Short Communication
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Enhanced Antibacterial Activity of Manuka Honey with Higher Methylglyoxal Concentration Against *Staphylococcus aureus*: *in vitro* Study

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Abstract
Objective: This study aimed to investigate the antibacterial activity of two types of Manuka honey with varying concentrations of methylglyoxal (MGO), 70 and 1050 mg/kg against *S. aureus* in vitro.
Methods: Two types of Manuka honey were purchased and tested for their antimicrobial activities. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the broth tube dilution method.
Results: The results revealed that both concentrations of Manuka honey exhibited antibacterial activity against *S. aureus*, with the higher concentration (1050 mg/kg) exhibiting enhanced antibacterial activity. The MIC was determined to be 100% v/v (undiluted) for the initial concentration and 1:2 (50% v/v) for the higher concentration. The MBC was not reached at the dilution level used in this experiment for the initial concentration, but it was inferred to be at a dilution of 1:2 for the higher concentration.
Conclusion: These findings suggest that Manuka honey with a higher concentration of MGO may be more effective in inhibiting the growth of *S. aureus* and potentially killing bacteria present. The results of this study highlight the potential use of a high concentration of Manuka honey as an alternative antimicrobial agent against *S. aureus*. Further investigation into its efficacy in various clinical settings is required.
Keywords: Manuka honey, *S. aureus*, methylglyoxal, minimum inhibitory concentration

Introduction

Staphylococcus aureus is a significant global contributor to morbidity and mortality from infections, causing a range of conditions from skin infections to severe pneumonia and septicemia. The challenge of managing *S. aureus* infections is heightened by rising antibiotic resistance and the lack of an effective vaccine.[1] This situation underscores the urgent need for alternative antimicrobial treatments, prompting a reevaluation of traditional remedies, such as honey.[2] Honey's complex chemical composition varies with its botanical origin, climate, and harvesting practices, contributing to its antimicrobial effectiveness. Although the exact mechanism behind honey's antimicrobial properties is not fully understood, key factors include high sugar content, low water content, acidic pH, and hydrogen peroxide production upon dilution.[3] Notably, no bacterial resistance to honey as an antimicrobial agent has been reported, likely due to its unique blend of natural components.[4]

Manuka honey, derived from the blossoms of the Manuka tree in New Zealand, is renowned for its antibacterial properties and is used in various therapeutic applications, particularly for wound healing. Its strong antibacterial activity is largely attributed to methylglyoxal (MGO), with commercial Manuka honey products containing MGO concentrations ranging from 70 mg/kg to over 1100 mg/kg.[5] While many components contribute to its efficacy, MGO is often highlighted as the key active ingredient.[6] However, the relationship between MGO concentration and Manuka honey's antibacterial effectiveness is still not fully understood. Research indicates that MGO modifies the structure of bacterial fimbriae and flagella, potentially reducing bacterial adherence and motility.[7] This study aims to compare the effects of two different concentrations of Manuka honey on the *S. aureus* ATCC 25923 strain to assess if higher MGO levels enhance antibacterial activity.

Materials and Methods

All sample processing and honey preparation was done in Al Baha University, Faculty of Medicine, Microbiology laboratory. The used method is based on Kacaniova et al., and Payveld studies.[8,9]

Honey Preparation and Processing

Two types of Manuka honey with initial MGO concentrations were used for the experiments: 70 mg/l for the first concentration and 1050 mg/l for the second. A spoonful of each honey sample was placed in sterile screw-cap containers and stored in the laboratory. Initial filtration was performed through a sterile mesh to remove particulate matter, after which the clarified samples were refrigerated at 2–8°C for later use. Both honey samples followed the same processing protocols according to the experimental design.

Honey Quality Control

Honey samples were assessed for bacterial presence. Each stock tube was cultured on nutrient agar plates and incubated at 37°C for 48 hours to confirm sterility.

Preparation of Bacterial Isolates

The reference *S. aureus* ATCC 25923 was used in this study. Three to five pure colonies were picked from the nutrient agar plate with an inoculating wire loop, suspended in 4–5 ml of nutrient broth, and incubated at 37°C for 24 h. The bacteria suspension was diluted with sterile distilled water

until it matched the turbidity of 0.5 McFarland Standards (10^5 CFU/ml).

