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Targeting Intracellular *S. aureus* in Infection Modelling by Ikarugamycin Compound and their Toxicity toward Bovine Mammary Epithelial Cells

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Abstract: *Staphylococcus aureus* is a versatile pathogen causing a wide variety of disease .In animals these bacteria is main causative agent of bovine mastitis ,leading to huge economic loose in diary industry. Beside the development of antibiotic resistance ,the intracellular survival of *S.aureus* within udder cells has rendered many antibiotic ineffective, leading to therapeutic failure .Therefore, the study aims to investigate the antibacterial activities of the Ikarugamycin(IKA) compound against intracellular *S. aureus* and determine the cytotoxicity of the compound toward bovine mammary epithelial cells (Mac-T cells). Minimum inhibitory concentration (MIC) was used to determine the antibacterial activity of IkA and Mac-T cells were infected with *S. aureus* using *in-vitro* infection models' assay. Ikarugamycin intracellular antibacterial activity assays were used to determine the bactericidal activity of IkA against intracellular *S. aureus*. The cytotoxicity of IkA against Mac-T cells was evaluated using the Alamar blue assay. We showed that *S. aureus* is susceptible to Ikarugamyicin with MIC value of 0.6 μg/mL. Ikarugamycin at 4 MIC and 8MIC have bactericidal activity by reducing 3 and 5 logs 10 CFU/ml of *S.aureus* in the first six-hour respectively. Moreover, the Ikarugamycin compound possesses intracellular killing activity, at 5 μg/mL killed 90% of intracellular *S. aureus*. The concentration of Ikarugamycin that inhibits 50% of Mac-T cells (IC50) was 9.2 μg/mL; therefore, the IC50 is far from the concentration required to kill 90% of intracellular *S aureus*. The study highlighted that Ikarugamycin antibiotic could be used to deal with infections caused by intracellular and multi-drug resistance *S. aureus* in the case of mastitis.

Keywords: antimicrobial resistance; intracellular bacteria; *S. aureus*; mastitis; Ikarugamycin compound; antimicrobials

1. Introduction

Staphylococcus aureus is one of the major pathogenic agents of bovine mastitis in lactating dairy cows. [1]. It is considered the most frequent disease, leading to huge economic loss in dairy industries due to the reduction of milk production, increased death and culling rates, and increased treatment costs. [2,3].The disease is also associated with profound welfare issues due to the associated morbidity and impact on livestock health [2,3]. Antibiotics administration is considered the most common strategy for treatment and control of bovine mastitis [4]. However, the use of antibiotics has become less effective due to the development of antibiotic resistance against common

antibiotics used for mastitis treatment [5][6][4]. Also, S. aureus can invade and survive inside udder cells [7], which are more challenging to combat due to the restriction of their contact with antibiotics. This has been associated with poor treatment outcomes in S. aureus mastitis, where the percentage of cure using currently approved antibiotics is approximately 10–30% [5]. Intracellular S.aureus causes mastitis is less susceptible to common conventional antimicrobial agents such β-lactams, aminoglycosides, macrolides, and fluoroquinolones due to the inability to penetrate and accumulate in the mammalian cells [8]. The delivery of antibacterial into desired locations in the body is one of the main challenges for successful therapeutics; antimicrobial need to cross the host cell membranes either through diffusion or endocytosis. More than two-thirds of antibiotics fail against intracellular pathogens. The presence of S. aureus in cells, therefore, provides privileged reservoirs from which re-infection can occur [9] resulting in long-term and repeated infection[10]. The intracellular survival strategies of S. aureus are associated with the subclinical and relapsing infection of bovine mastitis[11]. The facultative intracellular parasitism and biofilm production of S. aureus, therefore, protects them from host immune responses and the effect of antibiotics [12], and this poses huge treatment challenges for the global public and livestock health. This has thus necessitated the need for better antimicrobials with an impact on mechanistic endocytic uptake (such as ikarugamycin) to achieve therapeutic intracellular level [13].

