International Journal of Multidisciplinary Research and Publications
ISSN (Online): 2581-6187

Discovering of Peptide Contributions to Drug Developments

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***Abstract*—** *Rusticyanin, a protein of 155 amino acids obtained from Thiobacillus ferrooxidans was used to design RP131-155 peptide (Mw=2688.21). The aim of this study was to investigate the antimicrobial effect of various concentrations of RP131-155, a peptide derived from the N-terminal of rusticyanin on Enterobacter aerogenes, Salmonella arizonae, Escherichia coli and Staphylococcus aureus. The antimicrobial activity was determined by using disk diffusion method. A higher inhibition zone was observed for E. coli, E. aerogenes, S. arizonae and S. aureus respectively. The minimum inhibition concentration (MIC) value of RP131-155 against E. coli, E. aerogenes and S. arizonae was shown to range between0.08-0.16mg/ml whilst that of S.aureus was between 0.32-0.63mg/ml.*

***Keywords*—** *Rusticyanin, peptide, antimicrobial.*

I. INTRODUCTION

A. Microorganisms and Infection Disease

Micro-organisms are believed to be the most extended form of life on Earth, they have demonstrated a great flexibility and can be found everywhere, evolved mechanisms for survival in such environments [1]. It is estimated that 2 million people died by infection disease. Since the introduction of antibiotics into the field of medicine, they have been utilised for the treatment of infectious diseases as well as medical procedures such as surgery and chemotherapy. However, years after the discovery and the use of the first antibiotics, some organisms still survived the effects of these agents. The era of antimicrobial resistance begins when Staphylococci aureus was observed to have survived in the presence of penicillin, this occurrence was reported to be due to the inappropriate widespread use of penicillin. The inappropriate use of antibiotics leading to resistance include the abuse in the use of these drugs in clinical practice leading to selective pressure; and also the unnecessary use of antibiotics in agriculture and animal feed [2]. Unfortunately, the emergence of microbial resistance strains and multi-resistance strains (superbugs) is a major cause of treatment failure of infectious disease.

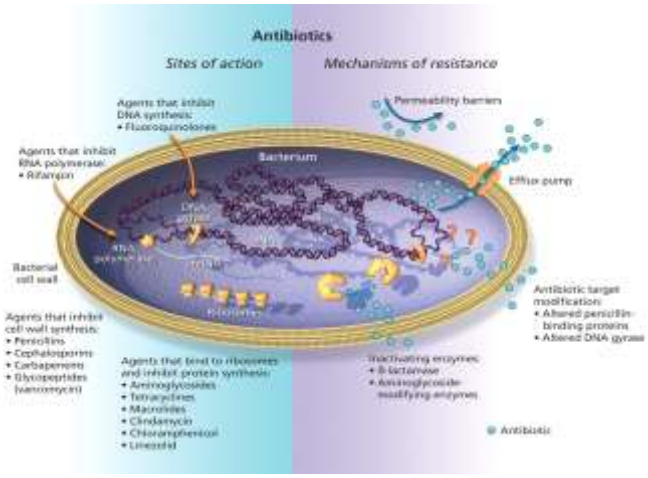
B. Development of antibiotic

The discovery of antibiotic serves as a gateway for the treatment of infection. These compounds vary in terms of their chemical nature and mode of action as well as their effect on the animal body.

II. ANTIBIOTIC RESISTANCE

Antimicrobial resistance could be natural or acquired, natural resistance can be observed in Gram negative organisms whose cell walls are protected by outer membrane bilayer. Certain organisms such as the staphylococcus spp. have the ability to secrete β-lactamase, an enzyme that inactivates penicillin molecule [3]. While acquired resistance to antibiotic occurs through certain mechanisms including spontaneous mutation of bacterial chromosomes (s), recombination of DNA, and exchange of genetic material with other bacteria.

A. Mechanism of Antimicrobial Resistance



B. Mechanism of Action of AMPs

The mechanism of action of AMPs is not very well established, though many antimicrobial peptides are reported to act directly on the membrane of the bacterial cell. The activity of AMPs depends on the amino acid sequence, membrane lipids and peptide concentration [4].

