



## Effect of Stingless-Bee (*Trigona Itama*) Honey Against Selected Gram-Positive Oral Pathogens

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### ABSTRACT

**Objective:** Honey is a naturally occurring resource with a long history of being utilized as complementary medicine. The generation of hydrogen peroxide by enzymes in most kinds of honey provides antibacterial action. However, another species of honey has significant antibacterial properties against some microorganisms. Therefore, this study aims to assess the antimicrobial effect of stingless bee (*trigona itama*) honey (kelulut) on *S. aureus*, *E. faecalis*, *S. mutans*, and *E. coli*.

**Methods:** The honey was sourced locally in raw form and sent for decontamination by gamma irradiation which was achieved by exposing it to a dose of 25 kGy. The antimicrobial screening was carried out using the agar well diffusion technique, and concentrations of honey selected for this purpose were 1%, 5%, 10%, and 20%.

**Results:** The results demonstrated that Kelulut honey did not show any antimicrobial effect against the tested pathogens, which disagrees with numerous studies reported earlier. This preliminary data provides valuable insights as specific Gram-positive oral pathogens are resistant to the antimicrobial capability of Kelulut honey.

**Conclusion:** Kelulut honey could not demonstrate antimicrobial properties on the disc diffusion method. Further research needs to be conducted to determine the accurate concentration of KH needed to inhibit virulent bacteria. Further research should be conducted to evaluate KH at different manuka levels and different temperatures.

**Keywords:** Community; Data; Health; Patient; Public; Vaccines

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### Introduction

Honey is a natural substance produced by a variety of bees (*Apis mellifera* L.) who pollinate succulent flowers and use their nectar.<sup>1</sup> Honeybees accumulate the collected nectar in honeycombs. Once collected, the nectar undergoes processing in an internal pouch called the crop, where various enzymes break down sugars. The resulting solution is then released by the bees into the honeycombs. Finally, through the flapping of bee wings, air currents facilitate the evaporation of excess liquid.<sup>2</sup> The entire process creates a thick, viscous liquid with 80% sugars, 17% water, and 3% other

substituents, even though the exact composition depends on the source of nectar.

Besides its high nutritional value and worldwide use as a source of carbohydrates, its medicinal properties have been highly acknowledged around the globe and throughout history. Ancient literature referencing the medicinal properties of honey can be found in Sumerian Clay tablets, Veda, Egyptian papyri, the Bible, and the Holy Quran.<sup>3</sup> In modern medicine, honey has been mentioned in literature to have antibacterial properties, antioxidant properties, healing properties, and anti-inflammatory and anti-hypertensive properties.<sup>4</sup>

Researchers have shown great interest in

the anti-bacterial properties of honey. Previous literature is available on its action against a variety of bacterial species including *Staphylococcus aureus*, *Escherichia faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Lactobacillus acidophilus*, and *Salmonella typhimurium*.<sup>5</sup> However, vast research is being conducted to determine the mechanism of action and mode of use. Based on their clinical and pathological significance, this study was conducted to investigate the antibacterial properties of honey against *Staphylococcus aureus* (*S. aureus*), *Streptococcus mutans* (*S. mutans*), *Enterococcus faecalis* (*E. faecalis*) and *Escherichia coli* (*E. coli*). After decades, *S. aureus* remains one of the most versatile and dangerous pathogens in humans. The likelihood of acquiring both community-acquired and hospital-acquired *Staphylococcus* infections has increased with little change in mortality. Treating such infections has become difficult due to the emergence of drug-resistant strains.<sup>6</sup> *S. mutans* is a facultative anaerobic and Gram-positive coccus commonly found in the oral cavity and is an important contributor to dental tooth decay. *S. mutans* are naturally present in the human oral microbiota; nevertheless, oral streptococci have harmless and harmful bacteria. Under the circumstances, commensal streptococci can turn into opportunistic pathogens, ultimately initiating disease and harming the host.<sup>7</sup> *E. faecalis* is a Gram-positive, facultatively anaerobic, and commensal bacterium that inhabits the gastrointestinal tracts of humans. It is usually found in healthy humans but can contribute to life-threatening infections, especially in hospital environments. As referenced in this study, a non-oral pathogen used *E. coli* is a Gram-negative, facultatively anaerobic rod-shaped bacterium belonging to the genus *Escherichia*. It is commonly found in the human intestine. Most *E. coli* strains are harmless to the host, but sometimes they can cause severe food poisoning.<sup>8</sup>

