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Safety Evaluation of Secretome Proteins from *Paenibacillus polymyxa* Kp10 and *Lactococcus lactis* Gh1 as the Potential Antimicrobial Therapeutic Agent

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Received: 22/11/2022
Accepted: 16/2/2023
Published: 31/7/2023

Keywords:

Secretome proteins,
Paenibacillus
polymyxa Kp10,
Lactococcus lactis
Gh1, Safety
evaluation and
Antibiotic resistance

Abstract

The secretome proteins of *Paenibacillus polymyxa* Kp10 and *Lactococcus lactis* Gh1 were previously found to have the potential for controlling the antibiotic-resistant pathogen. Therefore, their safety evaluation to ensure consumer health is deemed important. This study was to evaluate the toxicity effect of both secretome proteins in human cells against Medical Research Council cell strain 5 (MRC5). Then, their antibacterial activities were tested in human serum to assess the stability of both secretome proteins. The results showed no cytotoxic effects of either secretome protein when MRC5 cells were treated up to the determined concentrations. Therefore, no IC₅₀ was determined. In addition, human serum did not affect the antibacterial activity of both secretome proteins against Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE). In conclusion, both secretome proteins are potentially safe for future development as a potential therapeutic agent.

1. Introduction

Today, the uncontrolled use of conventional antibiotics has caused various problems in the medical field, resulting in the widespread emergence of antibiotic resistance worldwide. Vancomycin-resistant *Enterococcus* (VRE) and Methicillin-resistant *Staphylococcus aureus* (MRSA) are prominent antibiotic-resistant bacteria. They have resulted in significant patient deaths and financial burdens on healthcare systems (Ventola, 2015; Dadgostar, 2019; Zainal Baharin *et al.*, 2021). In addition, antibiotic resistance poses a significant risk to current medical advances (Ventola, 2015; Zainal Baharin *et al.*, 2021; Golkar, Bagasra & Pace, 2014). The efficacy of conventional antibiotics has dramatically deteriorated over time, and more effective therapeutics against infections caused by antibiotic-resistant bacteria are urgently needed (Golkar, Bagasra & Pace, 2014; Sengupta, Chattopadhyay & Grossart, 2013; Wright, 2014). Several studies are searching for alternative compounds that could replace existing antibiotics.

Secretome protein is known as an antimicrobial substance that has the ability to inhibit the growth of bacteria (Damayanti, Rusdiana & Wathoni, 2021) by releasing antimicrobial peptides (AMPs) such as cathelicidin, RNase3, human β -defensins, and

calprotectin (Kasiri *et al.*, 2016). Secretome proteins appear to have enormous potential to replace the role of antibiotics (Damayanti, Rusdiana & Wathoni, 2021). Generally, AMPs released by secretome proteins act as host defences, where many of them have been isolated from living entities, such as bacteria, animals, plants, and fungi (Zainal Baharin *et al.*, 2021; Kumar, Kizhakkedathu & Straus, 2018). In previous studies, secretome proteins from *Paenibacillus polymyxa* Kp10 (Kp10) and *Lactococcus lactis* Gh1 (Gh1) were shown to display antimicrobial activity and could potentially replace antibiotics (Zainal Baharin *et al.*, 2022; Mokhtar *et al.*, 2020; Jawan *et al.*, 2021). A recent study showed that Gh1 exhibited antimicrobial activity against pathogenic *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* and *Bacillus cereus* species (Jawan *et al.*, 2020), while Kp10 was shown to demonstrate antimicrobial activity against *Escherichia coli* (Mokhtar *et al.*, 2020).

