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Antibacterial Activity of Honeybee Venom Against Pathogenic Bacteria in Comparison with Common Antibiotics

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Abstract

Antibiotic resistance has dramatically spread in recent decades and has become a serious problem in modern medicine. Honeybee venom or bee venom (BV) has anticancer, anti-inflammation, and antimicrobial properties. This study aimed to evaluate the influence of BV on certain pathogenic bacteria. The effects of different concentrations of BV against *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were evaluated by disk diffusion method (inhibition zone, IZ), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Additionally, the antibacterial activity of BV was compared with that of ampicillin, penicillin, and tetracycline. The results indicated that different concentrations of BV had an inhibitory effect on the pathogen species. The MIC and MBC of BV were 18 and 24 μg/mL for *K. pneumonia*, respectively, 38 and 42 μg/mL for *P. aeruginosa*, respectively, and 21 and 31 μg/mL for *S. aureus*, respectively. Among the bacterial species tested, BV was the most effective against *K. pneumonia*. Meanwhile, *P. aeruginosa* was the most resistant against BV. The comparison revealed that 100 μg/mL BV had a greater effect on IZ against *P. aeruginosa* and *K. pneumonia* compared with evaluated common antibiotics ($p < 0.05$). This study showed that BV demonstrated antibacterial effects. Precise toxicology examinations are required for the development of new antibiotics.

Keywords: antibacterial activity, disk diffusion method, honeybee venom, MBC, MIC

Introduction

Antibiotics resistance is a global problem, threatening human health and causing high mortality. Researchers in different countries are exploring and producing new antibiotics [1]. Humans have faced various diseases since the beginning and are attempting to find treatments for some of the most challenging diseases using natural products. Natural toxins have been used for the treatment of diseases since time immemorial [2]. Honeybee venom or bee venom (BV) has been widely applied to treat various diseases, such as rheumatism, arthritis, multiple sclerosis, and infectious and inflammatory diseases. BV, released from the venom gland in the abdominal cavity of a honeybee, is a pharmacological and enzymatic composition of biogenic amines, peptides, and enzymes. It contains active peptides, such as melittin [3], adolapin, apamin, mast cell degranulating peptide; enzymes, including phospholipase A2 and hyaluronidase [4]. It also contains nonpeptide components, such as dopamine, histamine, and norepinephrine. Norepinephrine, melittin, and phospholipase A2 are the main compositions of BV. Melittin is a peptide made up of 26 amino acids and comprises 50%–60% of the venom in dry weight [5]. Melittin breaks the membrane phospholipids, making the cell slippery, leading to the integration loss of bilayer phospholipids and synthesis [6]. The antimicrobial effect of BV against Gram-negative and -positive strains were previously investigated. Infectious diseases caused by microorganisms pose major health problems in developed and developing countries [7]. *Staphylococcus aureus* is a Gram-positive, cocci-shaped, facultative anaerobic bacterium that causes a variety of life-threatening infections [8]. *Pseudomonas aeruginosa* is a Gram-negative, aerobic bacterium that causes a wide range of clinical diseases and is recognized for its antibiotic-resistance characteristics [9]. This pathogen is common in most environments and can be found in small numbers in water, normal intestinal flora, and the skin [10]. *Klebsiella pneumoniae* is a Gram-negative, facultative, anaerobic bacillus bacterium that can cause a variety of diseases, including liver abscess, pneumonia, and meningitis. It can be found in the normal flora of the mouth, skin, and intestines [11]. In this study, the

antibacterial activity of BV against three pathogenic bacteria (*K. pneumonia, P. aeruginosa, and S. aureus*) was evaluated and compared with three common antibiotics (ampicillin, penicillin, and tetracycline).

Materials and Methods

Preparation of BV. Honey bees (*Apis mellifera meda*) were galvanized with electric shock without harming them [12]. After they bit the collector disk, BV was collected, dried on a crystal disk, and kept in a freezer at −20 °C [13].

Antimicrobial Activity Assays

Microorganisms. *S. aureus* (ATCC 25923), *P. aeruginosa* (PTCC 1310), and *K. pneumonia* (PTCC 1053), were prepared in the Traditional Medicine Institute of Isfahan (Isfahan, Iran). Muller–Hinton agar (MHA) medium was transferred to sterilized Petri dishes (5 cm thick). The bacteria samples were removed from the basal culture by an applicator and inoculated in the medium under aseptic conditions.

