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Combining Bacteriophage Lysin LYSGH15 and Celery (*Apium graveolens* L.) Apigenin for Treating *Staphylococcus aureus* Pneumonia in Children

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ABSTRACT

Pneumonia is one of the devastating diseases that affect children under five and can result in high incidence and significant mortality rates. A substantial subset of these cases is caused by *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacteria that can cause severe complications and even death. Additionally, antibiotic resistance complicates this problem further. In order to combat this issue, combining flavonoids and bacteriophage products may be a critical solution in decreasing morbidity and mortality rates of pneumonia. Apigenin, extracted from celery (*Apium graveolens L.*), which has anti-inflammatory and antioxidant effects. Combined with bacteriophage lysin, it can work synergistically against infections and inflammation without being easily resisted by bacteria. This literature review focuses on the use of apigenin and phage lysin, LysGH15, as a treatment for children's pneumonia, sourced from Google Scholar and PubMed, using several combined keywords such as "new treatment", "children pneumonia", "*Staphylococcus aureus*", "MRSA", "new antibacterial", and "therapy". Combination therapy shows potential as a new treatment candidate for children's pneumonia.

Keywords: Apigenin, celery, children's pneumonia, herbal medicine, MRSA, phage lysin

ABSTRAK

Pneumonia dikenal sebagai salah satu penyakit paling mematikan pada anak di bawah 5 tahun, dengan insiden 156.000.000 kasus per tahun, dan angka kematian yang signifikan. Salah satu penyebab penting penyakit ini adalah bakteri Gram-positif *Staphylococcus aureus (S. aureus)*, yang sering menyebabkan komplikasi dan kematian. Peningkatan resistensi *S. aureus* terhadap antibiotik juga memperumit kondisi ini. Kombinasi perawatan yang berasal dari produk flavonoid dan bakteriofag mungkin dapat menjadi kunci untuk menurunkan morbiditas dan mortalitas. Apigenin adalah salah satu jenis flavonoid yang dapat diekstrak dari seledri *(Apium graveolans L.)*. Bersama dengan lisin dari bakteriofag, kedua komponen ini bekerja secara sinergis dalam kasus infeksi dan peradangan, dengan keunggulan berupa tidak mudah mengalami resistensi oleh bakteri. Tinjauan literatur berasal dari Google Scholar dan PubMed menggunakan beberapa kata kunci: "pengobatan baru", "pneumonia anak", "*Staphylococcus aureus*", "MRSA", "antibakteri baru", dan "terapi" berfokus pada penggunaan apigenin dan lisin bakteriofag, LysGH15, sebagai pengobatan untuk pneumonia anak. Terapi kombinasi ini diharapkan berpotensi untuk dikembangkan sebagai pengobatan pneumonia anak.
Muhammad Habiburrahman, Stefanus Sutopo, Delly Chipta Lestari. Potensi Terapi Kombinasi Bakteriofag LYSGH15 dan Ekstrak Apigenin Seledri (*Apium graveolans* L.) sebagai Strategi Baru Eradikasi Pneumonia Anak Akibat Infeksi *Staphylococcus aureus*

Kata kunci: Apigenin, seledri, pneumonia anak, pengobatan herbal, MRSA, lisin fage

INTRODUCTION

Pneumonia is a major cause of fatality in children, especially those under five years old. In a recent epidemiological study in the United States of America, the incidence of community-acquired pneumonia in children requiring hospitalisation is estimated to be 15.7 cases/10,000 children, especially among children younger than two years old.[1,2] In Indonesia, the incidence of community-associated pneumonia is 988/100,000, with a case fatality rate (CFR) of 1.4%-4.2%, while the healthcare-associated pneumonia incidence is around 538/100,000, with a CFR of 9.1%–25.5%.[3]