C. Alternative Mechanism of Action of AMPs

Recent studies indicate evidence that AMPs may interact with the putative intracellular target, beside their ability to interact with microbial membrane. AMPs alternative mechanisms of action may act independently or together with membrane permeabilization. They might target intracellular molecule such as DNA, RNA, proteins and enzymes. They have been reported to interfere with microbe cell wall synthesis, nucleic acid synthesis, enzyme activity and protein

Amana Asoudani "Discovering of Peptide Contributions to Drug Developments" *International Journal of Multidisciplinary Research and Publications (IJMRAP)*, Volume 6, Issue 7, pp. 18-23, 2023.

synthesis since they are capable of spontaneously traversing bacterial outer and inner membranes [5].

III. Antimicrobial Peptides in Drug Development

Natural AMPs have been isolated and characterized from organisms ranging from prokaryotes (eubacteria, protozoans and algae) to eukaryotes (plants, insects, fish, reptiles, birds and mammals). Natural AMPs are expensive and difficult to create on a large scale. Therefore, more modern approaches have been developed to identify AMPs and as well reduce the long hours (days, weeks or months) spent on identification, isolation, purification and characterization of active AMPs from natural source. Some of the derived and synthetic AMPs have more antimicrobial properties than the natural occurring peptides; some examples include hLF-1-11, histatin 5 (P-113) and Pexiganan.

IV. Methods Used For Assessment Antimicrobial Activity

The antimicrobial assay was used evaluate the antimicrobial activity of of Rusticyanin Peptide (P131-155).

A. Antimicrobial Assays Used to evaluate the antimicrobial activity

Different antimicrobial assay are used in order to evaluate the antimicrobial activity, among which include minimum bactericidal concentrations (MBCs) and minimum inhibitory concentrations (MIC) (ATL, 2012). The MBC is considered as the lowest concentration of a specific antimicrobial agent required to kill a particular micro-organism; however, MICs is more common than MBCs (ATL, 2012. MICs are considered as the gold standard for determining the susceptibility of antimicrobial agents against microorganisms [6]. This can be determined by methods such as Kirby-Bauer Disk Susceptibility Test, antimicrobial gradient method, agar well diffusion method, microtiter broth dilution method, automated instrument system and broth dilution methods [7]. In this study, the antimicrobial activity of the rusticyanin sequence 131-155 (RP131-155) was determined using disk diffusion method. The disk diffusion method was developed in the early 1950s, due to the time consumption of the original method of susceptibility of antimicrobials "broth dilution methods". However, each laboratory modified the disk diffusion procedure to suit its own need. The modified procedures included inoculums concentration, incubation time and temperature, type of media used and concentration of the antimicrobial compound. This apparently led to a widespread confusion due to published variations in the protocol. A standard procedure for MICs "Kirby-Bauer disk diffusion test" was late developed in the early 1960s by William M. Kirby and A. W. Bauer. Presently, only the Clinical Laboratory Standards Institute is responsible for updating and modifying the original procedure of Kirby and Bauer through a global consensus process.

B. Source of Synthetic Peptide Was Used in this Study

Previously, the chemically synthesis peptides of bovine lactoferrin (BLF) showed antimicrobial activity against Gram-positive and Gram-negative bacteria. Different previous studies demonstrated that rusticyanin has antimicrobial activity against Gram-positive bacteria (S. thermophilus, M. luteus and S. aureus), Gram-negative bacteria (S. arizonae, E. aerogenes and E. coli) and yeast strains (C. albicans, C. tropicalis, R. rubra and S. cerevisiae) [8]. However, no work has been reported in the literature on the antimicrobial activity of Synthetic peptide obtained from Rusticyanin position 131-155 (RP131-155). The antimicrobial activity of RP131-155 was determined in the current study.