Antibacterial assay. Together with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), the disk diffusion method (also known as Kirby–Bauer) as the most common form of the antimicrobial assay [7, 14] was used to evaluate the antimicrobial effect of BV. After being incubated at 37 °C for 18 h, the suspension containing bacteria (1×10^6 CFU/mL) was adjusted to 0.5 MacFarland in MHA medium. In brief, 500 µL of the suspension was gently distributed on the surface of MHA via a sterile loop. Blank disks (6 mm in diameter) consisting of 30 µL of 2.5, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/disk BV concentrations were incorporated with MHA medium. Disks containing ampicillin (10 µg/disk), penicillin (10 µg/disk), and tetracycline (30 µg/disk) were applied as a positive control. The diameter of the inhibition zone (IZ) was measured 24, 48, and 72 h after incubation at 37 °C. Microwell method was used to evaluate MIC and MBC on *S. aureus*, *P. aeruginosa,* and *K. pneumonia*. The suspension of bacterial strains was prepared in a standard darkness of 0.5 MacFarland. Honey BV in different concentrations was added to the pipes containing 10 mL of these suspensions. For the determination of the MIC value, 95 µL of Mueller–Hinton broth (MHB) and 5 µL of microbial suspension were added to each well of a 96-well plate. In the first well, 100 µL of honey BV with a concentration of 400 µg/µL was added. Afterward, 100 µL was taken from the first well and transferred to the second well. This process continued until the sixth well was reached. As a negative control, the last well was not added with honey BV. The ingredients of every well were mixed for 20 min, and the plate was incubated at 37 °C for 24 h. At 600 nm, microbial growth was measured using a microtiter plate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). The MBC of BV was defined as the last dilution that resulted in bacterial death. This process was carried out in triplicate.

Statistical analysis. The IZ caused by different concentrations of BV and the MIC and MBC of BV (100 µg/mL) and common antibiotics were analyzed by one-way analysis of variance, followed by Tukey's posthoc test. Comparison between BV and common antibiotics in three different bacterial strains was analyzed by two-way analysis of variance, followed by Tukey's posthoc test using GraphPad Prism software version 7. Data were reported as mean± standard deviation.

Results and Discussion

Antimicrobial resistance has become a new challenge for human life. Researchers are trying to explore new materials with antimicrobial properties for the treatment of diseases. Natural products, including BV, have been traditionally used and recognized as an antibacterial agent against several Gram-positive and -negative to treat a wide range of infectious diseases.

Results showed that the different concentrations of BV had an inhibitory effect on all three species of *S. aureus*, *K. pneumonia*, and *P. aeruginosa* at 24, 48, and 72 h after treatment. Meanwhile, 5 µg/mL BV had no effect on *P. aeruginosa*. A lethal toxic effect on the three pathogens was caused by high concentrations of BV (Figure 1). Among the three pathogens, BV in high concentrations had the most antibacterial effect on *S. aureus* and *K. pneumonia* ($p < 0.0001$).

A comparison of IZ for the three bacterial strains between BV (100 µg/mL) and three common antibiotics showed that the BV had a high effect on *K. pneumonia*. Meanwhile, ampicillin and penicillin had a lower effect on *K. pneumonia* and *P. aeruginosa* compared with BV and tetracycline (Figure 2, $p < 0.05$). BV showed a lower effect on *P. aeruginosa* compared with *S. aureus* and *K. pneumonia.* However, BV had a greater IZ for *P. aeruginosa* compared with the other antibiotics ($p < 0.05$). Tetracycline showed a better effect on *S. aureus* than BV and the other antibiotics (Figure 2, $p < 0.05$).