Ikarugamycin (IKA) is a natural product antimicrobial agent (with both antibiotic and antiprotozoal activity) that was first isolated from cultures of *Streptomyces phaeochromogenes subsp. ikaruganensis* [14]. A wide range of biological properties of IKA has been reported include antimicrobials activity[15], antiprotozoal[14] (antitumor [16], immune regulation, and cytotoxic properties[17]. There are however limited data on the antibacterial properties and the potential benefit in the treatment of intracellular infection in the case of *S. aureus* associated bovine mastitis. This study was therefore we carried out to investigate the potential antibacterial activity of IKA against intracellular *S. aureus* in infection model of bovine mammary epithelial cells (Mac-T cells) and to assess the potential cytotoxicity.

1. Results

1.1. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration was used to evaluate the antimicrobial susceptibility of *S. aureus* to Ikarugamycin. The result indicates that the S. aureus with susceptible to Ikarugamyicin with MIC value 0.6 μg/ml and 5 μg/ml MBC value.

1.2. Time kills Assay

The results of the time curves assay show that the Ikarugamycin has bactericidal activity against *S. aureus* (reducing more than 3 $\log_{10}$) in initial time at 3 and 4 MIC. Ikarugamycin at 0.6 μg/ml inhibited bacteria's growth in 3 and 6 hours and at 2.5, and 5 μg/ml shows a bactericidal effect

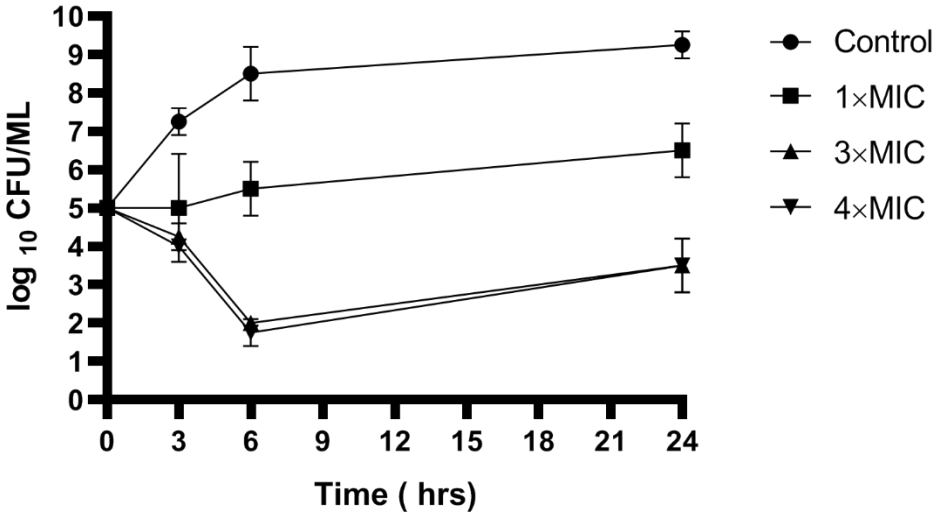

Figure 1. Time – Kill curves of S. aureus incubated with Ikarugamycin for 24 h at 37 °C. Each point represents the mean ± standard deviation. Measurements were performed in triplicates.

1.3. Invasion of MAC-T cells by S. aureus

To test the antibacterial activity of Ikarugamycin against intracellular S. aureus, and in vitro infection modeling of Mac-T cells by S. aureus were done using an Intracellular invasion assay. Two isolates were confirmed to invade Mac-T cells as indicated by the increase of survival of S. aureus after gentamicin explore and lysis Mac-T cells. Lysis of Mac-T cells released 10^5 CFU/ml of *S.aureus* 15 AL **(A)** , and 10^3cfu/ml of F3 3D **(C).**