The Minimum Inhibitory Concentration Preparation

The minimum inhibitory concentration (MIC) of various honeys was determined using the broth tube dilution method. A total of twenty sterile test tubes were arranged into two racks, each containing ten labeled tubes numbered 1 to 8. Quality controls, namely Honey Control (HC) and Growth Control (GC) tubes, were included at the end of the series.

One ml of freshly prepared nutrient broth was dispensed into all test tubes. Next, one ml of the first concentration honey solution was added to tube 1 of rack one and the HC tube using sterile micropipettes. A two-fold serial dilution was then performed by transferring one ml of the honey stock into tube 2, followed by thorough vortexing. This was repeated up to tube 8, achieving a dilution of 1:128, after which one ml was removed and discarded from tube 8. The GC tube contained no honey, while the HC tube was devoid of bacterial inoculum. All tubes, except the HC, were inoculated with one ml of cultured *S. aureus* suspension. This process was replicated for the second concentration on the second rack. After inoculation, all twenty tubes were incubated at 37°C for 24 hours, with periodic inspections to check for growth indicated by turbidity levels (Figure 1).

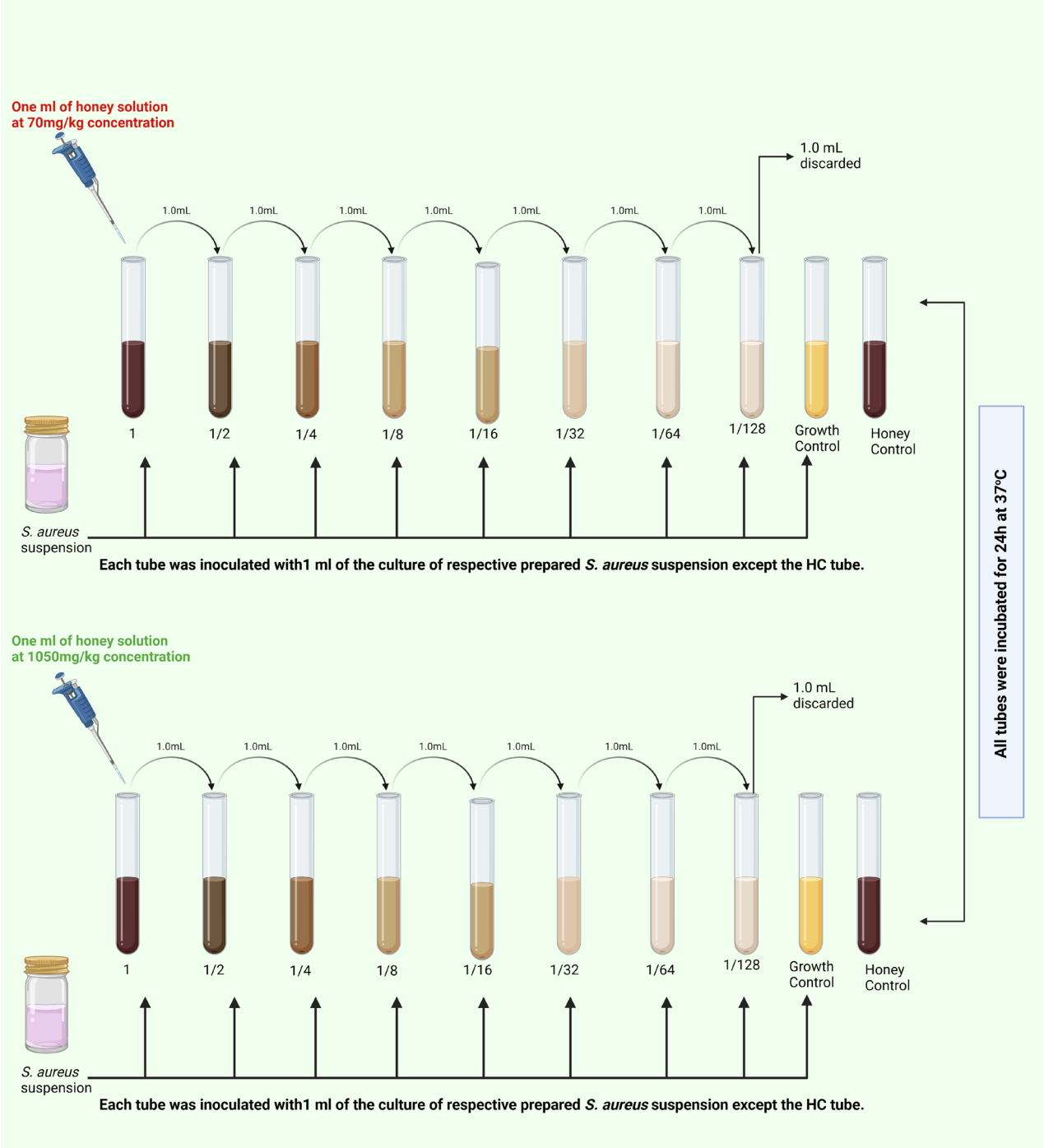

Fig. 1 **The minimum inhibitory concentration (MIC) preparation for both concentration.**

Minimum Bacterial Concentration

The minimum bacterial concentration (MBC) was ascertained through the subculturing of all incubated nutrient broth tubes of the two different concentrations. The plated samples were subsequently incubated aerobically at 37°C for a period of 24 hours. This procedure was undertaken to validate the minimal honey concentration at which microbial growth was absent in the tubes, thus defining the determined MBC value on the plates. The impact of honey on bacterial growth was categorized as bactericidal if no growth was observed on the plates, bacteriostatic if there was light to moderate growth, and ineffective if heavy growth was detected (Figure 2).