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Around 6% of community-acquired pneumonia (CAP) in children with the multidrug-resistant pathogen is caused by *Staphylococcus aureus (S. aureus)*, especially with methicilin resistance.[4] Pneumonia associated with this bacteria is often fatal and often complicated by lung necrosis, lung cavitation, empyema, pleural effusion, pneumatocele, and pneumothorax.[5] Two cohorts of patients with *S. aureus* pneumonia in Utah and Argentina were shown to have various but significant levels of methicilin resistance (21%, and 85%, respectively).[6,7] The technical implications of increased methicillin-resistant *S. aureus* (MRSA) infections are related to treatment complications due to resistance to commonly used antibiotics, such as gentamicin, erythromycin, fluoroquinolones, and ofloxacin.[8] Indonesia, in particular, suffers a moderate prevalence of MRSA (around 28%).[9] Treatment failure due to invasive MRSA infection is often reported, and current antibiotics used against resistant bacteria, such as vancomycin and linezolid, are riddled with drawbacks and side effects.[10] This disease is also associated with prolonged hospitalisation that correlates with increasing costs and risks of healthcare-associated infections, further compounded by the bleak prognosis, with a mortality rate of over 50%.[11–13]

The urgent need for new therapy options led researchers to discover new forms of therapy against *S. aureus* pneumonia. LysGH15, one of the lytic enzymes (lysins) produced by the GH15 bacteriophage, is proven both in vitro and in vivo to lyse *S. aureus* in a specific and immediate fashion in low concentrations.[14,15] However, the disintegration of bacteria en masse caused by this new antibacterial may lead to systemic inflammation and cytokine storm due to the rapid release of intracellular materials, including various bacterial endotoxins.[16–18]

Complementary therapy with anti-inflammatory properties, such as flavonoid apigenin, might be needed to reduce the risks of systemic inflammation. This flavonoid, produced from the celery plant (Apium graveolens L.), is seemingly able to complement LysGH15 due to its ability to down-regulate transmission of bacterial virulence factors and its capability to prevent inflammation.[19,20] This study attempted to explore the potential benefits of a combination therapy involving LysGH15 and apigenin as an alternative therapy for *S. aureus* pneumonia in children in an effective and cost-efficient manner.[21]

METHODS

This presented work is a literature review focused on elaborating the latest evidence on the potential of new antibacterials in fighting pneumonia in children caused by *S. aureus*. As illustrated in **Figure 1**, firstly, a literature search was conducted using the search engines Google Scholar and PubMed with the keywords: "children pneumonia", "*Staphylococcus aureus*", "MRSA", "new treatment", "new antibacterial", and "new candidate of therapy". After finding the idea of apigenin and LysGH15, those keywords were further explored and focused in search engines. Articles were selected based on several inclusion criteria (relevant topic, full text available, and published within ten years prior to the review) and exclusion criteria (not journal articles, original articles, nor studies of these study designs: cell-line studies, animal model studies, and clinical observational and randomized controlled trial studies; not in the English language, and not aligning with the patient population being reviewed). The methods in constructing the idea, conducting a search for evidence, and presenting the findings for this narrative review followed recommendations by Ferrari 2015,[22] Green, *et al*, 2006,[23] and Gasparyan, *et al,* 2011.[24]

The immediate result was an original article examining the combination of bacteriophage lysin LysGH15 and apigenin to combat *S. aureus* infection (Xia, *et al,* 2015).[21] Other studies on antibacterial and anti-inflammatory effects of LysGH15 (Zhang, *et al*, 2016),[25] apigenin (Wang, *et al*, 2014, Li, *et al*, 2013, and Dong, *et al*, 2013),[26–28] and various miscellaneous data points required to discuss their therapeutic applications were also found via Google Scholar and Pubmed, mainly to review pharmacokinetics, pharmacodynamics, and practical use for both agents. Forty-eight references are used for this review, including textbooks, reviews, and papers documenting original research. A narrative review is then formed out of these materials.