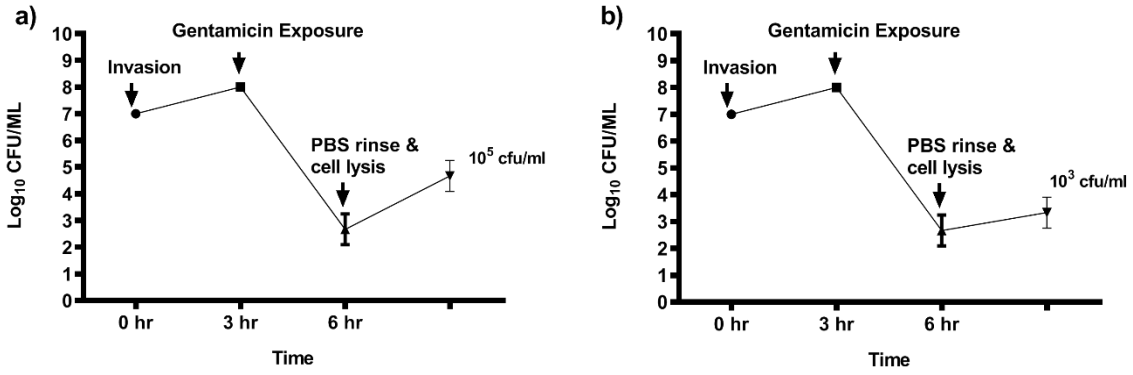

Figure 2. Figure Intracellular survival assay of S. aureus in Mac-T cells. Following gentamicin exposure, lysis of Mac-T cells released approximately a) 105 CFU/mL of S. aureus 15AL (a) and 103cfu/ml of F3 3D. (b).

1.4. Bactericidal Activities of Ikarugamycin against Intracellular Staphylococcus aureus

To determine the antibacterial activity of Ikarugamycin against intracellular *S. aureus*, bovine mammary epithelial cells were infected with *S. aureus* by using Gentamicin protection assay. Infected MAC-T cells were treated with Ikarugamycin with different concentrations (0.6, 2.5, 200, and 5 μg/ ml) for 3 hours for the Ikarugamycin to enter into the host cells. The Ikarugamycin was removed and the cells lysed to calculate the CFU of surviving intracellular bacteria. The results indicated that Ikarugamycin at 5 μg/ ml killed between 85 to 90 % of intracellular *S. aureus* (Figure (4))

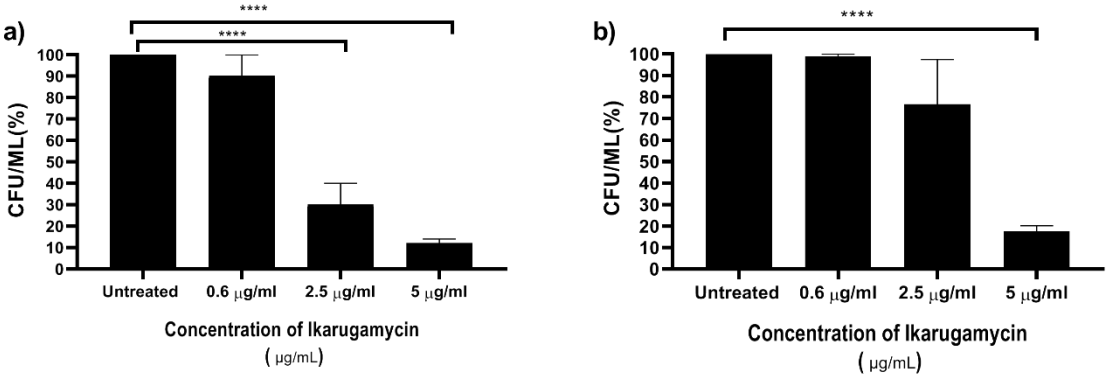

Figure 3. Antibacterial activity of IKA against intracellular S. aureus 15 AL(A) and S. aureus F53B(B), MAC-T cells infected with S. aureus then exposure to different concentration of GO, untreated infected cells were used as a control to establish cfu value.