Our recent study has shown that secretome proteins of Kp10 and Gh1 have antibacterial activity against MRSA and VRE (Zainal Baharin *et al.*, 2022). Given their potential as an alternative antibiotic, we further conducted a preliminary safety evaluation on both the secretome proteins by using agar well diffusion assay in the presence of serum for their stability test and cytotoxicity test in MRC5 cells. In common practice,

registered pharmaceutical products are tested for safety and quality by the Ministry of Health through the Pharmaceutical Services Division (Department of Standards Malaysia, 2012). This coincides with the concept of *Halalan Toyyiban*, according to which Allah has commanded us to eat food that is not only halal but also wholesome (Hamdan & Hashim, 2022). Based on the great potential found in both secretome proteins of Kp10 and Gh1, the proteins are expected to be stable in the presence of human serum, and there will be no cytotoxicity effect when tested on human cells. Therefore, the safety assessments conducted in this study would fulfil the halal aspect that the secretome proteins are safe for humans and can be further developed as the potential therapeutic agent.

2. Materials and methods

2.1 Bacterial culture, growth, and cell conditions

Kp10 and Gh1 isolates producing secretome proteins were obtained from Professor Dr. Arbakariya Ariff of the Bioprocessing and Biomanufacturing Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Kp10 was cultured in M17 broth at 37°C while Gh1 were cultured in de Man Rogosa and Sharpe (MRS) broth at 30°C (Mokhtar *et al.*, 2020; Kasimin *et al.*, 2020). For Medical Research Council cell strain 5 (MRC5), the cells were obtained from UPM-MAKNA Cancer Research Laboratory (CanRes) Institute of Bioscience, Universiti Putra Malaysia and were cultured in Dulbecco's Modified Eagle Medium (DMEM) broth at 37°C. Two antibiotic resistant bacteria used in this study, Methicillin resistant *S. aureus* (MRSA) ATCC 700699 and Vancomycin resistant *Enterococci* (VRE) ATCC 700221 were obtained from the American Type Culture Collection (ATCC). The inoculates of MRSA and VRE were prepared using the colony suspension method, in which colonies were picked from cultures previously grown on Mannitol Salt Agar (MSA) and sheep blood agar for 24 hrs at 37°C (Arjyal, Kc & Neupane, 2020; Özsoy & Arzu, 2017), and transferred to Brain Heart Infusion (BHI) broth before incubating for 24 hrs at 37°C. All media were purchased from Oxoid, UK, except for DMEM, purchased from Thermo Fisher Scientific, USA.

2.2 Preparation of secretome proteins from cell-free culture supernatants of Kp10 and Gh1

Secretome proteins of Kp10 and Gh1 were isolated and purified from the bacteria's cell-free culture supernatant using a modified version of the ammonia sulphate precipitation technique (Duong-Ly & Gabelli, 2014). First, Kp10 and Gh1 were cultivated for 24 hrs at 37°C in M17 and MRS broth, respectively. Then, 1000 mL of a suspension of cultivated bacteria was aliquoted into 50 mL falcon tubes and centrifuged at 10,000 x g for 15 mins at 4°C. With the use of a magnetic stirrer, all cell-free supernatants were mixed into a sterile bottle, and 567 g (NH₄)₂SO₄ was added until it was dissolved (Thermo Fisher Scientific, USA). After that, the solvents were aliquoted into 50 mL tubes and incubated at 4°C overnight. After incubation, the tubes underwent a 15-minute, 10,000 x g centrifugation at 4°C. Upon removing the supernatant, the pellet was dissolved with distilled water and kept overnight at 4°C. Finally, all the tube contents were combined into one sizable beaker to prepare the inoculum.