Results

After incubation, bacterial growth was confirmed in the growth control (GC) tube, indicating turbidity, while the no growth control (HC) tube demonstrated the absence of growth. All other tubes were compared to the GC tube to assess turbidity. A positive mark (+) was assigned to tubes exhibiting growth, whereas a negative mark (–) was assigned to those without turbidity. The minimum inhibitory concentration (MIC) was identified as the lowest concentration that completely inhibits growth.

In the initial concentration of 70 mg/kg, no growth was noted in the first tube, with light growth in the second tube and heavy growth in subsequent dilutions. Only two colonies appeared on the agar plate for the second tube, while heavy growth was observed in all other plates, increasing with decreasing dilution (Table 1). For the second concentration of 1050 mg/kg, complete inhibition of bacterial growth occurred in the first two dilutions (1/2), marking the MIC, while growth was present in the remaining tubes. Similar patterns were observed in the minimum bactericidal concentration (MBC) testing, with only the first two plates showing no growth and the others exhibiting increased growth (Table 2).

Discussion

Honey is well-known for its antimicrobial properties, demonstrating both bacteriostatic and bactericidal effects on various bacteria, as shown in this study, where specific concentrations of honey inhibited bacterial growth. These results align with other studies highlighting honey's antimicrobial properties.[10,11] However, honey's effectiveness can vary based on its type and floral source. Different honey types from various regions contain varying levels of bioactive compounds that influence their antimicrobial activity.[12,13] Notably, Manuka