1.5. Cytotoxicity Assay

Toxicity test was performed using Resazurin reduction assay, and the test is based on measuring the metabolic activity of cells. The viable cells with metabolic activity can reduce resazurin (deep blue color in solution) into the resorfin product, pink, and fluorescence. Our results highlighted that the concentration of Ikarugamycin that inhibits 50% of MAC-T cells (IC50) was 9.2

μg/mL; therefore, the IC50 is far from the concentration required to kill 90% of intracellular S. aureus in Mac-T cells.

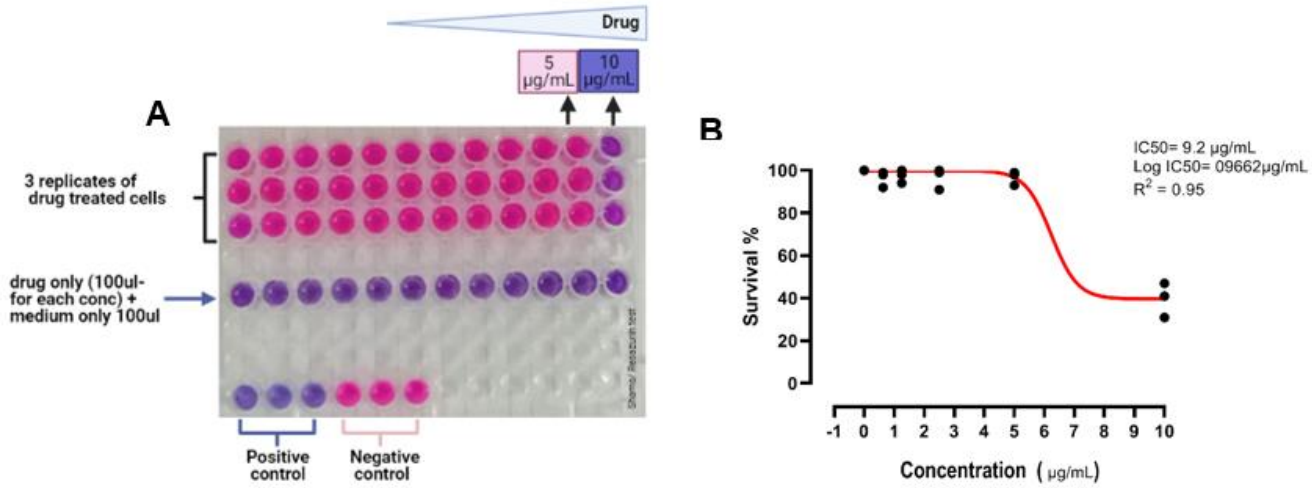

Figure 4. A) 96-well plate showing resazurin reduction assay after 48 hours. B) A dose-response curve showing the IC50 of Ikarugamycin toward MAC-T cells.

2. Discussion

Staphylococcus aureus mastitis is challenging to combat using conventional antibiotics and antimicrobials due to the development of antimicrobial resistance, the ability of the bacteria to invade and hide in the host cells, and biofilm formation. In this present study, we investigate the antibacterial activities of the Ikarugamycin compound against intracellular *S. aureus* and determine the cytotoxicity of the compound toward bovine mammary epithelial cells (MAC-T cells). IKA has antibacterial activity against *S. aureus* when the MIC was 0.6 μg/mL while at 4 MIC and 8 MIC, it has anti bactericidal activity. Besides, IKA possesses considerable intracellular antibacterial activity at 5 μg/mL killing 90% of intracellular *S. aureus.* This is similar to previous studies about the biologically active nature of the IKA, with notable biological activities including antibacterial [15], antiprotozoal [14], and antitumor effects [16].

