity of Esculentin 1–21, a linear antimicrobial peptide from frog skin against bacteria causing bovine mastitis. In another study, Barreras-Serrano et al. (2017) evaluated the activity of antimicrobial peptide K9CATH in a murine model of mastitis. Li et al. (2017a, b) assessed the antimicrobial activities of NZ2114 and MP1102 against *S. aureus* in milk, in cultured mammary epithelial cells, and in a mouse model in order to evaluate their potentials as anti-mastitis agents.

## Conclusion

Mastitis is a communicable disease of the mammary glands of dairy cattle, with high worldwide prevalence at both the cow and udder quarter levels. Apart from losses incurred in production the treatment of mastitis poses more economic burden. Excessive, unmonitored and careless usage of antibiotics for treatment of mastitis has led to the development of antimicrobial resistance (AMR) in many mastitis causing micro-organisms. The present study effectively established the potential of Polybia MP-1 as a potent antimicrobial peptide against different MDR isolates of *S. aureus*, *E. coli* and *K. pneumoniae* isolated from mastitic milk samples. The peptide showed very low haemolytic activity against RBCs of different mammals and no cytotoxicity even at a high concentration of 160 µM when tested in Vero cells. Toxicity studies in *G. mellonella* larvae showed the peptide to be safe up to 40 mg/kg body weight with no mortality even after 72 h. The peptide effectively disrupted both the outer



and inner *E. coli* (ATCC 29,522) bacterial membrane as was observed by NPN uptake and SYTOX Green assay. Hence, Polybia MP-1 with its potent antimicrobial activity and very low toxicity has immense potential for development as a therapeutic intervention against mastitis. Further validation in animal models can help formulate intramammary or topical antimicrobial preparations based on Polybia MP-1 for the therapeutic management of bovine mastitis. Studying synergism between AMP and different antibiotics may also be taken up to ascertain if the inclusion of some AMPs in the antibiotic preparation can reduce the therapeutic dose of the existing antibiotics. If this can be achieved, it will be another good contribution in reducing the AMR due to use of high doses of different antibiotics in the treatment of mastitis.

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**Data Availability** All data related to this work are presented in the MS hence, no supplementary data is provided.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical Approval** In this study, no invasive procedure was performed on human or any other animal species, except collection of whole blood from rabbit and mice for performing the haemolytic assay, for which the permission from the Institute Animal Ethics Committee was obtained vide Reference No: F.26–1/2015–16/JD(R) dated 28th Feb, 2019.

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