The antimicrobial effect of BV is a complex process due to the presence of various bioactive compounds, including enzymes, peptides, nonpeptides, and biogenic amines [15]. Furthermore, BV contains phospholipase A_2 (PLA_2) that exhibits antimicrobial effects. Hence, PLA_2 may be the cause of the antibacterial effects of BV; however, BV also contains melittin, which may amplify these effects . Gram-positive bacteria are typically more sensitive to antimicrobial agents than Gram-negative bacteria due to the variations in their cell wall compositions. Gram-positive bacteria have mucopeptide compositions, and Gram-negative bacteria have a thin

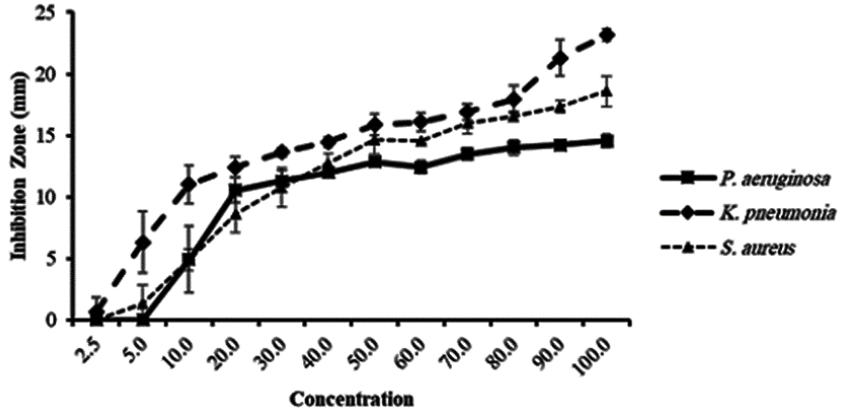

Figure 1. Inhibition Zone Effect of Different Concentrations of Honey Bee Venom on Three Species of Bacteria