Several studies have reported the cytotoxic properties of IKA, and this has been explored for its anti-tumor property [17]. No data are, however, available on the cytotoxicity using MAC-T cells and the precise mechanism of toxicity. Our study also found that the concentration of IKA that inhibited 50% of MAC-T cells (the IC_{50}) was 9.2 μg/mL. This IC_{50} is comparatively higher than the concentration required to kill 90% of intracellular *S. aureus* in MAC-T cells. This highlights the comparatively lower concentration required for an effective bactericidal effect against intracellular *S. aureus* compared to the concentration required for a considerable cytotoxic effect. This finding is consistent with previous findings in which IKA has shown promisinging antimicrobial activitiy [18]. The potential antibacterial effect against intracellular bacterial seen in our study is also consistent with the findings of the important antimicrobial (antibacterial, antifungal, and antiprotozoal) effect of IKA and the related members of the polycyclic tetramate macrolactams (PTMs) group (including dihydromaltophilin and frontalamide) [19]. These groups are also important potential anticancer drug candidates as highlighted by their cytotoxic roles [20][16]

S. aureus is a typical facultative intracellular bacteria [3]. The invasion of host cell start by the adheres of bacteria to surface of the body such as mucosal membrane and skin, S. aureus secreted some factor may help to adhesion [21] followed adhesion S. aureus start to colonization and many factors secreted by bacteria are involved) to resist the immune response of hosts and thus achieve successful colonization[22]. After successful adhesion and colonization, S. aureus invades cells and survive within it. The bacteria can internalization the cell and reside in special compartments using some mechanisms, to escape from host immune defense and targeting by antimicrobials Figure (6)

The intracellular survival strategies of *S. aureus* are associated with the subclinical and relapsing infection of bovine mastitis and lead to treatment challenges, and many antibiotics are failed to combat the intracellular infection

