ORIGINAL ARTICLE
Journal of Science Technology Education Art and Medicine
DOI: 10.63137/jsteam.256955

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

Rizwan Mahmood[a], Wan Nazatul Shima Shahidan[a]*

[a]School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu, 16150, Kelantan, Malaysia

*Corresponding address: *School of Dental Sciences, Universiti Sains Malaysia, Kota Bharu, 16150, Kelantan, Malaysia*
Email: shima@usm.my

Received: 28 February 2024 / **Revised:** 29 April 2024 / **Accepted:** 08 May 2024 / **Available Online:** 10 June 2024

ABSTRACT

Objective: Honey is a naturally occurring resource with a long history of being utilized as a 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 agar well diffusion method. Further research needs to be conducted to determine the accurate concentration of KH needed to inhibit virulent bacteria. Future research should evaluate KH samples with characterised bioactive profiles (phenolics, flavonoids, organic acids) at concentrations of 30–100%, both in raw and gamma-irradiated forms, to determine whether the null result reflects sub-threshold dosing, irradiation-related degradation of bioactive compounds, or inherent species-specific limitations.
Keywords: Honey; Trigona itama; Stingless Bee; Antimicrobial Activity; Agar Well Diffusion; Gram-Positive Bacteria; Oral Pathogens

Copyright: © 2024 by the authors. This is an open-access article licensed under the terms of the Creative Commons Attribution 4.0 International, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Citation: Mahmood R, Shahidan WNS. Effect of Stingless-Bee (*Trigona itama*) Honey Against Selected Gram-Positive Oral Pathogens. J Sci Technol Educ Art Med. 2024;1(1):9-15

Introduction

Honey is a natural substance produced by a variety of bees (*Apis mellifera* L.) who pollinate succulent flowers and use their nectar. [1] 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. [2] 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. [3] In modern medicine, honey has been mentioned in literature to have antibacterial properties, antioxidant properties, healing properties, and anti-inflammatory and anti-hypertensive properties. [4]

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*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa, Streptococcus pyogenes, Lactobacillus acidophilus,* and *Salmonella typhimurium.* [5] 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. [6] *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. [7] *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. [8]

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. [9] 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%. [10]

These concentrations were selected to evaluate whether sub-inhibitory doses of KH exhibited any detectable antimicrobial effect. Although subsequent literature has reported minimum inhibitory concentrations in the range of 30–50% for similar honey types against comparable organisms, the present concentration range was established prior to the availability of those data and was designed to screen for activity at lower, more clinically practical dilutions. The absence of activity at 20% supports the need for future studies extending the concentration range to 30%, 50%, and undiluted (100%) KH.

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 6ºC. 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
E. coli	Medium: BA, BHIB Atmosphere: Facultative anaerobic Temperature: 37ºC, 24h
E. faecalis	Medium: BA, BHIB Atmosphere: Facultative anaerobic Temperature: 37ºC, 24h
S. mutans	Medium: BA, BHIB Atmosphere: Facultative anaerobic Temperature: 37ºC for 24-48h
S. aureus	Medium: BA, BHIB Atmosphere: Aerobic Temperature: 37ºC, 24h

† BA = Blood Agar; BHIB = Brain Heart Infusion Broth. All organisms were obtained as ATCC reference strains

Antimicrobial assessment by Agar well diffusion

The antimicrobial properties of KH were assessed using the agar well diffusion assay technique. [11] 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 x 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, producing wells of approximately 6 mm diameter. 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 incubated aerobically at 37 °C for 24 h (*S. aureus*, *E. coli*) or 24–48 h (*S. mutans*, *E. faecalis*). 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

OPEN ACCESS

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.

Statistical Analysis

All experiments were performed in triplicate on three separate occasions, yielding nine measurements per organism per concentration. Data are expressed as mean ± standard deviation (SD) of the zone of inhibition diameter (mm). Where no zone of inhibition was observed, the value was recorded as 0.00 mm. Given the uniformly null result across all KH concentrations and all organisms (0.00 mm in all replicates), no inferential statistical testing was warranted. Descriptive data were tabulated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

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).