RESULTS AND DISCUSSION

1. Pathogenesis of Pneumonia Caused *by Staphylococcus aureus*

As a pathogen, *S. aureus* has several virulence factors which play a role in its pathogenesis, including: 1) Surface protein A, which supports adhesion and colonisation; 2) Several invasion proteins, such as leukocidin, kinase, and hyaluronidase; 3) Pore-forming toxins (hemolysin, leukotoxin, and leukocidin), which can lyse the cell membranes of mammalian cells, including erythrocytes, keratinocytes, fibroblasts, as well as endothelial and epithelial cells; 4) Exotoxins which can damage the host tissue (SAE-G, TSST-1, exfoliatin, and Panton-Valentine leukocidin 1 (PVL-1)); and 5) Resistance to antimicrobials, both inherent and acquired.[29,30]

Several regulatory genes, such as the agr gene and the spa, sar, and sigB proteins, affect these virulence factors' expression. The spa protein regulates the synthesis of protein A, while the agr gene is essential in synthesising hemolysin, leukocidin, and PVL.[31,32] Staphylococcal bacteria have also developed methicillin resistance using the gene mecA spread through the genetic element SCC-mec (staphylococcal cassette chromosome mec) to encode penicillin-binding proteins and lower the affinity of beta-lactam antibiotics. All these pathogenic mechanisms cause a decrease in the effectiveness of such antibiotics in curing staphylococcal pneumonia.[29,30]

2. The Potential of Bacteriophage LysGH15 as an Antibacterial and Anti-inflammatory Agent

Endolysins are enzymes encoded by the bacteriophage genome and synthesised at the end of the lytic cycle to lyse host cells. The use of lysins is advantageous because of their high specificity for targeting bacterial cells, even for specific species. Moreover, this substance does not disrupt the composition of normal flora.[33–35] Lysins can enter and split the covalent bonds in cell wall peptidoglycans, causing hypotonic lysis. The specific areas which are attacked by these enzymes are the region between the D-alanine stem peptide and the glycine peptide cross-bridge and the regions with N-acetyl muramyl-L-alanine amidase in the centre.[25] Various lysins have been proven to destroy species of pathogenic bacteria, including Streptococcus pyogenes, Streptococcus pneumoniae, and Bacillus anthracis.[36–38] Therefore, phage lysins are suggested as a promising alternative to antibiotics in curing bacterial infections.

LysGH15 is a lysin found exclusively in the lytic bacteriophage strain GH15. It has shown solid lytic activity against MRSA in vitro and in vivo. The minimum inhibitory concentration (MIC) of LysGH15 needed against MRSA, and methicilin-sensitive *S. aureus* (MSSA) is 15.625

μg/mL and 31.25 μg/mL, respectively. Therapy using LysGH15 caused staphylococcal colony-forming units (CFU) to decline. After six hours, the CFU of staphylococci in the therapy wing is log unit 1.8, compared to log unit 8.9 in the control group. The pro-inflammatory cytokines (tumour necrosis factor (TNF) -α, interferon (IFN) -γ, interleukin (IL)-1β, and IL-5) induced in response to infection also