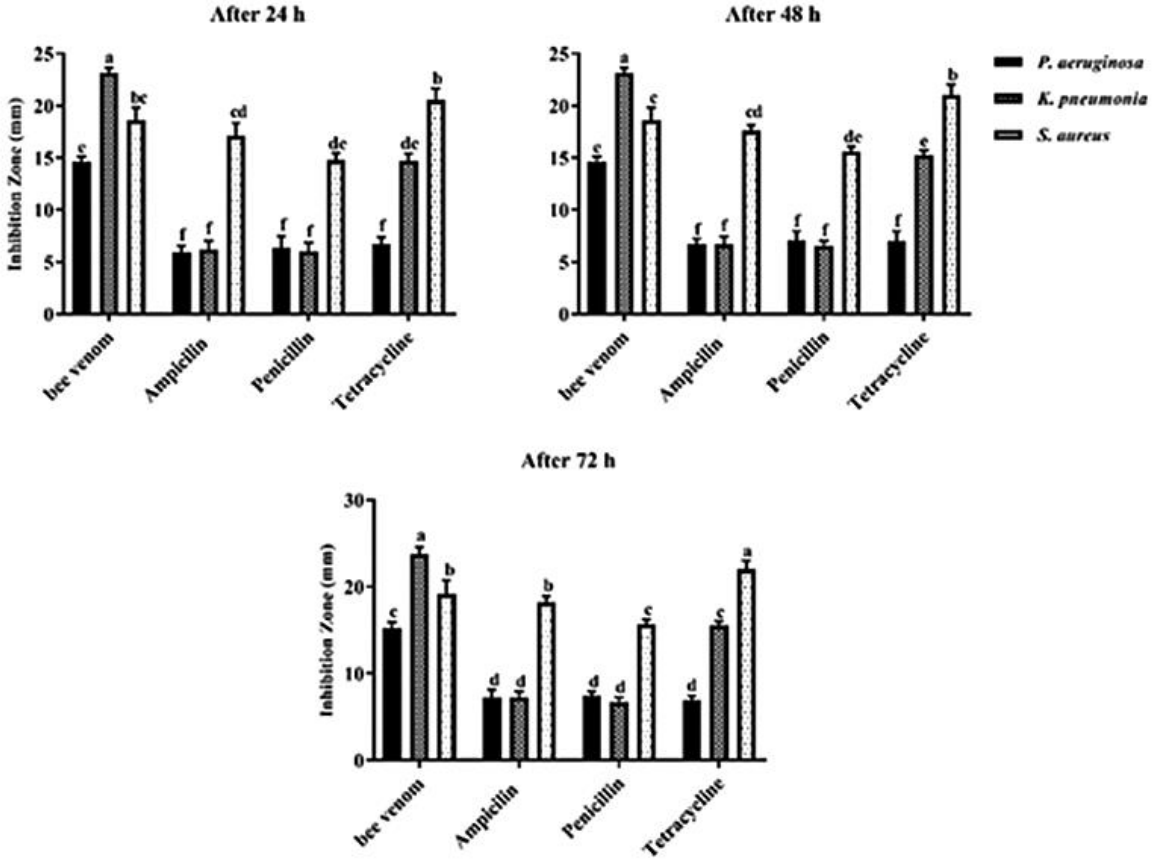

Figure 2. Comparison of the Inhibition Zone for *S. aureus*, *K. pneumonia*, and *P. aeruginosa* between Honey Bee Venom (100 µg/mL) and Common Antibiotics after 24, 48, and 72 h

layer of mucopeptides and most of their cell walls are made of lipoprotein and lipopolysaccharide. As a result, Gram-negative bacteria are more resistant to antibacterial agents than Gram-positive bacteria [16]. Our research showed that BV had better inhibitory effects against *K. pneumonia* compared with *P. aeruginosa*, even though both are Gram-negative bacteria. The inhibitory differences are probably due to the antimicrobial resistance pattern and require further investigations. Our results are compatible with those obtained by Zolfagharian *et al*. [17], who evaluated the effect of BV on several Gram-positive and -negative bacteria

including *S. aureus*, *S. typhimurium*, and *E. coli*. The results indicated that BV had a better inhibitory effect on *E. coli* than on other Gram-positive bacteria.

A comparison of MIC for the three species of bacteria between BV and common antibiotics was conducted by two-way ANOVA. The findings showed that BV and two antibiotics (ampicillin and penicillin) had a low effect on *P. aeruginosa*, with MIC of 38, 24, and 25 µg/mL for BV, ampicillin, and penicillin, respectively. The lowest MIC values of BV were observed for *K. pneumonia* and *S. aureus* at 18 and 21 µg/mL, respectively.

The lower MBC value of BV on *K. pneumonia* than on *S. aureus* and *P. aeruginosa* was related to the higher antibacterial activity of BV on this pathogen than on the other two bacteria.

BV also had a high MIC, showing that it was less effective on the three species of bacteria compared with the three antibiotics. The results of MIC showed that *S. aureus* was more sensitive to BV and all three common antibiotics than the other two pathogens ($p < 0.05$, Figure 3). The highest MIC was observed for BV against *P. aeruginosa*, and the lowest MIC was found for the common antibiotics against *S. aureus* ($p < 0.05$, Figure 3).

The results of MBC showed that BV had higher MBC and showed less inhibitory effect on pathogens compared with the three common antibiotics ($p < 0.05$). Ampicillin, penicillin, and tetracycline had a greater inhibitory effect on *S. aureus* than on the two bacteria ($p < 0.05$). The highest MBC was observed for BV against *P. aeruginosa* ($p < 0.05$, Figure 4).

In this study, we evaluated the antibacterial activity of BV against *S. aureus*, *P. aeruginosa*, and *K. pneumonia* and compared it with common antibiotics. Results showed that BV had the highest effect on *K. pneumonia* and *S. aureus* and the lowest effect *on P. aeruginosa*. The highest effect of BV was observed against *K. pneumonia* with a MBC of 24 µg/mL. In 2015, AL-Ani *et al*. [32] reported that 30 µg/mL BV had the best antimicrobial activities on K. *pneumonia*. Our results were compatible with those obtained by AL-Ani *et al*. and Jalaei *et al*. [18], who reported that MIC of wasp (*Vespa orientalis*) venom was 128 and 64 µg/mL on *K. pneumonia* and *S. aureus*, respectively. Their study also indicated that wasp venom had potential inhibitory effects on Gram-positive and -negative bacteria. In our study, the MIC of BV for *K. pneumonia* and *S. aureus* was 18 and 21 µg/mL, respectively. BV was more effective in terms of its inhibitory effect on *K. pneumonia* and *S. aureus* when

