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Research Article

# Antibacterial Activity of Malaysian Trigona itama and Trigona thoracica Honey Against Gram-Negative and Gram-Positive Bacteria

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**Malaysia is among many tropical countries where stingless bees and their products are abundantly available and extensively used for human health. Stingless bee honey can be collected either directly from bee farms or harvested from tropical forests. Stingless bee products, especially honey, are traditionally consumed mainly by the local Asian people for therapeutic purposes. This study was conducted to determine the antibacterial properties of Malaysian stingless bee honey from the species *Trigona itama* and *Trigona thoracica* against selected Gram-negative and Gram-positive bacterial samples. Agar well diffusion and micro broth dilution assays were conducted to determine the antibacterial activity of four stingless bee honey samples from the *Trigona* genus. The *Trigona* honey of the four samples demonstrated vital zones of inhibition against *Staphylococcus aureus* (ATCC 9144), *Staphylococcus epidermidis* (ATCC 14990), *Streptococcus pyogenes* (ATCC 19615), *Escherichia coli* (ATCC 85218), *Salmonella* Typhi (ATCC 19430), and *Klebsiella pneumoniae* (ATCC 10273). The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values of the four honey samples against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *E. coli*, *Salmonella* Typhi, and *K. pneumonia* were successfully obtained at lower honey concentrations but higher sample dilutions. This study justified that Malaysian stingless bee honey has broad-spectrum antibacterial activity against Gram-negative and Gram-positive bacteria and possesses promising antibacterial therapy for future health regimens.**

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## Introduction

Stingless bee honey is a desirable natural product that has been consumed for ages in its natural form. Many Asian people appreciate honey for the treatment of diseases and pursue honey in every part of the region where it is available, such as Central and South America, Africa, Western Asia, Northern Australia[1] and Southeast Asia, especially Malaysia[2]. During the ancient era, honey is believed to have played a major role in folk medicine with significant healing properties. It has been used alone or in combination with other natural products as the main ingredient for preparing several traditional remedies and was ultimately used for the treatment of various ailments.

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Previous researchers have indicated the application of stingless bee honey in the treatment of cough and throat infections, infertility, fever, skin bruises, ulcers, wounds, and eye and ear infections[3]. Various tribes and cultures from suburban communities believe in the superior therapeutic properties of stingless bee honey compared to other types of honey. The Aboriginal people of Australia and the Latin American honey practitioners trust the fact that honey produced by the *Meliponini* tribe is more valuable and has a stronger medicinal effect than honey produced by other tribes, including *Apis* honey[4] Stingless bee honey is generally referred to as pot honey[5] meanwhile Malaysians refer to that honey as "Madu Kelulut". Products of Malaysian stingless bees, whether honey, propolis, or bee bread, are in high demand throughout the year {Al-kafaween, 2021 #17}. Stingless bee honey from *Trigona itama* and *Trigona thoracica* are the most available species in Malaysia, which have been used for fruit plant pollination. To date, bees from the *Trigona* genus are domestically farmed in villages or rural territories. Compared to other natural supplements, stingless bee honey is considered affordable, delightful, and appetizing due to its sweet and sour flavor[6]. Based on these characteristics, Malaysian stingless bee honey has commercial potential for national and international markets. Altogether, the socioeconomic status of stingless bee farmers may be increased and is also beneficial to villagers as their additional income. Unfortunately, the medicinal grade value of Malaysian stingless bee honey is less documented, and its therapeutic potential against Gram-negative and Gram-positive bacteria has never been investigated. Therefore, we aim to investigate the antibacterial activity of honey from the species of *Trigona itama* and *Trigona thoracica.* The future findings may be crucial for health regimes and support the stingless bee industry among villagers.