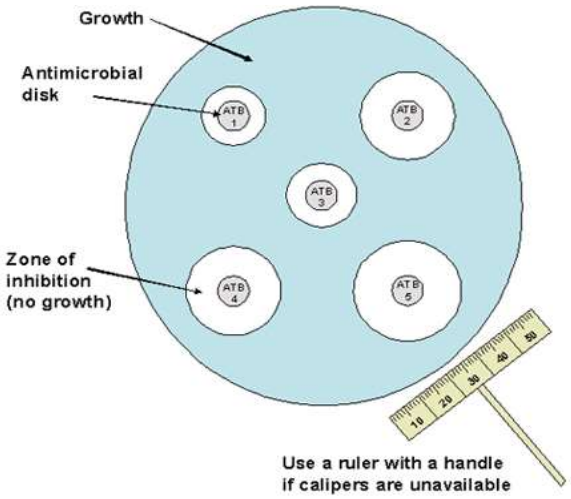


Fig. 1. Component of minimum inhibitory concentrations assay

Rusticyanin (RCN) is a copper-containing metalloprotein (154 amino acids) obtained from Thiobacillus ferrooxidans; an organism involved in the oxidization of Fe2+ to Fe3+. The structure of RCN has been studied by many methods and spectroscopic techniques such as X-ray, NMR and EPR [9]. The sequence analysis showed that it has a molecular weight is 16.5KD and contains 155 amino acid residues [10]. A Pfam search indicates that RCN sequence belongs to the clan CU oxidase (CL0026) which contains a family of nine members including Copper-bind, COX2, Cu-oxidase, Cu-oxidase2, Cu-oxidase3, Cu bind like, Cupredoxin 1, Ephrin and SoxE. While the PDBsum search of Rcn indicated the presence of a plastocyanin-like motif and cupredoxin domain (Figure 2). modifying the original procedure of Kirby and Bauer through a global consensus process.

The copper-binding site of rusticyanin is located within a hydrophobic region at one end of the molecule. surrounded by a number of aromatic rings and hydrophobic residues [9, 10]. This configuration is believed to contribute to the acid stability of the copper site, since close association of the aromatic rings with the histidine ligands would sterically hinder their dissociation from the copper. Among the factors determining the redox potential of Rcn include the contributions from charged side-chain particularly in the ligand cysteine residue [11]. In vitro study on Rcn, demonstrated it has anticancer activity against breast cancer cell line (MCF-7) and human melanoma (J774).

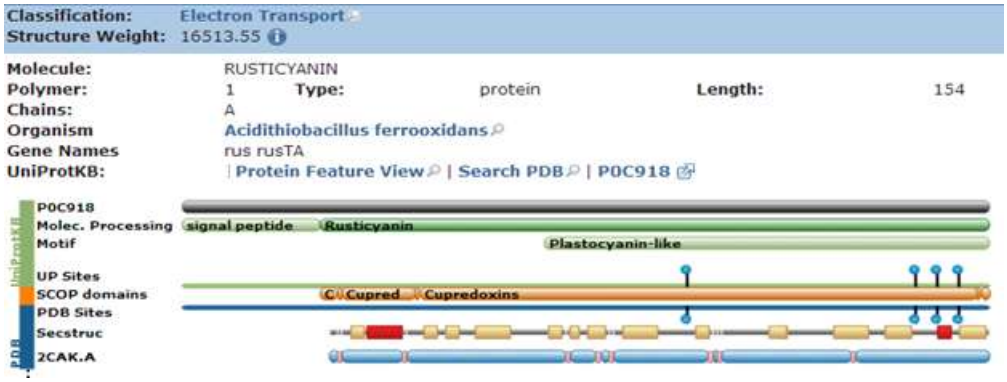


Fig. 2. Molecular description of rusticyanin with a resulotion of 1.27 obtained from X-RAY diffraction [10].

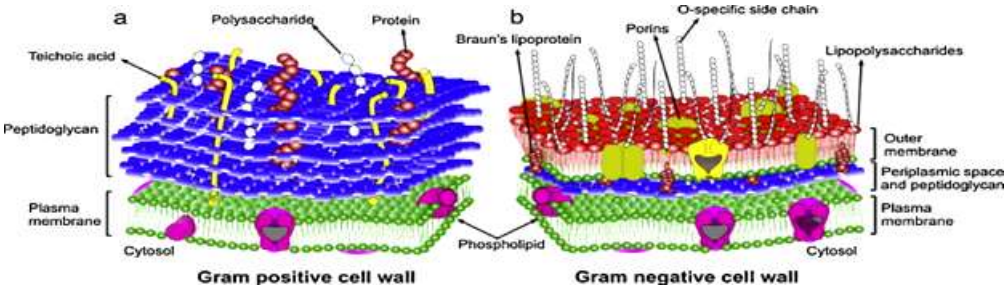


Fig. 3. The bacterial cell wall. (a) The Gram-positive envelope. (b) The Gram-negative envelope.