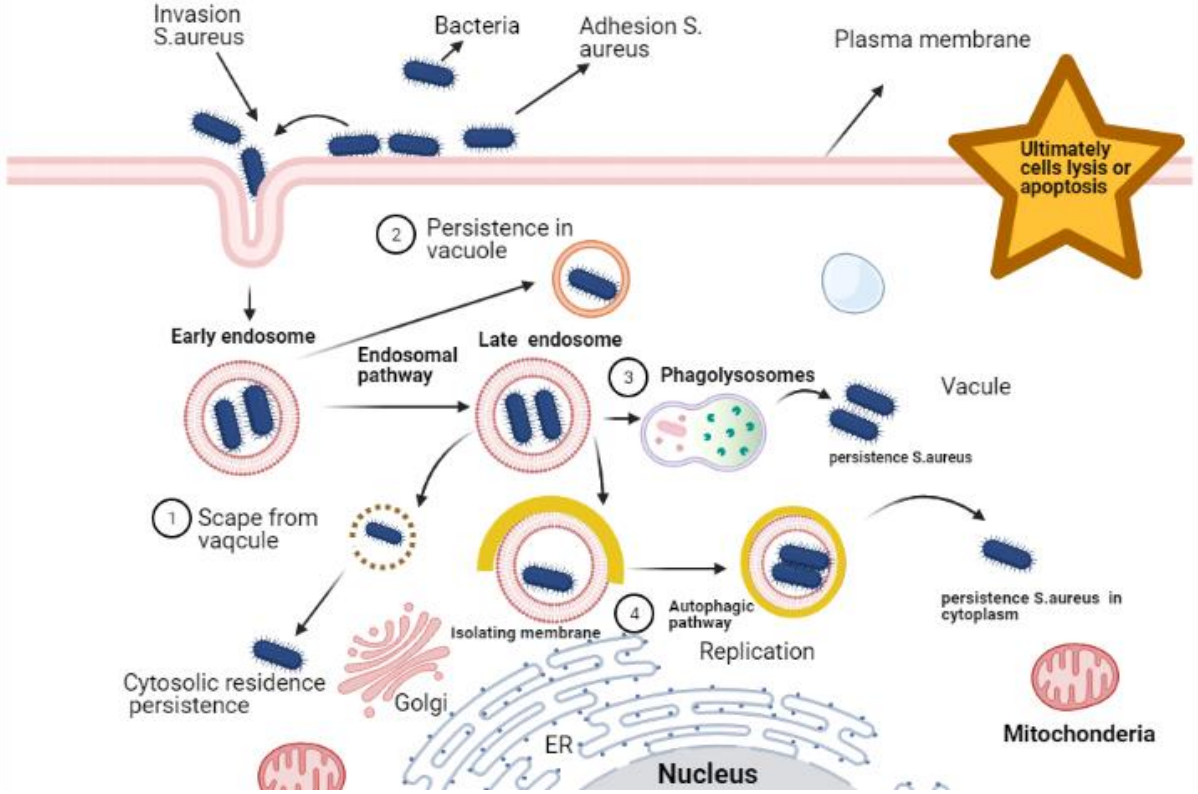

Figure 5. Summary diagram illustrates the mechanisms of intracellular invasion of S.aureus to mammalian cells and cellular fate. The possible fate includes (1) escape from the endosomal compartment, (2) persistence in vacuoles, (3) survival within the lysosome.

3. Materials and Methods

3.1. Reagent

Ikarugamycin(IKA) was purchased from the company (Toku-E, Tokyo, Japan). The compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). IKA prepared as a 1 mg/mL stock solution in DMSO (dimethyl sulfoxide) and stored at –80 and further diluted in phosphate-buffered saline before use. The final concentration of the DMSO in the solution was less than 0.01%.

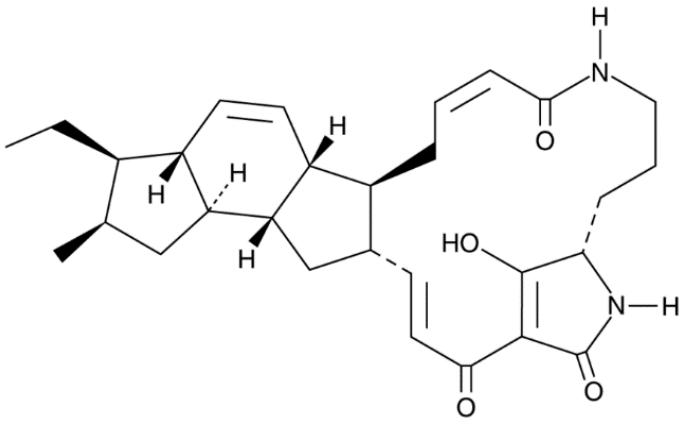

FIGURE 6. Structure of Ikarugamycin, which consist of macrolactam ring, tetramic acids, and carbocycles (Antosch, Schaefers, and Gulder 2014)

3.2. Bacterial Strains and Culture Conditions

Staphylococcus aureus F51B was isolated from cases of subclinical mastitis on dairy farms on the east coast of Malaysia. In addition, *S. aureus* strain 15 AL sample was obtained from Dr. Shan Goh, Royal Veterinary College, London. All these isolates are knowns as antibiotic resistance and able to invade the udder cells. The isolates were maintained in − 80 °C freezer stock. Before the experiment, bacteria were cultured in Mannitol Salt Agar (MSA) followed by grown in Nutrient agar (NA, Oxoid, UK) at 37 °C for 24 hours in shake incubator at 200 rpm.

3.3. Determination of Minimum Inhibitory Concentrations (MIC) of IKA

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to CLSI 2016. Briefly, a range of concentrations of antimicrobials was prepared in a 96-well microplate, followed by inoculation of bacteria to yield 5×10^5 cfu/mL in a 200 ml final volume. The plate was then incubated at 37 °C for 18 h. The lowest concentration of an antimicrobial that inhibited the growth of bacteria was scored as the MIC [23].

3.4. Time-kill Assays

Time-kill assays were performed using the broth macrodilution method (CLSI 2016). Briefly, overnight *S.aureus* was diluted to obtained 5×10^5 cfu/mL final concentration.