## Materials and Methods

### *Sample Collection*

Stingless bee honey from *Trigona itama* and *Trigona thoracica* were collected from stingless bee farmers in Kelantan and commercially purchased from Terengganu. Honey samples used in this study are shown in Table 1. All samples were kept at room temperature and labeled accordingly.

| Honey Sample | Bee Specie |
|---|---|
| **A** | *Trigona thoracica* from Kelantan |
| **B** | *Trigona itama* from Kelantan |
| **C** | *Trigona thoracica* from Terengganu |
| **D** | *Trigona itama* from Terengganu |

**Table 1.** Profile of Honey Samples

## *Test Organisms*

The test organisms were collected from the microbiology laboratory, Medical Campus, Universiti Sultan Zainal Abidin (UniSZA). Three Gram-positive and three Gram-negative American Type Culture Collection (ATCC) samples were used. They were *Staphylococcus aureus* (ATCC 9144), *Staphylococcus epidermidis* (ATCC 14990), *Streptococcus pyogenes*(ATCC 19615), *Escherichia coli* (ATCC 85218), *Salmonella* Typhi (ATCC 19430), and *Klebsiella pneumoniae* (ATCC 10273) respectively. Inoculum for each test organism was prepared as described by[7] which was adopted from[8], by sub-culturing the test organism in Muller Hinton agar (MHA) and incubating at 37°C for 24 hours. A total of 3-5 morphologically identical colonies from the fresh overnight culture media were suspended into 5 mL of sterile Muller Hinton Broth. By using a spectrophotometer, the optical density (OD) was adjusted by sterile peptone water to match 0.5 McFarland standards ($1.5\times10^8$ CFU/mL), within the absorbance range of 0.08 to 0.13. The prepared inoculums were utilized immediately after completing the sample preparation. Positive and negative controls were used to validate every experiment. The positive control was conducted by placing Ampicillin 10 mcg (CT0003B), Ciprofloxacin 5 mcg (CT0425B), and freshly prepared 2%, 4%, and 8% of phenol on agar plates that had been inoculated with the individual test organism, and then they were incubated at 37°C for 24 hours. Sterile distilled water was used as a negative control.

## *Honey Preparation for Agar Well Diffusion Technique*

Sterile distilled water and honey were used to prepare the different weight/volume percentage solutions (w/v %). Absolute honey was used for the 100%, 75% w/v honey solution was prepared by adding 1.5 g of honey into 2 mL of sterile distilled water. The 50% w/v honey solution was prepared by adding 1 g of honey into 2 mL of sterile distilled water, the 25% w/v honey solution was prepared by adding 0.5 g of honey into 2 mL of sterile distilled water, and the 10% w/v honey solution was prepared by adding 0.2 g of honey into 2 mL of sterile distilled water. The mixtures were prepared in the preparation room and away from direct sunlight. The tubes were vortexed to ensure the solution was well mixed and was freshly prepared.

## *Agar Plate Preparation*

MHA was used for this analysis by preparing 150 mL according to the manufacturer's instructions. After autoclaving, the MHA was allowed to cool at room temperature. A laser thermometer was used to check the temperature of the broths until they achieved 45°C - 50°C. The broth was immediately added with 100 µL of the individual test organism that had already been prepared and adjusted to 0.5 McFarland standards. The seeded MHA was gently swirled to ensure complete mixture between the test organism and the culture media. The MHA was aseptically and slowly poured into the petri dish while observing the presence of air bubbles before continuing to the next experiment. The plates were placed upside-down at 4°C overnight.

### *Agar Well Diffusion Technique*

Six wells were created for every petri dish containing culture media that were prepared overnight by using a sterile 7 mm cork borer. The wells in the petri dish were labeled, and 200 µL of the five different honey concentrations (100%, 75%, 50%, 25%, and 10%) were filled accordingly. The last well was filled with sterile distilled water as a negative control. The prepared plates were then incubated at 37ºC for 24 hours. A digital vernier caliper was used to measure the zones of inhibition, which were recorded properly. The assay was carried out in triplicate for each of the test organisms.

### *Honey Preparation for MIC Analysis*

The micro broth dilution technique was performed to quantify the minimum inhibitory concentration (MIC) of the stingless bee honey. The MIC of honey is defined as the lowest concentration inhibiting visible growth of bacteria after overnight incubation at 37°C. The honey for MIC was freshly prepared before conducting the assay. A weight by volume percentage of stock solutions for each honey sample was prepared by gently and carefully weighing 7.5 g of honey and then pouring it into a clearly labeled, clean, and sterile graduated cylinder. Honey samples were diluted with sterile Mueller Hinton II broth (MHB). The final volume of 15 mL was achieved by adding the broth into the graduated cylinder containing the honey sample, ultimately achieving a 50% w/v stock solution. Two other stock solutions of 40% and 30% w/v were also prepared. All solutions were well mixed using a vortex device. Further subsequent honey dilutions such as 25%, 12.5%, 15%, 7.5%, 1.875%, and 0.3125% were acquired by preparing a two-fold serial dilution.