2.3 Agar well diffusion assay for antibacterial activity and serum stability test

Modified agarose diffusion assay detected the antibacterial activities and serum stability test of secretome proteins from Kp10 and Gh1. Briefly, a single colony of each MRSA and VRE was grown in trypticase soy broth (TSB, 30 g L⁻¹) overnight at 37°C under aerobic conditions. 2 x 10⁸ CFU mL⁻¹ of the bacterial culture of each strain was added to warm (50–55°C) sterile agarose [1% agarose (low EEO, Sigma, St. Louis, MO), 0.03% nutrient broth, and 10 mM Phosphate Buffer Saline (PBS), pH 7.4 (1:100 v/v)]. For antibacterial activity, 10 µL samples of secretome proteins from Kp10 and Gh1 were added to 3 mm wells punched by agar punch (BioRad Laboratories, Hercules, Canada). Then, 0.2 mol L⁻¹ sodium acetate (solvent) and 20000 IU penicillin-streptomycin solution of the same volume were added as a negative and positive control, respectively. After overnight incubation at 37°C, the diameter of each clean zone of growth inhibition was measured, indicating the antibacterial activity of Kp10 and Gh1 against MRSA and VRE strains. A stock of human serum was obtained from the Haematology Laboratory, Faculty of Medicine and Health Science UPM for the stability test. Then, the secretome proteins were diluted in PBS or human serum for sample preparation at a different minimum inhibitory concentration (MIC). Then, 10 µL of each sample was again added to 3 mm wells punched by agar punch (BioRad Laboratories, Hercules, Canada). Human serum without the addition of secretome proteins served as a negative control. The inhibition zone was measured after 24 hrs incubation at 37°C.

2.4 Determination of Minimum Inhibitory Concentration (MIC)

The Resazurin-based 96-well plate microdilution method was used to determine the MIC of each secretome protein (Elshikh *et al.*, 2016). Resazurin was prepared in distilled water at 0.02% (wt/vol), sterilised by filtration, and stored at 4°C for up to two weeks after preparation. The direct colony suspension method was used to prepare the organism's saline suspension at the McFarland 0.5 turbidity standard density, which corresponded to 1-2 x 10⁸ CFU/ml. Plates were prepared aseptically, and a sterile 96 well plate was properly labelled. A volume of 100 µl of the test material was pipetted into the first row of the plate (Well 1). In the case of the other wells, 50 µl of Muller Hinton broth (MHB) was added (Wells 2-12). Serial dilution was performed using a multichannel pipette, starting from Well 1 and finishing at Well 10. Well 11 and 12 were used as negative control. The tested concentrations of the different samples were achieved through doubling serial dilution. Finally, 1 µl of tested bacteria was added to each well. Following overnight incubation at 37°C, 30 µl of resazurin (0.02%) was added to all wells. This was followed by further incubation for 2-4 hrs to observe any colour change. The MIC is defined as the lowest concentration of the test material to inhibit growth. Columns with no colour change (the blue resazurin colour remained unchanged in no growth condition) were scored as MIC value after incubation. The presence of pink and purple colours indicated growth. The test was run in triplicate.

2.5 Cytotoxicity assays

MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was used to determine the potential cytotoxicity of the secretome proteins. Cell culture with 2 x 10³ cells/ml concentration was prepared and plated (100 µl/well) onto 96-well plates. The diluted ranges of sample extracts (100, 50, 25, 12.5, 5, 1, 0.5 (µg/ml)) were added to each

well and further incubated for 72 hrs. MTT solution was added to the cell suspension by the end of incubation and continued for another 3-hrs incubation. After the solubilisation of the purple formazan crystals using Dimethyl sulfoxide (DMSO) was completed, the Optical Density (OD) of the plant extract was measured using an ELISA reader at a wavelength of 570 nm. The cytotoxicity was recorded as the drug concentration causing 50% growth inhibition of the cells (IC₅₀ value) using the formula (Zahra *et al.*, 2020) given below. After determining the percentage of cell viability, graphs were plotted with the percentage of cell viability against their respective concentrations. The test was run in triplicate.