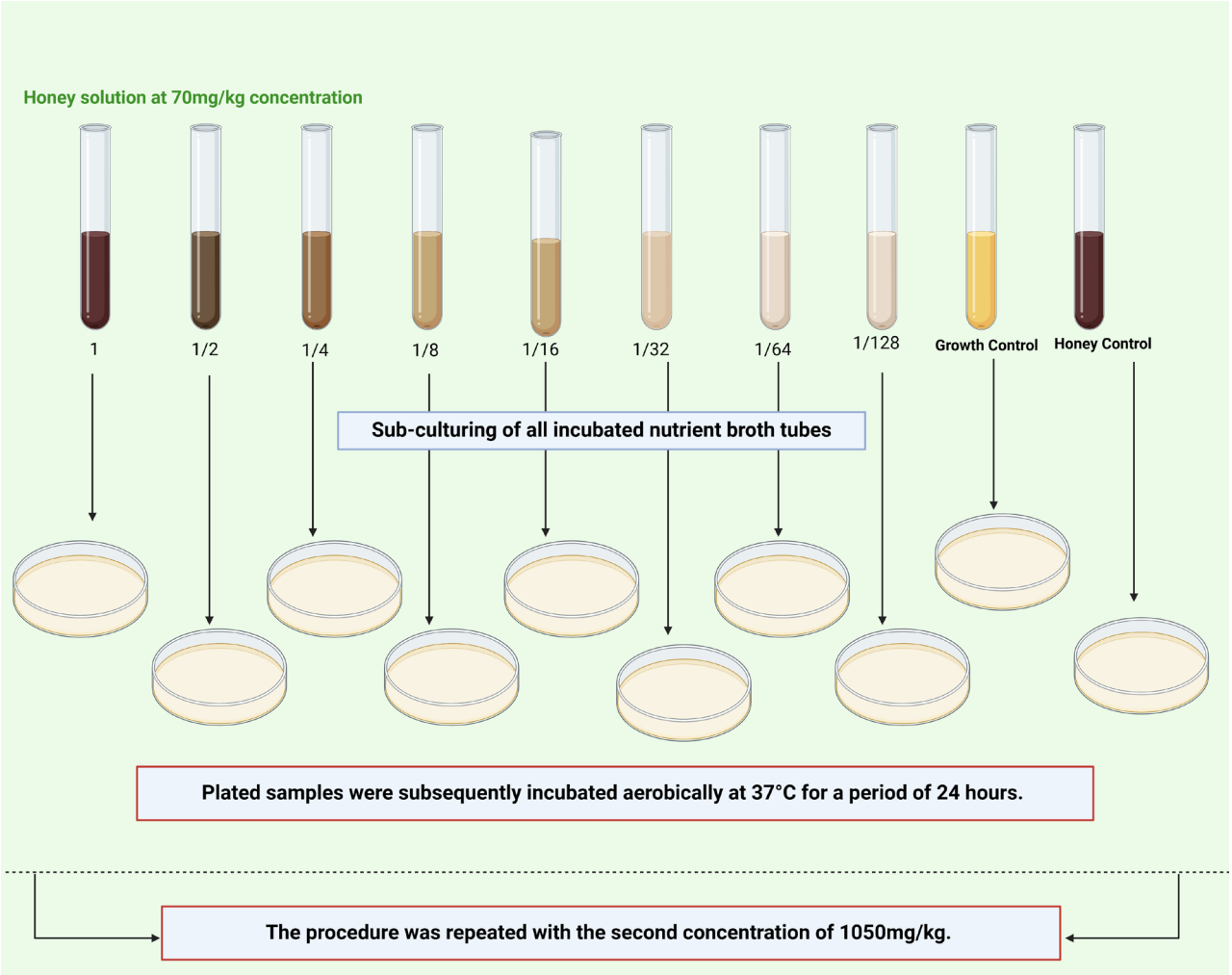

Fig. 2 **Measurement of the minimum bacterial concentration (MBC).**

Table 1. **MIC% and MBC (v/v) of Manuka honey containing 70 mg/kg of MGO**

	Honey Dilutions									
Bacteria	**Net 1**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**MIC% (v/v)**	**MBC%(v/v)**
S. aureus	–	+/–	+	+	+	+	+	+	100	50

+, Bacterial growth; –, No bacterial growth; +/–, a trace of growth.

Table 2. **MIC% and MBC (v/v) of Manuka honey containing 1050 mg/kg of MGO**

	Honey Dilutions									
Bacteria	**Net 1**	**1/2**	**1/4**	**1/8**	**1/16**	**1/32**	**1/64**	**1/128**	**MIC% (v/v)**	**MBC%(v/v)**
S. aureus	–	–	+	+	+	+	+	+	50	50

+, Bacterial growth; –, No bacterial growth.

honey is particularly rich in methylglyoxal (MGO), which exhibits enhanced antimicrobial activity compared to other honey types.[3,14]

S. aureus is a prominent human pathogen responsible for a diverse array of clinical infections, ranging from superficial infections like skin and soft tissue infections, wound infections, to more serious infections such as bacteremia, infective endocarditis, osteoarticular, pleuropulmonary, and device-related infections.[15] Given the effectiveness of Manuka honey in inhibiting the growth of bacteria, MGO has been identified as the main component responsible for its antibacterial effect.[2,16] However, it remains unknown whether the efficacy will increase with higher concentrations of MGO or not. Therefore, this study aims to compare two types of Manuka honey in vitro, one with the lowest concentration of MGO and the other with the highest found in the market. This study result showed both concentrations (70 and 1050 mg/Kg) have an antibacterial effect on *S. aureus*. Though the initial concentration, visual inspections revealed no growth in the first tube and a trace of growth (+/–) in the second tube. Subsequent tubes exhibited increasing turbidity with dilution, indicating the growth of *S. aureus*.