### *MIC Assay*

A 96-well flat-bottom microtiter plate was used for the assay. Wells of the test sample contained inoculated bacteria with honey solution from four honey types. Assay growth control wells contained bacteria and broth, sterility control wells contained broth only, corresponding negative control wells contained honey only, and test wells contained inoculum and honey. All wells were investigated in a horizontal direction (A1 to A12). A volume of 200 µL of each honey concentration, broth only, and inoculum only was carefully and aseptically dispensed into the wells using a micropipette. A solution containing 190 µL of honey at different concentrations and 10 µL of selected bacterial inoculums was aseptically transferred into the test wells. The plates were incubated at 37°C for 24 hours in a shaker incubator set at 120 rpm. After 24 hours of incubation, the plates were observed visually for the presence of turbidity. The absorbance of optical density was measured at 590 nm by a microtiter plate reader. The percentages of inhibition of bacterial growth were calculated using the following formula:

$$\text{Inhibition percentage} = \frac{1 - \text{OD of test well} - \text{OD of corresponding negative control}}{\text{OD of assay growth control} - \text{OD of sterility control}} \times 100\%$$

### *Minimum Bactericidal Concentration (MBC) Assay*

The MBC is the lowest concentration of honey that results in killing 99.9% of the tested bacteria. It was conducted after obtaining the MIC results by observing the overnight incubated 96-well plates. Each well that was free from visible turbidity was selected for MBC determination. A sterile wire loop was gently and aseptically dipped at one time into the well of clear solution. Sterilization before dipping the wire loop into a different well must be properly done to avoid possible contamination. Sub-culturing the bacteria from the wire loop was conducted on a fresh MHA petri dish. The plates were clearly labeled and incubated at 37ºC for 24 hours. Bacterial growth on the plates was observed. Petri dishes free of any bacterial growth were confirmed as an MBC value[9].

### *Statistical Analysis*

For each data set, three replicates were performed. Statistical Package for the Social Sciences software (SPSS 20) was used to analyze the data sets. All data were expressed as mean ± standard deviation.

## Results and Discussion

### *Antibacterial activity by Agar Well Diffusion*

The antibacterial activity of honey was assessed by the inhibited clear zone of bacterial growth around the wells. The clear zone was measured and recorded as the zones of inhibition. The size of the inhibition zone describes the quality of the honey. The larger inhibition zones justified the greater antibacterial activity of the honey compared to smaller zones. The clear zone justified the absence of bacterial growth or less antibacterial activity from the honey[10]. The stingless bee honey from four samples has revealed promising antibacterial activity against selected Gram-positive and Gram-negative bacteria. All four types of honey from *Trigona itama* and *Trigona thoracica* demonstrated the largest inhibition zone at 100% concentration compared to 75%, 50%, and 25% against *S. aureus, S. pyogenes*, *S. epidermidis*, *E. coli*, *K. pneumoniae* and *S.* Typhi. *Trigona itama* from Kelantan demonstrated the largest inhibition zone against *S.aureus* by inhibiting 31.41±4.14 mm of inoculated bacteria after exposure to crude 100% honey. Meanwhile, *Trigona itama* and *Trigona thoracica* from Terengganu were unable to inhibit the growth of *E. coli* and *K. pneumonia* at 25% honey. However, *Trigona thoracica* from Kelantan was unable to inhibit the growth of *K. pneumonia* at 25% honey. There is a great relationship between the concentration of honey and the antibacterial activity of honey against the tested bacteria. Among all the test organisms, *S. aureus* was the most susceptible after exposure to 25% honey, as shown in Table 2.