Following that, bacterial was added into tubes containing designated concentration of IKA (1MIC, 3MIC, and 4MIC) , and untreated bacteria served as control. followed by incubation at 37°C in incubator shaker , and 100µl of suspension was takeout at various times (3, 6,12,24 hours) followed by serial dilution, and plated on nutrient agar. Plates were incubated for 18 hours at 37°C, followed by colony counting to assess the time killing kinetics. The data were plotted with time against the logarithm of the viable count. Each experiment was performed in triplicate[24]

3.5. Bovine Mammary Epithelial cells culture

An immortalized bovine mammary epithelial (MAC-T) cell line (obtained from Dr. Amanda Gibson (Royal Veterinary College, London)) was used as a host for in vitro intracellular infection. The MAC-T cells were maintained in Dulbecco's modified Eagle's medium DMEM) with 10 % fetal bovine serum(FBS, supplemented with 5% penicillin-streptomycin and 1% insulin. The cells were incubated in 5% CO_2 at 37 °C.

3.6. Intracellular invasion Assay

In- vitro infection of MAC-T cells was performed using gentamicin protection assay as previously described [7]. Aliquots of *S.aureus* diluted to 5×10^7 to CFU/mL were co-incubated with host cells for 3 hours for intracellular internalization, followed by gentamicin exposure at 200 mg/l for 3 hours to elucidate the intracellular bacteria. Finally the infected Mac-T cell was lysed using 0.5% Triton X-100; the lysed cells were serial diluted and plated on nutrient agar to obtained CFU/ml of intracellular bacteria. [7]

3.7. Intracellular antibacterial activity tests of IKA

The host cells were infected with *S. aureus* followed by incubation with gentamicin to kill the extracellular bacteria. Following gentamicin exposure, host cells were rinsed with PBS. Subsequently, the wells containing infected cells were treated with IKA at 1MIC, 3MIC, and 4MIC, and cells without IKA treated were used as control. Plates were incubated for 3 hours to kill intracellular bacteria. Afterward, the IKA was removed, and the cells were lysed after rinsing. Lysed cells were serially diluted and plated on nutrient agar to quantify internalized bacteria,

3.8. Cytotoxicity of IKA

The cytotoxicity of IKA toward Mac-T cells was assessed using resazurin assay. Briefly, the host cells line 4 × 10^4 cells/well were seeded in 96 well plates for 48 h, followed by co-incubated with IKA with increasing concentration up to 10 µl/ml. The plate was incubated at 37°C for 3 h. Non-treated cells and medium only will be used as controls. Finally, Resazurin sodium salt (Sigma-Aldrich, UK) at 44 µM final concentration was added to each well, then the plate was incubated

for 48 h. After the 48 h, the optical density (OD) was measured using POLARstar Omega microplate reader (BMG, Labtech, Germany) at 550 nm and 630 nm. The OD value change (or % dye reduction) is proportional to the viable cell number. Survival curves were plotted, and the IC50 (inhibitory concentration 50%) for IKA was calculated using Graph Pad Prism version 8.00. All experiments were conducted in triplicate.

3.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (San Diego, CA, USA). Data are presented as means ± standard deviation (SD). Comparisons between the groups were performed using ANOVA with turkey tests. The differences were considered significant at $p \leq 0.05$. Each experiment was performed at least three times..

4. Conclusions

In conclusion, the present study demonstrated that IKA has antibacterial activity against intracellular *S. aureus* in in-vitro models in Mac-T cells with host cells tolerance of IKA at concentrations higher than the required concentration to kill 90% of intracellular *S. aureus*. This highlights the potential importance of IKA as an alternative drug candidate to be explored in the treatment of persistent bovine mastitis caused by *S. aureus*. In-vivo studies are needed to evaluate IKA potency and toxicity in bovine udder infected with *S. aureus* mastitis.

Author Contributions: SIS and NF designed the research and wrote the manuscript. SIS performed the experiment. AAA and AEM wrote the manuscript. EA, MM, and NFK supervised the work and revised the manuscript. All authors have read and approved the final version of the manuscript.

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