Based on these observations, the Minimum Inhibitory Concentration (MIC) can be determined as the lowest dilution of honey that completely inhibits the growth of *S. aureus*. The first tube with no growth, indicating that the MIC is the undiluted concentration (1:1) or 100% v/v. The MIC is the concentration at which no visible growth of the targeted microorganism is observed. Therefore, the MIC of honey against *S. aureus* in this experiment is identified as 100% v/v or undiluted, where no growth was detected. However, subsequent dilutions showed a decrease in efficacy, with faint growth evident, suggesting that the first dilution may only be bacteriostatic, as it impedes growth but does not fully inhibit it. A previous study by Brudzynski and Lannig[17] also observed the bacteriostatic effect of honey. Although there is a similarity in the observed effect of honey, it is important to note that honey preparation can vary depending on its source, initial dilution, and processing factors. Consequently, solely comparing results based on dilution may not be advisable at this point. Nevertheless, the confirmed antibacterial effect of honey in a bacteriostatic manner remains consistent with previous reports.[14]

In this experiment, honey's Minimum Bactericidal Concentration (MBC) was also evaluated. The first tube showed no growth during incubation, the second indicated trace growth (+/–), while the other tubes displayed increasing turbidity, suggesting *S. aureus* growth. On agar plating, the first tube had no colonies, the second had two, and the rest showed heavy growth with dilution. The MBC is the lowest dilution with no colonies, indicating bactericidal activity. Since the second plate had two colonies, a bacteriostatic effect was noted, and the MBC remains undetermined. Results suggest the MBC of honey against *S. aureus* at 75 mg/kg lies between the first and second dilutions.[11]

For the honey concentration at 1050 mg/kg, no growth was detected in the first two tubes, while growth occurred in all subsequent tubes (from the third onwards). This indicates that the concentration in the second tube represents the MIC, determined to be a dilution of 1:2. Thus, the MIC is 50% of the original concentration of honey in the nutrient broth, effectively inhibiting *S. aureus* growth. This experiment shows that a 50% v/v concentration of honey possesses antimicrobial properties, suggesting higher concentrations of Manuka honey are more effective against bacterial growth. Hence, concentration is crucial for honey's antimicrobial applications.[18]

When the contents of all the tubes were cultured on nutrient agar plates, the first two plates showed no growth, indicating that the honey concentrations in these tubes effectively inhibited and killed *S. aureus*. This suggests an inferred Minimum Bactericidal Concentration (MBC) at a dilution of 1:2, equivalent to a 50% v/v concentration of honey. These findings support the notion that manuka honey with high MGO concentrations may act as a natural and potent bactericidal antibiotic.[19,20]

The potential of methylglyoxal (MGO) as a therapeutic agent raises the question of whether isolating and purifying it from honey could be effective for treating skin and soft tissue infections. Assessing the safety and potential side effects of isolated MGO is essential; thus, initial studies on animal models should evaluate its efficacy before human trials. If successful, MGO could emerge as a novel topical antibiotic with minimal side effects. Its antimicrobial activity must also be tested against various pathogens to understand its broad-spectrum effectiveness. Expanding research to include diverse microbial strains will help clarify MGO's potential applications. Given the antimicrobial properties of manuka honey, particularly its high MGO levels, it offers a promising route for developing natural topical antibiotics, especially against pathogens like *Staphylococcus aureus*.[21] Further investigation into purified MGO's therapeutic potential and preclinical studies

is warranted to establish its safety and efficacy for clinical applications.

Conclusion

This study investigated the antibacterial activity of Manuka honey with varying methylglyoxal (MGO) concentrations against *Staphylococcus aureus* isolates in vitro. Results showed that both concentrations exhibited antibacterial properties, with the higher concentration (1050 mg/kg) displaying enhanced bactericidal activity. The Minimum Inhibitory Concentration (MIC) was 100% v/v (undiluted) for the initial concentration and 1:2 (50% v/v) for the higher concentration. The Minimum Bactericidal Concentration (MBC) was inferred to be 1:2 for the higher concentration, while it was not reached for the initial concentration. These findings suggest that Manuka honey with higher MGO may effectively inhibit and kill *S. aureus*. Further research is needed to purify MGO and evaluate its effectiveness for treating skin and soft tissue infections in animal models before progressing to human clinical trials.

Conflicts of Interest Disclosure

The author declare that he has no competing interests or conflicts of interest regarding this research.

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