V. Microorganisms Examined for Antimicrobial Activity

Bacteria are unicellular organism, having a thick, rigid cell wall, which is known to provide cell integrity. They are categorized into Gram-positive and Gram-negative based on their cell wall. In the current study, three Gram-negative bacteria and one Gram-positive bacterium were used to evaluate the antimicrobial activity of RP131-155. The Gram-negative bacteria were Enterobacter auergenes DMU 52, Salmonella Arizonae and Escherichia coli DMU, while the Gram-positive bacterium was Staphylococcus aureus MRSA.

A. Gram-Negative Bacteria

The cell walls of Gram-negative bacteria consist of a much thinner peptidoglycan layer, although the structure is more complex due to the presence of an outer membrane. The outer membrane consists of a layer of lipoprotein, polysaccharide and phospholipid. Unlike the plasma membrane which contains two layers of phospholipid, the outer membrane has only one, with the outer layer being made up of lipopolysaccharide (Figure 3) [12] [13].

B. Gram-Positive Bacteria

The cell walls of Gram-positive bacteria are thick, comprising of several layers of peptidoglycan which are connected to each other by cross-linkages forming a strong and rigid cell wall. Therefore, the plasma membrane of Gram-positive bacteria is embedded by proteins and covered by multilayered peptidoglycan shell, and they also contain polysaccharides, teichoic acids and proteins (Figure 3) [12] [13].

VI. Objectives of this Study

The main objectives of this study include Assessment Activity of synthetic Rusticyanin Peptide (P131-155) against some Gram-positive and Gram-negative bacteria.

VII. Methodology

A. Synthetic Rusticyanin (131-155) Peptide (RP131-155)

RP131-155 (Mw=2688.21 the peptide was reported to be 100% pure according to the high performance liquid chromatography (HPLC) analysis.

B. Antimicrobial Assay (RP131-155)

- Material and Reagent:

Bunsen burne, pipettes and pipette tips, sterile loop, eppendorfs tubes, antimicrobial peptide disk, agar plates, ruler and Mueller-Hinton agar. Rusticyanin (131-155) Peptide and the four different micro-organisms used.

- Micro-organisms:

Four bacterial species were used for this assay. The Gram-positive bacterium used was Staphylococcus aureus (MRSA) whereas the Gram negative bacteria used were Salmonella arizonae, Enterobacter aerogenes and Escherichia coli.

- Peptide preparation:

6000µg of RP (131-155) was added into 600µl distilled water giving a concentration of 10mg/ml and the Eppendorf tubes were accurately labelled. Nine Eppendorf tubes was accurately labelled (2-10), each containing 300µl distilled water. Then a serial dilution was conducted using a one in two dilution, the ultimate concentrations were 10.0mg/ml, 5.0mg/ml, 2.50mg/ml, 1.25mg/ml, 0.63mg/ml, 0.32mg/ml, 0.16mg/ml, 0.08mg/ml, 0.04mg/m and 0.02mg/ml.

Stock Solution Concentration = 6000µg/600µl

= 10µg/µl
=10mg/ml

Therefore, dilution factor= 300µl/600µl= ½ , which means a 2× dilution factor

- DISK DIFFUSION ASSAY:

From each of the four microorganisms stock solution, a loop was transferred into a fresh nutrient broth prepared by addition of 13g Luria Bertani (LB) Broth in 1L of distilled water. Cultures were appropriately labelled then incubated for 370C for 24hrs. 40 agar plates were prepared from a stock solution of 252gm of nutrient agar in 9L of distilled water. These agar plates were accurately labelled with assigned micro-organism. 10 agar plates were assigned to each organism (10 agar plates assigned to E. aerogenes; 10 agar plates assigned to S. arizonae; 10 agar plates assigned to E. coli and 10 agar plates assigned to .S aureus) and accurately labelled 1-10 then sterilised by autoclaving at 1210C for an hour. Therefore, each of the 10 agar plates were streaked with 100µl of it assigned micro-organisms using appropriate precautions to avoid contamination. Then three disks were then placed in each of the forty agar plates by the aid of a sterilised tong.

For every organism, the agar plate was labelled 1-10 in respect with the prepared concentrations of the RP(131-155) (10.0mg/ml, 5.0mg/ml, 2.50mg/ml, 1.25mg/ml, 0.63mg/ml, 0.32mg/ml, 0.16mg/ml, 0.08mg/ml, 0.04mg/ml and 0.02mg/ml.) In all the forty agar plates, each of the three disks was impregnated with 5µl of the prepared peptide, respectively. Then the plates were incubated for 48 hours at 370C, after which the zone diameter was measured using ruler. The whole procedure was repeated for impregnating each of the three disks with 10ul of RP(131-155).