3. Results and discussion

3.1 Antibacterial activity and the stability test of Kp10 and Gh1 secretome proteins against antibiotic resistant pathogens

MRSA and VRE were observed to have developed resistance to many antibacterial agents, most notably vancomycin, which was considered one of the last treatment options for MRSA and VRE infections, complicating treatment (Liu *et al.*, 2011). This issue has sparked worldwide concern because resistance to many available conventional antimicrobial agents makes treating bacterial infections difficult. Many deaths have been associated with VRE, which was first identified in the mid-1980s, and MRSA in 1961s; both have since then spread rapidly to become a major health threat all over the world (Zainal Baharin *et al.*, 2021; Cetinkaya, Falk & Mayhall, 2000). The antibacterial activity of secretome proteins derived from Kp10 and Gh1 against MRSA and VRE, as demonstrated in our previous study (Zainal Baharin *et al.*, 2022), may highlight a new potential in overcoming the problem of antibiotic resistance.

Their antimicrobial susceptibility was tested using agar well diffusion method and MIC to demonstrate the potential use of the Kp10 and Gh1 secretome proteins. Both secretome proteins were active against both MRSA and VRE strains as shown in Figure 1 and 2. The secretome protein of Kp10 recorded the highest inhibition zone of 21.3 mm against MRSA, and 17.0 mm was recorded against VRE. Meanwhile, the secretome protein of Gh1 also recorded the highest inhibition zone of 18.0 mm against MRSA and the least inhibition zone of 6.0 mm against VRE. Secretome protein of Kp10 gave MIC of $0.1563 \pm 0 \mu\text{g/ml}$ for MRSA and MIC of $0.3648 \pm 0 \mu\text{g/ml}$ for VRE, whereas the secretome protein of Gh1 gave MIC of $2.95325 \pm 0 \mu\text{g/ml}$ for MRSA and MIC of $1.47662 \pm 0 \mu\text{g/ml}$ for VRE. These results show the antibacterial potentials of the secretome proteins as the new agents to combat antimicrobial resistance.

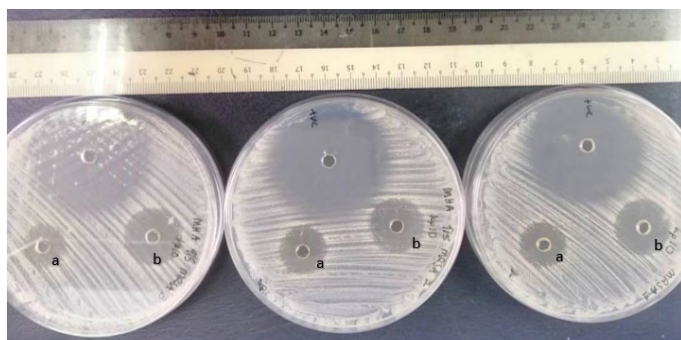


Figure 1: Agar well diffusion assay of secretome proteins extract of Gh1 (a) and Kp10 (b) against MRSA as indicator strain. Assay was conducted in triplicate.

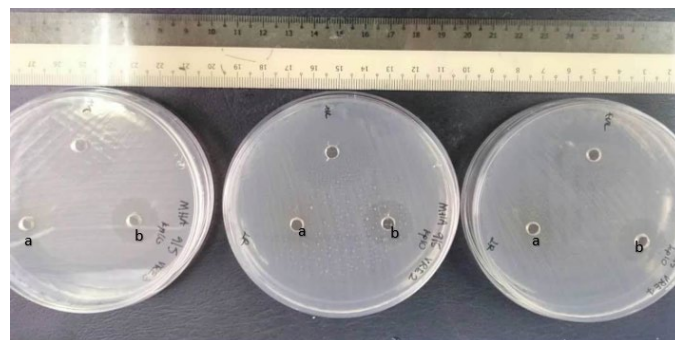


Figure 2: Agar well diffusion assay of secretome proteins extract of Gh1 (a) and Kp10 (b) against VRE as indicator strain. Assay was conducted in triplicate.