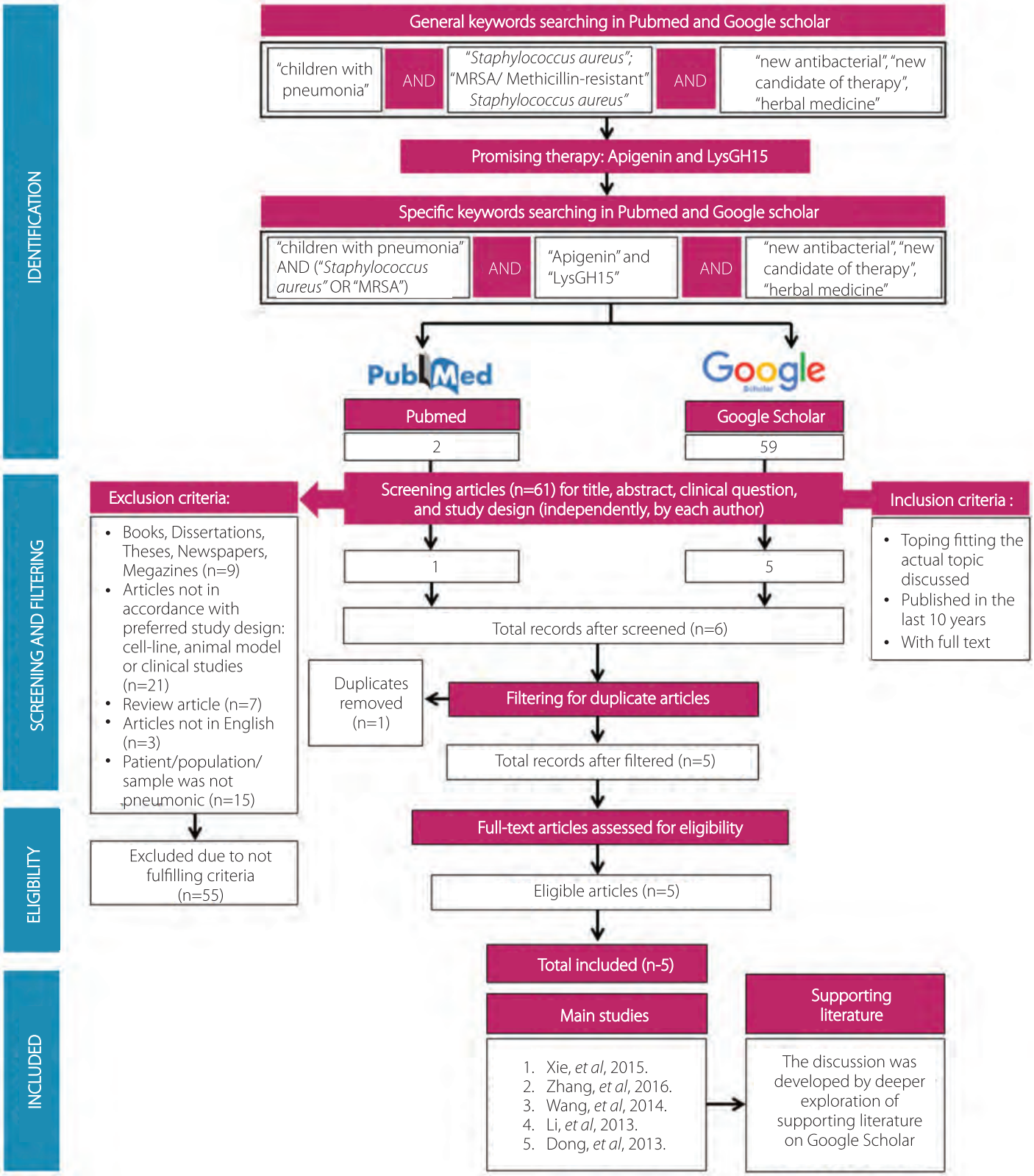

Figure 1. Search strategy for the literature used in this study.


decreased significantly. However, LysGH15 is an immunogenic protein that can induce a specific antibody's creation. LysGH15 is a safe and efficient antibacterial, causing less resistance risk in *S. aureus* strains after repeated uses or side effects and tolerance issues after high dose administrations.[25]

3. The Potential of Apigenin from *Apium graveolens L.* as an Antibacterial and Anti-inflammatory Agent

Celery (*Apium graveolens* L.) enjoys use in various societies, especially in Indonesian society, as a vegetable, spice, or condiment in soups. Indonesia, in particular, is one of the most prolific celery exporters in the world, with 2.94% of vegetable exports and an export value of USD110,000.[39] Although not usually used as a medicinal plant, new research has focused on the flavonoid contents of this plant. One hundred grams of fresh celery leaves can contain 5.3–16 µmol of the flavonoid apigenin and 18-51 µmol of apigenin glycoside (apiin/apigenin-7-O-aminoglycoside), as detected through a system of high-performance liquid chromatography (HPLC) and a photodiode array.[40] Apigenin has a chemical formula of C15H10O5 with a molecular weight of 270.23 g/mol. Apigenin has various antibacterial impacts against Gram-positive and Gram-negative bacteria.[28,41,42] It also provides an anti-inflammatory effect by reducing the mediator nitric oxide (NO) and inducible NO synthase (INOS) both in vitro and in vivo.[26]

In an animal model of asthma and acute lung injury, apigenin has been proven to show an inhibitory effect on eosinophil infiltration of lung tissue.[26,27] These findings are consistent with the inhibition of various inflammatory cytokines, such as TNF-α, IL-6, IL-1 β1, nuclear factor-kappa beta (NF-κβ), and lung cyclooxygenase (COX)-2.[26,27] Additionally, apigenin also reduces the activity of matrix metalloproteinase and the activation of transforming growth factor (TGF)-β1, therefore reducing lung fibrosis.[26] Through its reduction of T-helper (Th)-17 cell activity, apigenin also has a protective impact on lung tissues, enhancing T-cell and B-cell function as part of an effective immune response.[27]