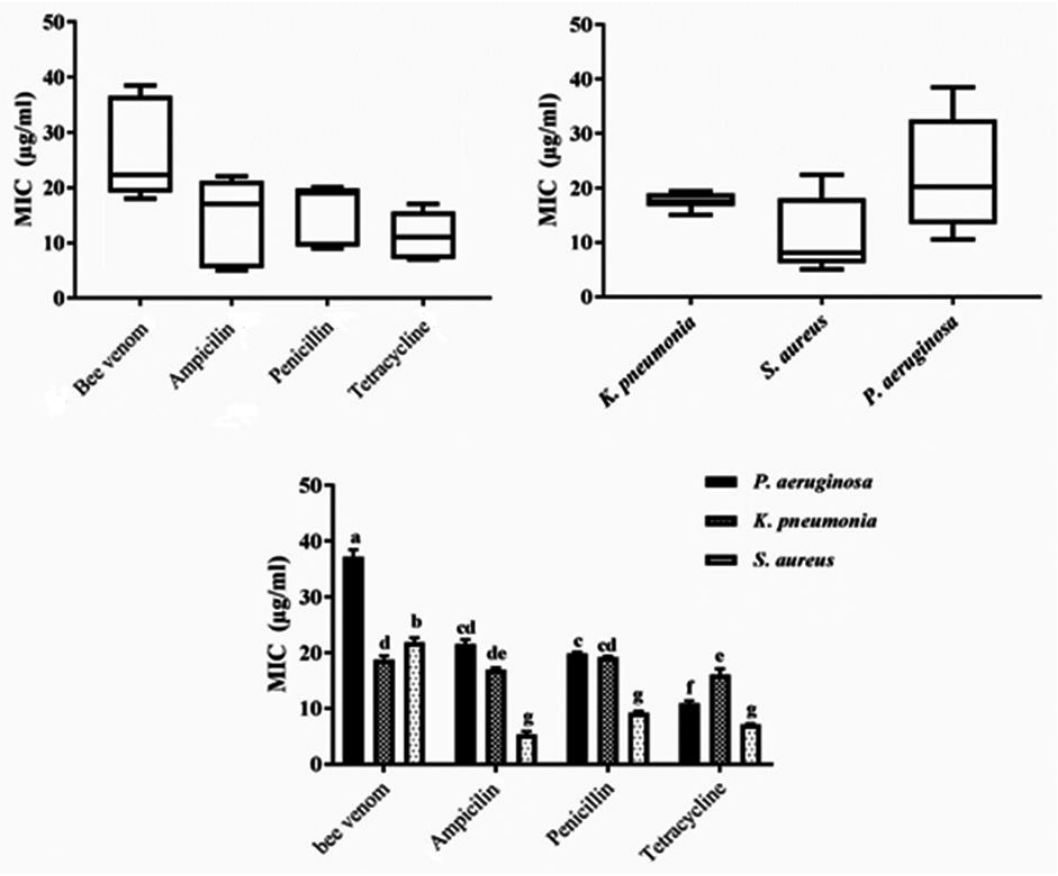

Figure 3. MIC Value of Honey Bee Venom and Common Antibiotics for *S. aureus, K. pneumonia,* and *P. aeruginosa*