To achieve maximum therapeutic benefits, honey is required to be free from any contaminating agents. There are many known sources of contamination, including pollen, the digestive tract of bees, dust, nectar, or air. Also, contamination after honey harvesting is frequently problematic.<sup>9</sup> To address this issue, various traditional techniques such as pasteurization or blanching have been employed to deal with microorganism contamination which often jeopardizes product characteristics. Gamma irradiation, utilized as a

phytosanitary measure, has demonstrated its safety and efficacy in enhancing the hygienic quality of diverse herbal materials and foods. According to pioneer scientists Allen and Molan, to ensure the sterility of honey, a dose of 25 kilograys (kGy) of gamma irradiation is adequate for phytosanitation purposes. In particular, another study revealed that acidity, pH, sugar, and minerals contents in their studied honey were not affected significantly by gamma irradiation, while moisture, 5-Hydroxymethylfurfural (HMF) level, and vitamin E contents decreased significantly with gamma irradiation. However, vitamin C and color intensity increased considerably after gamma irradiation. Thus, this study treated the honey with gamma irradiation to achieve its maximum antimicrobial benefit.

This study aims to assess gamma-irradiated *trigona itama* honey's antimicrobial effectiveness on well-documented pathogens. To do so, selected pathogens were treated with various concentrations of *trigona itama* honey, and the antimicrobial assessment was done using an agar well diffusion assay.

## Materials and Methods

The study was approved by the institutional review board (USM/JEPeM/21040344).

### Honey Sample

The local raw stingless bee honey (Kelulut) was sourced from Syamille Agro Farm & Resort Sdn Bhd., located in Kati Kuala Kangsar, Perak, Malaysia. Subsequently, the honey underwent sterilization for analytical purposes at Agensi Nuklear Malaysia, MINTec-Sinagama, using 25 kilograys (kGy) of gamma irradiation. For experimentation, treatment concentrations of kelulut honey (KH) (v/v) were prepared by diluting it with sterile distilled water, with fresh dilutions made before each experiment. The chosen KH concentrations included 1%, 5%, 10%, and 20%.<sup>10</sup>

### Test Organisms

Pathogens such as *E. coli* (ATCC® 25922, USA), *S. aureus* (ATCC® BAA-1026, USA), *S. mutans* (ATCC® 25175, USA), and *E. faecalis* (ATCC® 14506, USA) were selected. All organisms were grown on Blood agar (BA) (Sigma-Aldrich, USA), further maintained in Brain Heart

Infusion Broth (BHIB) (Oxoid, UK), and stored at 6°C. For *S. aureus* and *E. coli*, 500µg ampicillin (Lyka Labs, India) solution was prepared as positive control. Whereas for *S. mutans* and *E. faecalis*, 20µg ampicillin solution was prepared. The growth conditions of organisms are detailed in Table 1.

**Table 1:** Growth conditions of microorganisms used in the study

| Microbes           | Growth conditions                                                                     |
|--------------------|---------------------------------------------------------------------------------------|
| <i>E. coli</i>     | Medium: BA, BHIB<br>Atmosphere: Facultative anaerobic<br>Temperature: 37°C, 24h       |
| <i>E. faecalis</i> | Medium: BA, BHIB<br>Atmosphere: Facultative anaerobic<br>Temperature: 37°C, 24h       |
| <i>S. mutans</i>   | Medium: BA, BHIB<br>Atmosphere: Facultative anaerobic<br>Temperature: 37°C for 24-48h |
| <i>S. aureus</i>   | Medium: BA, BHIB<br>Atmosphere: Aerobic<br>Temperature: 37°C, 24h                     |

#### Assessment of antimicrobial properties

The antimicrobial properties of KL were assessed using the agar disc diffusion assay technique.<sup>11</sup> Before each experiment, 3 mL of Brain Heart Infusion Broth (BHIB) was warmed and dispensed into a sterile flat-ended standard McFarland glass tube. A small inoculation loop was sterilized by heating it over a flame till it turned red. The loop was then cooled and used to collect bacterial colonies from Blood Agar (BA) plates. Approximately three colonies of the test strain were selected from the agar plates and sub-cultured into fresh agar plates. From these subcultures, the colonies were then inoculated into the flat-ended glass tube containing BHIB. The turbidity of the microbial suspension was adjusted to  $1.5 \times 10^8$  colony-forming units per milliliter (CFU/mL) using a densitometer calibrated to the 0.5 McFarland turbidity standard.