Meanwhile, in developing new drugs, the stability and safety of the proposed therapeutic agent must first be established (Kang & Lee, 2009). Concerns have been raised regarding their stability in human serum, although peptides released by secretome proteins are generally considered rather stable in many test assays (Jenssen & Aspmo, 2008). In this study, the preliminary test on the effect of human serum on the antibacterial activity of Kp10 and Gh1 secretome proteins was done using the agar well diffusion method at different concentrations of MIC values. The results are summarised in Table 1 and 2. There were no significant changes in the inhibition zone of secretome proteins in the presence and absence of the human serum within 24 hrs of incubation.

Therefore, we concluded that both secretome proteins were not interfered or affected by human serum, reflecting some safety features. This also gives an initial impression that the protein is safe to use in the human body as it does not interact with serum. However, more detailed studies, such as using HPLC and other related tests, should be carried out to validate the accuracy and efficacy of the treatment (Jenssen & Aspmo, 2008).

3.2 Cytotoxicity study of Kp10 and Gh1 secretome proteins on human cells, MRC5

Interest in the pharmacological effects of antimicrobial peptides on antibiotic resistant pathogens has increased dramatically over the past few years. Secretome proteins containing AMPs have been shown to possess numerous antibacterial activities through different cytotoxic effects against bacterial cells without exhibiting considerable damage to normal host cells (Lei *et al.*, 2019). Our observations on toxicity to MRC5 cells showed that the secretome proteins derived from Kp10 and Gh1 showed no toxicity to MRC5. This highlights the notion that the secretome proteins of Kp10 and Gh1 are not harmful to humans and can serve as potential therapeutic agents.

Nevertheless, it has been reported elsewhere that high concentrations of AMPs may cause severe toxicity to normal tissues, thus causing a significant side effect (Ventola, 2015; Mohammad, Thangamani & Seleem, 2015). More studies are therefore required to find the optimum range for human use. This study studied the inhibitory effect of Kp10 and Gh1 secretome proteins on MRC5 cells at different concentrations for 72 hrs. Results, as shown in Figure 3, suggested that no cytotoxic effect of secretome protein derived from Kp10 and Gh1 was observed when treated on MRC5 cells up to the identified concentrations. Hence, the IC₅₀ was not determined

Table 1: Serum stability test of Kp10 and Gh1 against MRSA
(Data presented as mean $\pm$ standard deviation values of triplicate measurements)

Type of AMPs	Zone of inhibition on negative control (Human serum) (mm)	Zone of inhibition on 0.5 X MIC against MRSA (mm)	Zone of inhibition on 0.5 X MIC against MRSA with Human Serum (mm)	Zone of inhibition on 1 X MIC against MRSA (mm)	Zone of inhibition on 1 X MIC against MRSA with Human Serum (mm)	Zone of inhibition on 2 X MIC against MRSA (mm)	Zone of inhibition on 2 X MIC against MRSA with Human Serum (mm)
Kp10	-	12	11	21	22	40	42
Standard deviation	-	1	1	0.5	0.5	1	1
Gh1	-	9	9	17	18	30	29
Standard deviation	-	0.5	0.5	0	1	1	1

Table 2: Serum stability test of Kp10 and Gh1 secretome proteins against VRE
(Data presented as mean $\pm$ standard deviation values of triplicate measurements)

Type of AMPs	Zone of inhibition on negative control (Human serum) (mm)	Zone of inhibition on 0.5 X MIC against VRE (mm)	Zone of inhibition on 0.5 X MIC against VRE with Human Serum (mm)	Zone of inhibition on 1 X MIC against VRE (mm)	Zone of inhibition on 1 X MIC against VRE with Human Serum (mm)	Zone of inhibition on 2 X MIC against VRE (mm)	Zone of inhibition on 2 X MIC against VRE with Human Serum (mm)
Kp10	-	14	14	16	18	20	20
Standard deviation	-	1	0.5	0	1	1	1
Gh1	-	-	-	2	2	4	3
Standard deviation	-	-	-	1	0	1	0.5

in the tested secretome proteins. Thus, Kp10 and Gh1 secretome proteins that can induce apoptosis in MRSA and VRE (Zainal Baharin *et al.*, 2022) could be a potential therapeutic agent with a safety feature. Further studies *in vivo* environment and clinical trials need to be conducted to establish Kp10 and Gh1 secretome protein as safe agents for antibiotic resistance treatment.