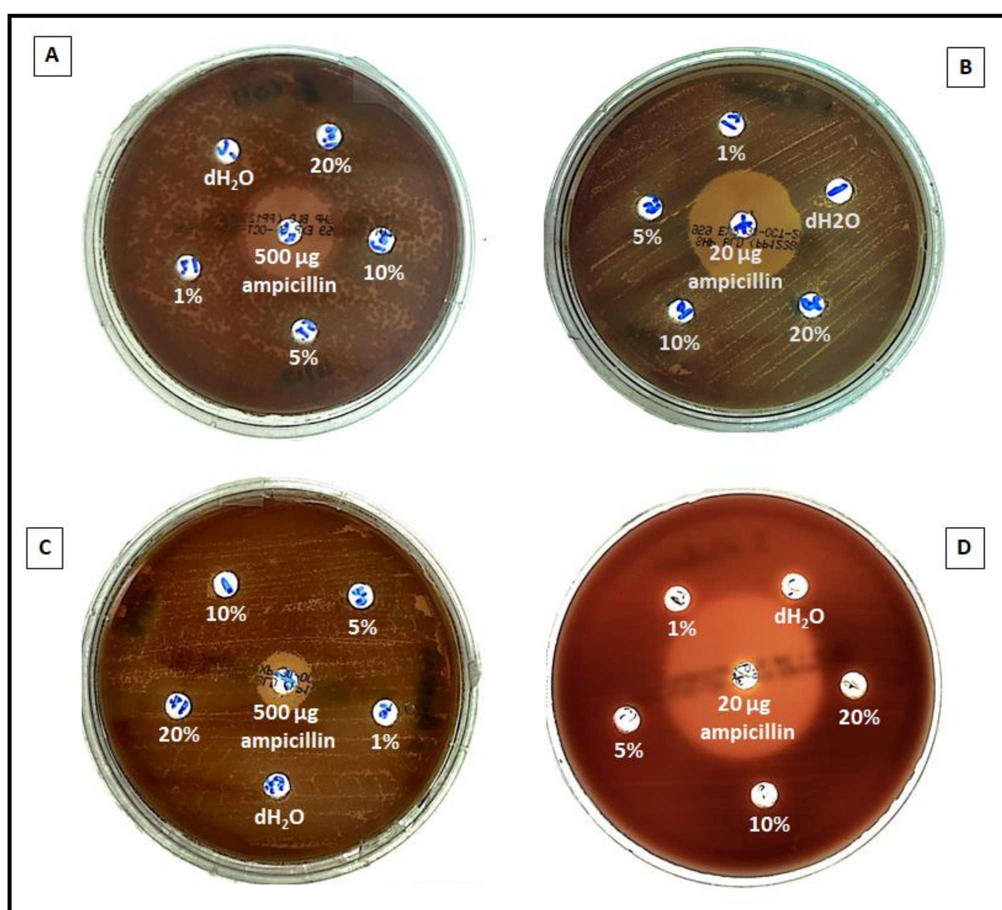

Figure 1: Inhibition zones were observed around wells containing the positive control (ampicillin), while no inhibition zones were visible around gamma-irradiated KH concentrations and the negative control (distilled water) against A. *E. coli*, B. *E. faecalis*, C. *S. aureus*, and D. *S. mutans*

Trace zones (< 1 mm beyond the well margin) were observed in isolated replicates but were not reproducible; mean inhibition across all replicates was 0.00 mm for all KH concentrations. 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.