Once the turbidity was adjusted to meet 0.5 McFarland standards, the microbial suspension was evenly distributed across the surface of the Blood Agar (BA) plates using a sterile cotton swab. Wells were then created on the agar surface using the blunt end of a sterile Pasteur pipette. Following the creation of the wells, 55µL of each of the four test concentrations, along with positive and negative controls, were introduced into the wells. Ampicillin served as the positive control, while sterile distilled water served as the negative control.

The agar plates were subsequently incubated in an incubator at 37°C for 24 hr. conducive to microbial growth. The plates were checked for microbial growth every day. After the designated incubation period, microbial growth became visible on the agar surface, and the antimicrobial activity was assessed based on the presence or absence of inhibition zones surrounding the wells. The diameter of inhibition zones around the wells was measured in millimeters using a ruler. All experiments were conducted in triplicate to ensure consistency and reliability of results.

#### Results

The antimicrobial effectiveness of gamma-irradiated KH was assessed under four concentrations (1%, 5%, 10%, 20%) against Gram-positive *S. mutans*, *S. aureus*, *E. faecalis*, and non-oral Gram-negative *E. coli*. The KH was not able to produce inhibition zones against tested concentrations (Figure 1). Small zones were found in a few samples, but the mean result was negatory. Likewise, the negative control wells containing distilled water showed no inhibition zones, while all positive control wells containing ampicillin effectively inhibited bacterial growth. The findings regarding the inhibition zones of KH, tested for its antimicrobial properties, are detailed in Table 2.

#### Discussion

This study tested the antimicrobial properties of gamma-radiated KH against four virulent microorganisms. KH was not able to prevent microbial growth on agar plates. The results contrast with other literature which demonstrates honey's effectiveness against these bacteria. In 2023, three different types of honey, namely Sidr honey, Tualang honey, and Manuka honey were tested against *S. aureus* and all three showed microbial inhibition on agar plates with Manuka honey showing the highest antibacterial capacity.<sup>12</sup>

Similarly, a study conducted on Malaysian Kelulut honey demonstrated a 39% reduction in *S. aureus* biofilm, a 41% reduction in *P. aeruginosa* biofilm, and a 37% reduction in *E. coli* biofilm at 30% KH concentration.<sup>13</sup> In a study conducted in Brazil, two honey samples derived from the stingless bee species *Scaptotrigona postica* and *Scaptotrigona bipunctata* were tested for their susceptibility to bacterial growth. The susceptibility testing,

performed using the agar well diffusion assay, revealed that both honey samples were effective in inhibiting the growth of both Gram-positive and Gram-negative bacteria, including *E. coli*, *E. faecalis*, *S. mutans*, and *S. aureus* at 50% honey concentration. However, very few studies were conducted with kelulut honey.

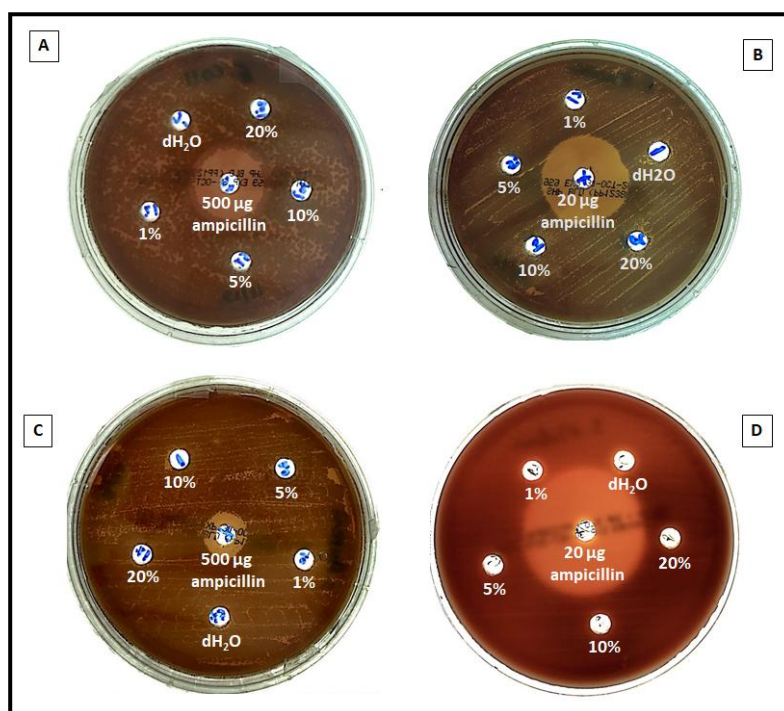
**Table 2:** Assessment of Pathogen Inhibition by Gamma-Irradiated KH