A previous study revealed that the extraction of apigenin could be done using the Soxhlet apparatus technique with 96% ethanol, continued by thin-layer chromatography and spectrophotometry. Meanwhile, its concentration could be measured through densitometry. Briefly, 5 grams of celery apigenin were used for extraction, and 7 mL of methanol was added to a sealed reaction tube. The mixture was subjected to sonication for 20 minutes and filtered using filter paper into a measuring flask. The extracted solution was then washed with methanol until it reached a volume of 10 mL. The exact process was repeated with this first extract to gain the second extract. This was compared with a solution of 10 mg of reference standard apigenin in 10 mL of methanol through suspension with a Langendorf pipette in a GF254 silica gel plate, then eluded in a chloroform-methanol-water (70:30:6.5 v/v) motile phase with a chromatograph length of 8 cm.[43] Various researchers have demonstrated the extraction of various concentrations of apigenin. In 2009, Gusniwati extracted 25 mg of apigenin from ethanol extraction of 200 g of dry celery leaves, and the resultant substance was confirmed to be apigenin through paper and thin-layer chromatography with infrared and UV-spectrum analysis.[44] In 2014, Zhu, *et al*, using ethanol dissolution, extracted 75.4 mg of apigenin from 1 g of celery leaves. This result was confirmed through solid-phase extraction high-performance liquid chromatography (SPE-HPLC) using a hybrid organic-inorganic monolith as the solid-phase adsorbent.[45]

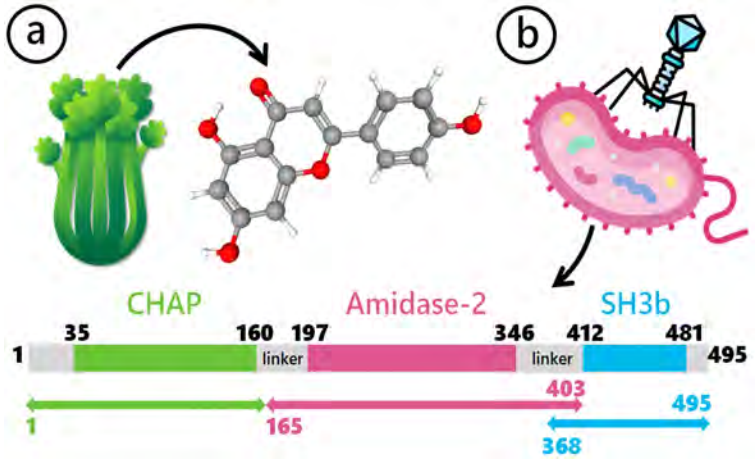

Figure 2. The structure of apigenin and LysGH15. (A) The chemical structure of apigenin.[46] (B) The domain structure of the LysGH15 protein.[47] (The figure was designed and modified from Pubchem[46] and Gu, et al.[47] Animation is from www.flaticon.com).
Abbreviation: CHAP: a cysteine, histidine-dependent amidohydrolases/peptidase domain; SH3b: N-terminal catalytic domain and a cell wall binding domain SH3b; **LysGH15**: Lysin GH15.

4. Combination Therapy of LysGH15 and Apigenin in the Treatment of *S. aureus* Pneumonia

Multiple therapeutic measures are the best option for treating various bacterial infections, including MSSA and MRSA. It has been discovered that LysGH15 has shown high efficiency and a broad lytic spectrum against MSSA and MRSA. Meanwhile, apigenin is capable of reducing *S. aureus* α-hemolysin, as well as host inflammatory responses. In order to prove the benefits of combination therapy, an *S. aureus* pneumonia mice model was created by infecting mice with 5x107 CFU of USA300 strain *S. aureus*. However, the research discussed here is limited to curing MSSA pneumonia with combination therapy. However, it is highly possible that this combination therapy also worked in curing MRSA pneumonia due to the differences in the mechanism of action of these substances compared to beta-lactam antibiotics.[21]