ORIGINAL ARTICLE
Journal of Science Technology Education Art and Medicine
DOI: 10.63137/jsteam.256955

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

Mean zones of inhibition (mm)				
KH Conc. (%)	***S. aureus***	***S. mutans***	***E. faecalis***	***E. coli***
1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
10	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
20	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Ampicillin (positive control)	14.22 ± 3.34	32.87 ± 2.55	26.00 ± 2.64	21.67 ± 1.86
Distilled water (negative control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

† KH = Kelulut honey (*Trigona itama*). Ampicillin concentrations: 500 µg/mL for *S. aureus* and *E. coli*; 20 µg/mL for *S. mutans* and *E. faecalis*. All values are mean ± SD of three independent experiments performed in triplicate (n = 9 per cell). A dash-free format is used; 0.00 indicates no detectable zone of inhibition. Wells were 6 mm in diameter; zone measurements include well diameter.

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. [12] 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. [13] In a study conducted in Brazil, honey from stingless bees *Scaptotrigona postica* and *Scaptotrigona bipunctata* inhibited the growth of both Gram-positive and Gram-negative bacteria, including *E. coli*, *E. faecalis*, *S. mutans*, and *S. aureus*, with MIC values ranging from 0.62 to 10% (v/v) and inhibition zones of 8–22 mm on agar well diffusion. [14] 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.

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%. [15] 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. [16] The present study did not characterize the bioactive composition (e.g., phenolic content, trehalulose, organic acid profile) of the KH sample, which may partly explain the null result, as antimicrobial potency in stingless bee honeys is closely linked to these constituents rather than to methylglyoxal, which is the marker underlying the Unique Manuka Factor (UMF) grading system specific to *Leptospermum*-derived honeys. 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.

Stingless bee honeys differ substantively from *Apis*-derived honeys in moisture content, water activity, sugar composition (notably higher trehalulose), and antioxidant capacity. These compositional differences may influence antimicrobial potency and should be controlled for

in future comparative studies. [17]

Overall, existing literature suggests that honey harbors antimicrobial properties against a wide range of pathogens. [18] 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 did not demonstrate antimicrobial activity against the tested organisms at concentrations of 1–20% (v/v) using the agar well diffusion method. Further research needs to be conducted to determine the accurate concentration of KH needed to inhibit virulent bacteria. Future research should evaluate KH samples with characterized bioactive profiles (phenolics, flavonoids, organic acids) at concentrations of 30–100%, both in raw and gamma-irradiated forms, to determine whether the null result reflects sub-threshold dosing, irradiation-related degradation of bioactive compounds, or inherent species-specific limitations.

Acknowledgments

We thank the Universiti Sains Malaysia research laboratory for providing chemicals and reagents for the experiment.

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.

Data Availability Statement

All relevant data are within the manuscript. Additional data supporting this study are available from the corresponding author upon reasonable request.

Ethical Considerations

This study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/21040344). As the study was an in vitro investigation using commercially sourced ATCC reference strains and a commercially available honey product, no human participants, human biological samples, or animal subjects were involved. All laboratory work was conducted under institutional biosafety guidelines at Biosafety Level 1.

Funding

The research was funded by Universiti Sains Malaysia [304/PPSG/6315144].

Conflict of Interest

The authors declare no conflicts of interest.

References

1. Hossain ML, Lim LY, Hammer K, Hettiarachchi D, Locher C. Honey-based medicinal formulations: a critical review. Appl Sci. 2021;11(11):5159. doi:10.3390/app11115159.
2. Mărgăoan R, Topal E, Balkanska R, Yücel B, Oravecz T, Cornea-Cipcigan M, et al. Monofloral honeys as a potential source of natural antioxidants, minerals and medicine. Antioxidants (Basel). 2021;10(7):1023. doi:10.3390/antiox10071023.
3. Kumar R. Historical and religious perspective of honey. In: Kumar R, Agrawal V, Hajam YA, editors. Honey: a miraculous product of nature. Boca Raton (FL): CRC Press; 2024. p. 1-16. doi:10.1201/9781003490180-1.
4. Boukraâ L, editor. Honey in traditional and modern medicine. Boca Raton (FL): CRC Press; 2013. doi:10.1201/b15608.
5. Mustafa G, Iqbal A, Javid A, Manzoor M, Aslam S, Ali A, et al. Antibacterial properties of Apis dorsata honey against some bacterial pathogens. Saudi J Biol Sci. 2022;29(2):730-4. doi:10.1016/j.sjbs.2021.09.059.
6. Combarros-Fuertes P, Fresno JM, Estevinho MM, Sousa-Pimenta M, Tornadijo ME, Estevinho LM. Honey: another alternative in the fight against antibiotic-resistant bacteria? Antibiotics (Basel). 2020;9(11):774. doi:10.3390/antibiotics9110774.
7. Zhang Q, Ma Q, Wang Y, Wu H, Zou J. Molecular mechanisms of inhibiting glucosyltransferases for biofilm formation in Streptococcus mutans. Int J Oral Sci. 2021;13(1):30. doi:10.1038/s41368-021-00137-1.
8. Swelum AA, Elbestawy AR, El-Saadony MT, Hussein EOS, Alhotan R, Suliman GM, et al. Ways