| Mean zones of inhibition (mm) |                  |                  |                    |                |
|-------------------------------|------------------|------------------|--------------------|----------------|
| KH Conc. (%)                  | <i>S. aureus</i> | <i>S. mutans</i> | <i>E. faecalis</i> | <i>E. coli</i> |
| Ampicillin                    | 14.22±3.34       | 32.87±2.55       | 26±2.64            | 21.67±1.86     |
| Distilled water               | -                | -                | -                  | -              |
| 1                             | -                | -                | -                  | -              |
| 5                             | -                | -                | -                  | -              |
| 10                            | -                | -                | -                  | -              |
| 20                            | -                | -                | -                  | -              |

**Note:** Positive control = Ampicillin (500µg/mL for *E. coli*, *S. aureus*; 20µg/mL for *S. mutans*, *E. faecalis*,)

Negative control = Distilled water

Data are reported as mean ± standard deviation.



**Figure 1:** Inhibition zones were observed around wells containing the positive control (ampicillin), while no inhibition zones were visible around gamma-irradiated KH concentrations and the negative control (distilled water) against **A.** *E. coli*, **B.** *E. faecalis*, **C.** *S. aureus*, and **D.** *S. mutans*

Few older studies have shown the in vitro antimicrobial effects of KH on lower



concentrations, e.g., Al Masaudi described in his paper that 10% KH was able to inhibit bacterial growth by 50%. However, in the follow-up studies, the minimum concentration documented for bacterial inhibition was 30-50%. This could be one of the main reasons that KH was unable to demonstrate antibacterial properties in this study because the concentrations used were below 30% KH. Some studies show the relevance of the manuka factor in the type of honey. A study conducted in Saudi Arabia compared four different types of honey with variable manuka factors. The type with the highest manuka factor showed the greatest antimicrobial properties.<sup>14</sup> The current study did not consider the manuka factor of KH which could be the reason for the null hypothesis. In addition, this study used gamma-radiation-treated honey, which is not used in other studies. In previous literature, commercial honey was procured and filtered for experimentation, which is different from our methodology. Thus, follow-up studies are required to enquire about the effects of gamma-radiation on the chemical composition of KH.

Indeed, recent research indicates that honey derived from different honeybee species may possess distinct biological characteristics. For instance, a study conducted in Malaysia aimed to identify the entomological sources of honey collected from various regions of the country. The honey analyzed originated from two primary sources: stingless bees (*Heterotrigona itama*) and conventional honeybees (*Apis* spp), including *Apis dorsata*, *Apis mellifera*, and *Apis cerana*. The findings of this study revealed noteworthy differences between the two types of honey. Honey produced by stingless bees exhibited higher levels of moisture content and water activity compared to honey derived from *Apis* spp. Moreover, the stingless bee honey demonstrated significantly superior antioxidant activity, indicating its potential for enhanced health benefits. These findings align with emerging research trends highlighting the diverse biological properties of honey originating from different bee species. Further investigation into the specific compounds and mechanisms underlying these differences could provide valuable insights into the potential therapeutic applications of stingless bee honey and contribute to the expanding body of knowledge on natural remedies.<sup>15</sup>

Overall, existing literature suggests that honey harbors antimicrobial properties against a wide range of pathogens. However, to establish a conclusive understanding, it's essential to explore the antimicrobial potential of Kelulut honey (KH)

in both its raw state and after sterilization using a filter. Additionally, it would be valuable to extract and examine bioactive compounds like phenols and flavonoids present in KH. These additional investigations can provide a more comprehensive insight into the antimicrobial efficacy of KH and elucidate the underlying mechanisms responsible for its therapeutic effects. Further studies could be conducted with different manuka levels of KH and KH with different temperatures. Such studies will give valuable insight into the chemical mechanisms underlying honey-microbial interactions.

## Conclusion

Kelulut honey could not demonstrate antimicrobial properties on the disc diffusion method. Further research needs to be conducted to determine the accurate concentration of KH needed to inhibit virulent bacteria. Further research should be conducted to evaluate KH at different manuka levels and temperatures.

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## Author Contribution

WNSS conceived the idea, collected data, and wrote the initial manuscript. RM collected data, analyzed the data, validated the results, and proofread the finalized manuscript.

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## Conflict of Interest

None

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