VIII. RESULTS

A. *Antimicrobial Susceptibility Test*

RP131-155 is shown to have antimicrobial activity against E. coli, E. aerogenes and S. aureus, however the peptide has no effect against S. arizonae at 5µl of all the concentrations. The peptide is more susceptible to E. coli compared to E. aerogenes and S. aureus (Figure 4).

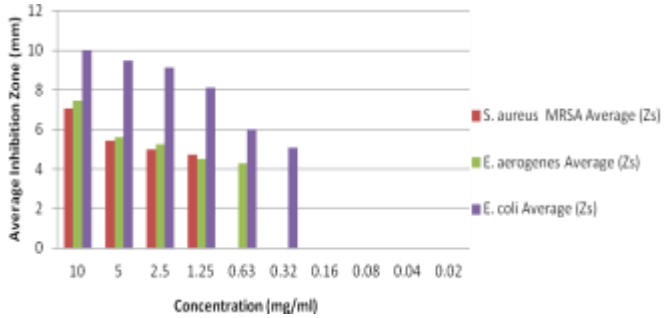


Fig. 4. Antimicrobial activity of various concentrations of 5µl RP131-155 against E.coli, E. aerogenes and S. aureus obtained by disk diffusion method. However, the peptide showed no effect against S. arizonae.

The antimicrobial activity of 10µl RP131-155 showed it effect against the four micro-organisms used for this study, though it is more effective against E. coli (Figure 5).

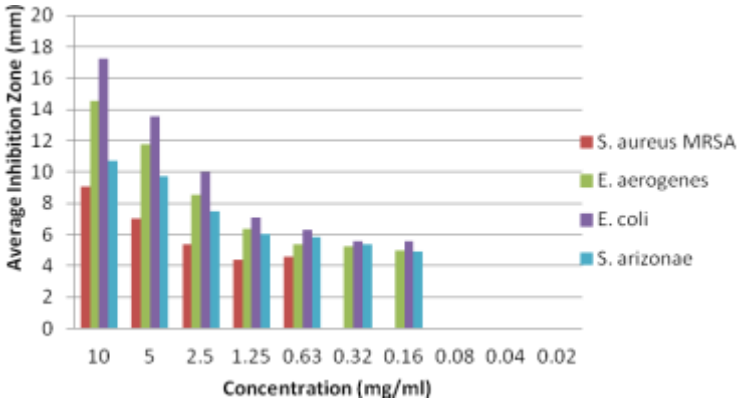


Fig. 5. Antimicrobial activity of various concentrations of 10µl RP131-155 against E.coli, E. aerogenes, S. arizonae and S. aureus obtained by disk diffusion method.

TABLE I. Antimicrobial activity of RP131-155 determined by disk diffusion method (values are in mean±S.D)

Concentration	Volume	Inhibition Zone Diameter (mm) (n = 3)			
µg/µl	µl	S. aureus MRSA	E. aerogene	E. coli	S. arizonae
10	5	7.05±0.06	7.48±0.49	10.00±0.00	N
	10	9.04±0.07	14.56±0.51	17.24±0.66	10.69±0.18
5	5	5.45±0.40	5.61±0.53	9.48±0.48	N
	10	7.04±0.07	11.79±0.22	13.56±0.50	9.73±0.25
2.5	5	4.99±0.01	5.27±0.57	9.16±0.17	N
	10	5.40±0.51	8.54±0.50	10.00±0.00	7.48±0.51
1.25	5	4.74±0.44	4.51±0.45	8.14±0.12	N
	10	4.41±0.41	6.37±0.54	7.10±0.09	6.02±0.28
0.63	5	N	4.28±0.38	6.00±0.00	N
	10	4.55±0.40	5.37±0.54	6.27±0.35	5.84±0.35
0.32	5	N	N	5.07±0.06	N
	10	N	5.21±0.48	5.56±0.44	5.40±0.41
0.16	5	N	N	0.00	N
	10	N	4.96±0.17	5.54±0.37	4.91±0.13
0.08	5	N	N	N	N
	10	N	N	N	N
0.04	5	N	N	N	N
	10	N	N	N	N
0.02	5	N	N	N	N
	10	N	N	N	N