to minimize bacterial infections, with special reference to Escherichia coli, to cope with the first-week mortality in chicks: an updated overview. Poult Sci. 2021;100(5):101039. doi:10.1016/j.psj.2021.101039.

9. Massous A, Ouchbani T, Lo Turco V, Litrenta F, Nava V, Albergamo A, et al. Monitoring Moroccan honeys: physicochemical properties and contamination pattern. Foods. 2023;12(5):969. doi:10.3390/foods12050969.
10. Hossain ML, Lim LY, Hammer K, Hettiarachchi D, Locher C. A review of commonly used methodologies for assessing the antibacterial activity of honey and honey products. Antibiotics (Basel). 2022;11(7):975. doi:10.3390/antibiotics11070975.
11. López-Malo A, Mani-López E, Davidson PM, Palou E. Methods for activity assay and evaluation of results. In: Davidson PM, Taylor TM, David JRD, editors. Antimicrobials in food. 4th ed. Boca Raton (FL): CRC Press; 2020. p. 13-40. doi:10.1201/9780429058196-2.
12. Al-Kafaween MA, Al-Groom RM, Hilmi ABM. Comparison of the antimicrobial and antivirulence activities of Sidr and Tualang honeys with Manuka honey against Staphylococcus aureus. Iran J Microbiol. 2023;15(1):89-101. doi:10.18502/ijm.v15i1.11923.
13. Al-Kafaween MA, Mohd Hilmi AB, Nagi Al-Jamal HA, Jaffar N, Al-Sayyed H, Zahri MK. Effects of selected Malaysian Kelulut honey on biofilm formation and the gene expression profile of Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli. Jordan J Pharm Sci. 2021;14(1):1-9.
14. Nishio EK, Ribeiro JM, Oliveira AG, Andrade CGTJ, Proni EA, Kobayashi RKT, et al. Antibacterial synergic effect of honey from two stingless bees: Scaptotrigona bipunctata Lepeletier, 1836, and S. postica Latreille, 1807. Sci Rep. 2016;6:21641. doi:10.1038/srep21641.
15. Almasaudi SB, Al-Nahari AAM, Abd El-Ghany ESM, Barbour E, Al Muhayawi SM, Al-Jaouni S, et al. Antimicrobial effect of different types of honey on Staphylococcus aureus. Saudi J Biol Sci. 2017;24(6):1255-61. doi:10.1016/j.sjbs.2016.08.007.
16. Aljaghwani A, Allemailem KS, Aljaghwani LF, Alrumaihi F, Joseph RJ, Khan AA, et al. Antimicrobial effect of different types of honey on selected ATCC bacterial strains. Pharmacogn J. 2021;13(1):217-25. doi:10.5530/pj.2021.13.30.
17. Homrani M, Escuredo O, Rodríguez-Flores MS, Fatiha D, Mohammed B, Homrani A, et al. Botanical origin, pollen profile, and physicochemical properties of Algerian honey from different bioclimatic areas. Foods. 2020;9(7):938. doi:10.3390/foods9070938.
18. Albaridi NA. Antibacterial potency of honey. Int J Microbiol. 2019;2019:2464507. doi:10.1155/2019/2464507.