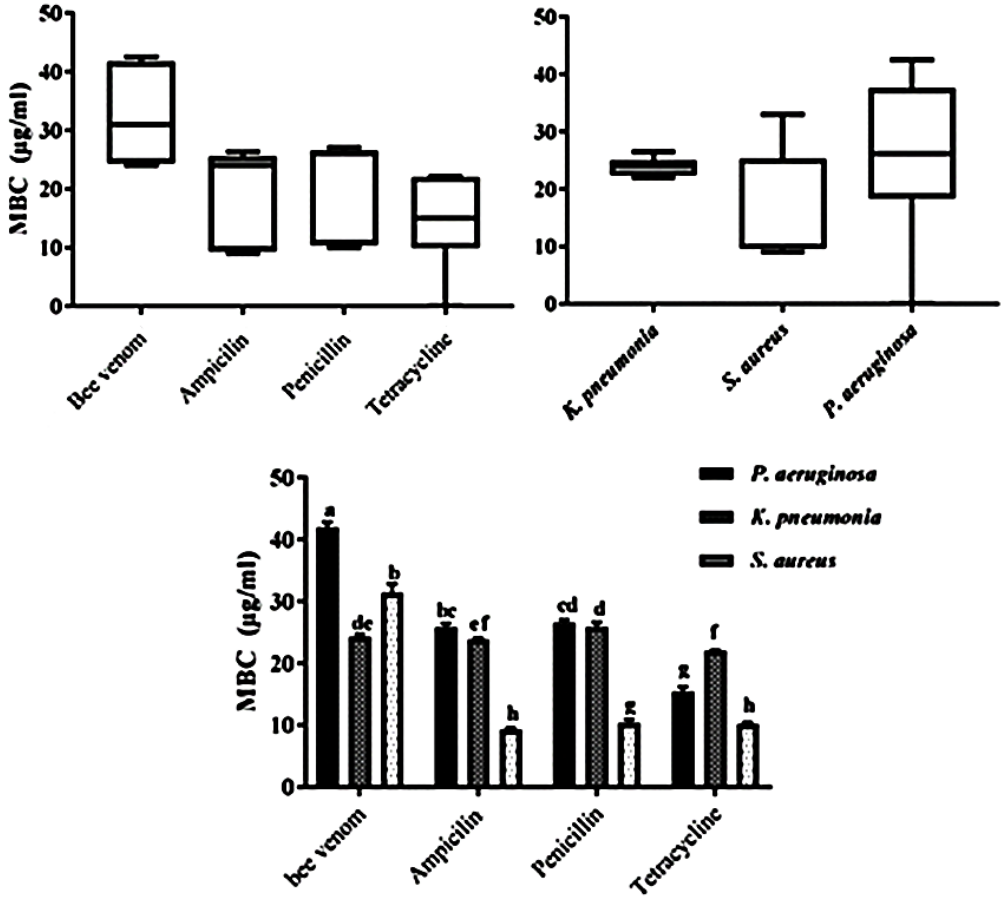

Figure 4. MBC of Honey Bee Venom and Common Antibiotics for *S. aureus*, *K. pneumonia*, and *P. aeruginosa*

compared with the result of Han *et al*. [19], who determined the effect of BV on *S. aureus* and found that the MIC was 15.5 µg/mL. Changes in the antimicrobial properties of BV against *K. pneumonia* and *S. aureus* can be caused by the different amounts of compositions and antibacterial resistance [20]. In our study, the highest MIC and MBC of BV were found for *P. aeruginosa* (38 and 42 µg/mL, respectively). This finding revealed that BV had a lower effect on *P. aeruginosa* than on *S. aureus* and *K. pneumonia*. Frangieh *et al*. [36] tested the effect of BV on *P. aeruginosa* and revealed that the antibacterial activity of BV was 38% at the MIC of 50 µg/mL. Zolfagharian *et al*. [21] showed that BV had a positive effect on *E. coli* and *S. typhimurium* but had no effect on *P. aeruginosa*. The current results suggested that honey BV generally has antimicrobial properties against pathogens, and tetracycline had a better effect on *P. aeruginosa* than BV and other antibiotics.

BV also had a high MIC, showing it was less effective on the three species of bacteria compared with the three antibiotics. The results of MIC showed that *S. aureus* was more sensitive to BV and all three common antibiotics than the other two pathogens ($p < 0.05$, Figure 3). The highest MIC was observed for BV against *P. aeruginosa*, and the lowest MIC was noted for common antibiotics against *S. aureus* ($p < 0.05$, Figure 3).

The antibacterial activities of natural compounds depend on different factors, including time, composition, temperature, and other parameters [22]. Although IZ sizes are measured after a few hours of incubation, many researchers measure the susceptibility of the bacteria to compounds after overnight microbial growth. In our study, the evaluation of IZs at different times demonstrated that different concentrations of BV had the greatest effect at 24 h than at 48 and 72 h (Figure 2).

The results of this study revealed that Gram-positive bacteria are more sensitive to BV than Gram-negative bacteria. This finding might be related to their different cell wall envelopes [23, 24]. According to Quistad *et al*. [25], the antimicrobial effect of wasp venoms is mostly due to the interaction between peptides and the anionic components of bacterial membranes that eventually destroy the bacterial cell. The primary target of peptides in venom is the bacterial cell envelope and the cell wall [26].

Conclusion

This study concluded that BV with remarkably antimicrobial properties can be a good candidate against pathogens, especially Gram-positive bacteria. MIC and MBC were measured in this work. Further studies on chemical, pharmacological, and molecular mechanisms are needed to enhance the efficacy of BV. New investigations on the toxicological effects of BV on normal cells in vitro are recommended.

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