4. Conclusion

All in all, data from this study is of interest, as it suggests that both secretome proteins of Kp10 and Gh1 are nontoxic and have safety characteristics on human cells, which fulfil the *Halalan Toyyiban* principle to be applied as therapeutic agents for antibiotic resistant pathogens. As Muslims, we comply that curing diseases is in the power of *Allah*. However, we must put

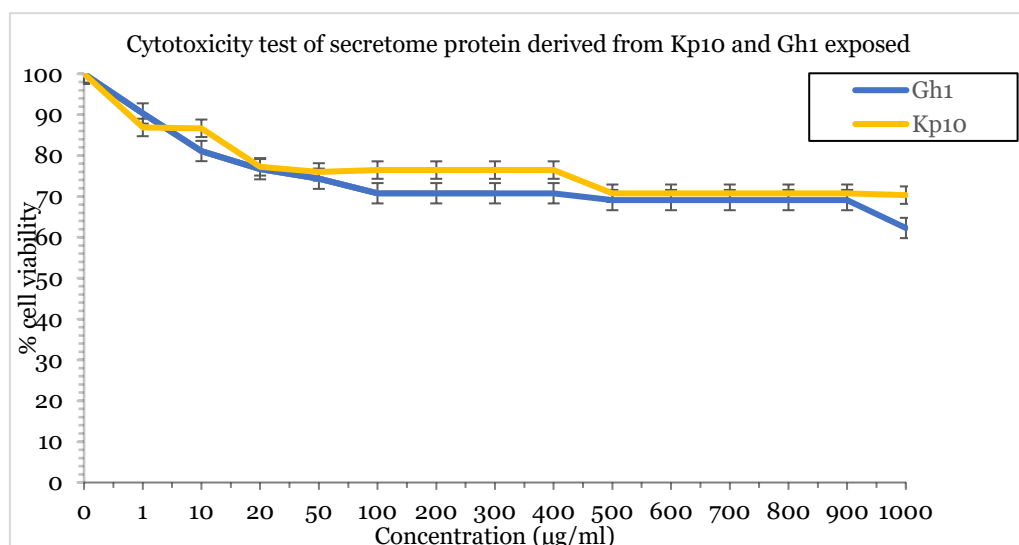


Figure 3: Cytotoxicity test to analyse the effect of secretome proteins of Kp10 and Gh1 on MRC5 cells after 72 hrs exposure. (Data presented as mean $\pm$ standard deviation values of triplicate measurements. There is no IC₅₀ determined in tested secretome proteins).

an effort to find a cure that does not contradict Islamic principles. The materials used as medicine must also be in accordance with Islamic philosophy, that is, clean and harmless.

To align with the halal concept, apart from being absence of haram elements, the safety assessment of the selected agent is also an important criterion before being used for human consumption. In this study, the safety status of the secretome proteins was furnished by the cytotoxicity and serum stability tests. We did not observe any effect of cytotoxicity of the secretome proteins on human cells. In addition, we also found that the serum did not affect the antibacterial activity of both secretome proteins. Further improvements are warranted to use the proteins, including the halal requirement. For the later, avoiding materials of non-animal origin in the process of the product may encourage the production of halal pharmaceutical commodities for medicinal use not only dedicated for use among Muslims who are concerned about the use of haram animal derivatives but this innovation can also benefit the public globally regardless of religion.

Acknowledgement

The authors are thankful to the Ministry of Higher Education, Malaysia (MOHE), for granting a Fundamental Research Grant Scheme (FRGS) (FRGS/1/2017/SKK11/UPM/01/1) for financial support. Special thanks to Halal Products Research Institute, Universiti Putra Malaysia, for providing the equipments, chemicals and instruments for this research.

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