Detection of bacterial load in lung tissue and blood of infected mice given LysGH15 monotherapy, apigenin monotherapy, or combination therapy one hour after S. aureus inoculation is provided by dilution and serial coating of blood homogenate and lung tissue. Histopathology of lung tissue of infected mice treated with buffer, monotherapy, combination therapy, and normal lung tissue. The ratio of wet lung tissue compared with dry lung tissue, total cell count in the tissue, lung protein analysis, and cytokine quantification from bronchoalveolar lavage fluid were also calculated after 24 h of inoculation.[21]

Purified LysGH15 was obtained from endolysin expression of cultured BL21 (DE3) strain *Escherichia coli*. According to the SAXS analysis, the structure of this enzyme is identified as a V-shaped monomer with three domains spatially separated from the envelope (**Figure 2b**). Apigenin is extracted from celery (Apium graveolens L.) and has a chemical structure shown in Figure 2a.[21]

A mice pneumonia model was made by intranasally injecting an increasing dose of *S. aureus* USA300 bacteria (5x107, 5x108, 5x109, and 5x1010 CFU). The average lethal dose is calculated, and it was discovered that 5x107 CFU of *S. aureus* is enough to kill all infected mice in three days, with clinical symptoms of pneumonia (increased breathing effort and lethargy), and deep red lung tissue with coarse and edematous texture. The lung also showed serious pathological injury, significant pooling of inflammatory cells (stained dark blue or purple), and pinkish liquid in the alveolar space. The alveolar walls show telangiectasia and congestion. All of these pathological changes are hallmarks of pneumonia. The combination therapy shows the capability to reduce injury and inflammation.

The lung tissue in the control population is hyperemic with a coarse and rigid texture, while those treated with the combination therapy show pink and flexible lung tissue. Histopathologically, no severe inflammation or other pathological changes existed in mice treated with combination therapy lung tissue. One minute after LysGH15 therapy, the bacterial cell morphology changed and began to look cracked in contrast to their typical morphology. Two minutes after administration, only bacterial cell debris could be observed.21 It was shown that there are no significant differences in lytic activity from LysGH15 given alone or with apigenin. Apigenin also did not affect the bactericidal activity of LysGH15 in *S. aureus*.

An experiment was also done to find substances that could counter the hemolysis of rabbit erythrocytes from supernatants of *S. aureus* culture containing α-hemolysin.[21] Apigenin (8 μg/mL) alone could not significantly stop hemolysis, while hemolytic activity after LysGH15 monotherapy with doses of 25, 50, and 100 μg/mL were respectively 86.5%, 67.4%, and 44.78%. Combinations of LysGH15 (50 or 100 μg/mL) and apigenin (8 μg/mL) could stop hemolysis.[21]

Monotherapy of LysGH15, apigenin, and combination therapy of both substances is given to determine the therapeutic effect one hour after MSSA infection. The paper suggested that by combining LysGH15 (60 μg) with apigenin (500 μg), the efficacy was enough to protect all mice against *S. aureus* pneumonia. The group treated with LysGH15 alone (60 μg) revealed a survival rate of 80%. However, all mice given only apigenin or buffer died in two and three days, respectively. Moreover, mice treated with combination therapy are healthier than mice given monotherapy 12 h after inoculation, with health defined from the general physical appearance of the mice semiquantitatively classified on a 0–5 scale.[21]

No significant difference in bacteremia was recorded between groups given LysGH15 monotherapy and combination therapy. The bacteria in the lung of the LysGH15 group and the combination group subjects both decreased to 300 CFU/mg and 35 CFU/mg, respectively. Bacteria in the lungs and blood of the group given apigenin monotherapy reached 105 CFU/mg and 320 CFU/mL in 24 h after therapy. However, the bacteria in the lungs and blood of mice not treated increased to 3.5x1010 CFU/mg and 5.7x105 CFU/mL, respectively, 24 h after therapy. This amount of bacteria eventually caused overwhelming bacteremia and death in three days.[21]