Fig. 6. Zone of inhibition of RP131-155 against S. aureus

IX. DISCUSSION

A large number of microbes have in the past few decades evolved to become resistant to currently used antibiotics. To worsen the case, some of the antibiotics that are still

considered more potent like amphotericin B are excessively toxic thus limiting their usage amongst patients receiving other therapies with toxic drugs e.g. anti-cancer therapy. As such, this issue has become a cause for global concern prompting a more rigorous research into alternative drugs that could potentially replace or be used synergistically with the available antibiotics. However, developing an alternative to antibiotics represent one of the greatest challenges of modern medicine. Antimicrobial peptides are considered as excellent candidates for circumventing this problem since they are rarely resisted by microbes which they are naturally exposed to and also show low toxicity to mammalian cells. Several strategies abound for the therapeutic application of AMPs including use as: immune-stimulatory agents to boost innate immunity, endotoxin neutralizing agents, single anti-infectives or even used synergistically with conventional antibiotics. AMPs work against microbes primarily through membrane permeation resulting in an efflux of nutrients and ions. The molecular pathways and mechanisms for permeation of different peptides vary mostly due to their amino acid sequence, peptide concentration and membrane lipids [14]. AMPs have in the past been isolated from numerous sources such as frogs, plants and microorganisms. More modern approaches have been developed to identify AMPs and as well reduce the long hours (weeks or months) spent on identification, isolation, purification and characterization of active AMPs from natural sources. One such methodology is to analyze genes and proteins in silico to determine or predict peptides that could potentially be AMPs by sequence similarity matches with naturally occurring ones like what is done by APD. Also antimicrobial assays were used to evaluate the antimicrobial activity.In this study, the disk diffusion method was used to determine the antimicrobial activity of RP131-155.

In the current study, disk diffusion method is used to determine the antimicrobial activity of RP131-155. This study appears to be the first study where disk diffusion method is used for proven the antimicrobial activity of RP131-155. Antimicrobial activity of RP131-155 was evaluated based on the diameters of clear inhibition zone surrounding the paper disks, and where there is no inhibition zone; it is assumed that there is no antimicrobial activity. Table 3 showed the antimicrobial activity of RP131-155 against E. coli, E. auergene, S. arizonae and S.aureus. With regards to the diameter of the inhibition zone, 10µl of the different concentrations (10.0mg/ml, 5.0mg/ml, 2.50mg/ml, 1.25mg/ml, 0.63mg/ml, 0.32mg/ml and 0.16mg/ml) of RP131-155 demonstrated effective inhibition on the growth of these bacteria when compared to 5µl of similar concentrations. However, the concentrations 0.08mg/ml, 0.04mg/ml and 0.02mg/ml have no effect against these bacteria at both 5µl and 10µl volume. The average size of inhibition zones varied from 10.00 to 5.07mm against E. coli at a volume of 5µl while at an increase volume of 10µl, the inhibition zone varied from 17.24 to 5.54mm; 7.48 to 4.28mm against E. auergene at a volume of 5µl while at an increase volume of 10µl, the inhibition zone varied from 14.56 to 4.96mm; 7.05 to 4.74 mm against S. aureus at a volume of 5µl while at an increase volume of 10µl, the inhibition zone varied from 9.04 to 4.55 mm; there was no inhibition zones observed for S. arizonae at a volume of 5µl while at an increase volume of 10µl the inhibition zone varied from 10.69 to 4.91mm (Figure 4 and Figure 5). This showed that inhibition zone sizes increased at a higher concentration and volume, indicating that RP131-155 was more effective at higher concentrations and volume. From Figure 5, the MIC of tested peptide “RP131-155” against E. coli, E. aerogenes and S. arizonae is between 0.16-0.08mg/ml whilst that of S.aureus is between 0.63-0.32mg/ml. In this case, there is no exact MIC which can be performed due to that the concentration diffusion taken between 0.16-0.08mg/ml and 0.63-0.32mg/ml is insufficient to identify the specific the latest inhibition zones.