To assess the condition of inflammation in pneumonia after therapy, the researchers used several parameters, including the ratio of wet lung tissue to dry lung tissue (W/D), total cell count, and bronchoalveolar lavage fluid protein content. The W/D ratio of the group treated with combination therapy (3.96) is most similar to the healthy control group (3.76), followed by the group treated with LysGH15 alone (4.93). The W/D ratio of the apigenin alone group (6.90) is similar to those treated with buffer (7.37). The total cell count and protein of the combination therapy group also decreased significantly compared to the untreated group and are lower than those given LysGH15 or apigenin alone. Acute pneumonia is often followed by the activation of the inflammatory system, commonly marked by infiltration of inflammatory cells and proteins in the alveolar space. This acute injury of the alveolocapillary barrier can cause increased permeability and exudative oedema, macroscopically found as a wet lung.[21]

The concentration of TNF-α, IL-1β, and IL-6 in the groups treated with combination therapy was very similar to healthy mice, and the difference is gradually higher in mice treated with only LysGH15, apigenin, and untreated mice, respectively. These cytokines are extensively involved in cell proliferation, inflammation, and immunity and may cause severe injury at the cellular and tissue levels. Compared to monotherapy results, the combination therapy indicated a decrease in the total cell count and protein levels and a reduction of pro-inflammatory cytokines, which may lead to less irreversible lung injury.[21]

5. Potential Therapeutic Applications

In humans, the oral administration of 84 mg apigenin in 5 g of dry celery will reach its maximum plasma concentration of 127±81 nmol/L in 7.2±1.3 h.[48] In mice, the injection of 60 mg/kg BW of apigenin will reach its maximum concentration of 1.33±0.24 μg/mL in 2.5±0.33 h, with a half-life of 4.198±0.29 h and relative bioavailability of 183%.49 Apigenin undergoes phase I metabolism in the liver by the enzymes CYP1A1, CYP2B, and CYP2E1, and phase II metabolism by the UDP-glucuronosyl-transferase UGT1A1, and at last secreted into its glucuronide and sulphate conjugate forms.[50,51] Apigenin in fresh celery (75 mg/100 g), orally administered, will be secreted via urine in 48–72 h and via faeces in 12–24 h.[52,53] Apigenin can also be given as a subcutaneous injection or intranasally.[21]

The pharmacological data for lysin LysGH15 is not yet available. However, the Food and Drugs Administration (FDA) has approved several clinical trials of phage-based therapies. A phase I clinical trial evaluated the safety of lysin-producing phage cocktails as an antibacterial against *S. aureus*. In 2009, an oral therapy of phages treating children's diarrhoea was also shown to be safe, well-tolerated, and had good efficacy. In 2015, a phase II clinical trial was recruiting cases to test a phage cocktail's efficacy in treating burn wounds.[54]

Bacteriophage lysins can be given through various routes (orally, topically, or via nebulisation); therefore, they can be applied to various infections.[55,56] They can also be used as a prophylaxis against MRSA and to destroy


MRSA colonies.[57] A case report shows that an oral phage preparation can eliminate intestinal MRSA infection.[58] The curative dose of apigenin versus *S. aureus* pneumonia is 50 mg/kg BW in mice, and it significantly boosts the capacity of LysGH15 in warding off *S. aureus* pneumonia.[21,28]

CONCLUSION AND RECOMMENDATIONS

Children pneumonia due to *S. aureus* is not overtly prevalent but very life-threatening. Moreover, some pneumonia cases due to *S. aureus* involved antibiotic-resistant strains, such as MRSA. This condition limits therapy choices and requires new, effective, affordable therapeutic agents or combinations. The studies shed light on the potential for combining the phage lysin LysGH15 and apigenin (from celery) in treating *S. aureus* pneumonia. Both components do not have any adverse interactions with each other and were proven to complement each other quite well, with benefits including better overall restoration and markedly less lung inflammation and injury. Developing countries have a significant burden of pneumonia in children, which increases the importance of these agents being of clinical use.

Controlled clinical trials are suggested to confirm this therapy's effectiveness and safety, and studies on the pharmacokinetic and pharmacodynamic features of phage lysins, in general, are also required. An in vitro and in vivo study on this combination therapy for MRSA pneumonia is also needed, as well as more efficient methods to extract and produce both therapeutic substances.

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