According to the results obtained (Figure 4 and Figure 5), a higher inhibition zone was observed for both 10µl and 5µl of RP131-155 against E. coli bacteria. This could be due to the fact that, E. coli is a gram negative bacterium containing a single layer of peptidoglycan surrounded by the outermembrane, while S. aureus is gram positive bacteria having several layers of peptidoglycan in their cell wall. However, S. arizonae and E. aerogene are also a gram negative bacterium but the disk diffusion results for this bacterium is not similar to that of E. coli. In the present study, RP131-155 was effective at higher concentrations against Escherichia coli, Enterobacter aerogenes, Salmonella arizonae and Staphylococcus aureus, this was demonstrated by observing the clear inhibition zone obtained.

X. Conclusion

Different concentrations of RP131-155 were used to demonstrate antimicrobial susceptibility against E. coli, S. aureus, E. auergene and S. arizonae, where RP131-155 is more effective against E. coli. Also, the APD prediction interface indicates that RP131-155 has antimicrobial properties, only 25 peptides have the best chance of being antimicrobial peptides.

References

[1] A. d. J. Sosa, D. K. Byarugaba, C. F. Amábile-Cuevas, P.-R. Hsueh, S. Kariuki, and I. N. Okeke, *Antimicrobial resistance in developing countries*: Springer, 2010.

[2] A. J. Alanis, "Resistance to antibiotics: are we in the post-antibiotic era?," *Archives of medical research*, vol. 36, pp. 697-705, 2005.

[3] J. Davies and D. Davies, "Origins and evolution of antibiotic resistance," *Microbiology and molecular biology reviews*, vol. 74, pp. 417-433, 2010.

[4] A. Giuliani, G. Pirri, and S. Nicoletto, "Antimicrobial peptides: an overview of a promising class of therapeutics," *Open Life Sciences*, vol. 2, pp. 1-33, 2007.

[5] Y. Li, Q. Xiang, Q. Zhang, Y. Huang, and Z. Su, "Overview on the recent study of antimicrobial peptides: origins, functions, relative mechanisms and application," *Peptides*, vol. 37, pp. 207-215, 2012.

[6] J. M. Andrews, "Determination of minimum inhibitory concentrations," *Journal of antimicrobial Chemotherapy*, vol. 48, pp. 5-16, 2001.

[7] L. B. Reller, M. Weinstein, J. H. Jorgensen, and M. J. Ferraro, "Antimicrobial susceptibility testing: a review of general principles and contemporary practices," *Clinical infectious diseases*, vol. 49, pp. 1749-1755, 2009.

[8] S. A. González-Chávez, S. Arévalo-Gallegos, and Q. Rascón-Cruz, "Lactoferrin: structure, function and applications," *International journal of antimicrobial agents*, vol. 33, pp. 301. e1-301. e8, 2009.

[9] M. V. Botuyan, A. Toy-Palmer, J. Chung, R. C. Blake II, P. Beroza, D. A. Case, *et al.*, "NMR Solution Structure of Cu (I) Rusticyanin fromThiobacillus ferrooxidans: Structural Basis for the Extreme Acid

Stability and Redox Potential," *Journal of molecular biology*, vol. 263, pp. 752-767, 1996.
[10] M. L. Barrett, I. Harvey, M. Sundararajan, R. Surendran, J. F. Hall, M. J. Ellis, *et al.*, "Atomic resolution crystal structures, EXAFS, and quantum chemical studies of rusticyanin and its two mutants provide insight into its unusual properties," *Biochemistry*, vol. 45, pp. 2927-2939, 2006.
[11] L. D. Kanbi, S. Antonyuk, M. A. Hough, J. F. Hall, F. E. Dodd, and S. S. Hasnain, "Crystal structures of the Met148Leu and Ser86Asp mutants of rusticyanin from Thiobacillus ferrooxidans: insights into the structural relationship with the cupredoxins and the multi copper proteins," *Journal of molecular biology*, vol. 320, pp. 263-275, 2002.
[12] S. Hogg, *Essential microbiology*: John Wiley & Sons, 2013.
[13] P. Tripathi, A. Beaussart, G. Andre, T. Rolain, S. Lebeer, J. Vanderleyden, *et al.*, "Towards a nanoscale view of lactic acid bacteria," *Micron*, vol. 43, pp. 1323-1330, 2012.
[14] B. M. Peters, M. E. Shirtliff, and M. A. Jabra-Rizk, "Antimicrobial peptides: primeval molecules or future drugs?," *PLoS pathogens*, vol. 6, p. e1001067, 2010.