| Test Organism | Sample | 100% | 75% | 50% | 25% | 0% |
|---|---|---|---|---|---|---|
| *S. aureus* | A | 29.04±1.50 | 22.52±0.62 | 17.95±0.25 | 11.57±1.20 | - |
| | B | 31.41±4.14 | 23.34±0.10 | 18.60±0.34 | 15.37±1.88 | - |
| | C | 23.50±5.04 | 19.46±1.19 | 16.24±1.00 | 13.93±2.03 | - |
| | D | 25.11±2.94 | 21.96±2.50 | 17.85±1.50 | 11.06±0.53 | - |
| *S. epidermidis* | A | 26.08±6.80 | 22.45±2.32 | 18.75± 1.22 | 12.05± 6.03 | - |
| | B | 30.23±1.20 | 25.07±0.00 | 21.11±4.25 | 13.11±3.35 | - |
| | C | 26.11±3.10 | 24.52±2.20 | 18.71±0.02 | 12.98±8.50 | - |
| | D | 27.49±1.31 | 24.96±2.50 | 19.21±1.73 | 11.96±2.40 | - |
| *S.pyogenes* | A | 24.01±058 | 21.02±0.62 | 16.94± 8.03 | 9.12± 3.70 | - |
| | B | 24.38±2.20 | 23.34±0.10 | 18.96±1.11 | 12.08±0.07 | - |
| | C | 24.55±1.90 | 17.52±2.20 | 14.92±0.02 | 10.81±0..50 | - |
| | D | 24.87±1.31 | 21.96±2.50 | 15.07±4.40 | 11.01±2.40 | - |
| *K. pneumoniae* | A | 21.28±2.70 | 16.6±0.57 | 13.32±0.95 | - | - |
| | B | 28.31±0.14 | 22.40±1.56 | 15.95±1.58 | 12.14±0.40 | - |
| | C | 17.68±0.22 | 15.96±0.31 | 11.72±1.70 | - | - |
| | D | 20.65±1.19 | 16.61±1.61 | 12.27±1.46 | - | - |
| *E. coli* | A | 23.51±2.28 | 15.07±1.00 | 13.41±2.47 | 9.93±0.20 | - |
| | D | 25.38±2.51 | 18.21±0.49 | 14.78±1.40 | 11.3±0.21 | - |
| | C | 20.19±0.55 | 12.37±0.35 | 9.16±0.27 | - | - |
| | D | 20.51±1.36 | 12.63±0.47 | 8.88±0.30 | - | - |
| *S.* Typhi | A | 26.58±2.13 | 18.58±1.74 | 16.59±1.43 | 10.83±0.25 | - |
| | B | 32.14±0.52 | 22.23±0.20 | 20.14±0.24 | 12.86±0.24 | - |
| | C | 17.05±2.73 | 14.91±1.82 | 11.83±0.19 | 8.52±0.61 | - |
| | D | 20.25±0.92 | 16.80±2.41 | 13.30±0.47 | 9.23±0.12 | - |

**Table 2.** Inhibition Zones (mm) of Honey Samples against the Pathogenic Bacteria Samples.

*Mean ± Standard Deviation*

## *Minimum Inhibitory Concentration*

The stingless bee honey samples demonstrated that the MIC for the Gram-positive *S. aureus, S. pyogenes*, and *S. epidermidis* was 0.625%. Meanwhile, for *E. coli*, it was 5%, and for *K. pneumoniae* and *S.* Typhi, it was 7.5%, as shown in Table 3. The entire honey samples tested achieved minimum inhibitory activity at higher dilutions but lower honey concentrations. Malaysian stingless bee honey showed exceptional quantitative activity, which agrees with the fact that the antibacterial activities of the honey samples are dose-dependent[11]. Interestingly, Malaysian stingless bee honey samples showed unique constant activity irrespective of the bee species, floral type, and geographic difference. The difference in susceptibility of the test bacteria was only identified based on the Gram stain characteristic. The antibacterial activity of the stingless bee honey against Gram-positive *S. aureus,*

*S. epidermidis,* and *S. pyogenes* was achieved at a lower concentration than that of the Gram-negative *E. coli, S.* Typhi and *K. pneumoniae*. A low MIC value is an indication of high antimicrobial property. The MIC range of 0.625% - 7.5% was recorded from the stingless bee honey samples. The antibacterial activity of the stingless bee honey is contributed by the type and origin of floral or pollen, the bee tribe, the acidity contents of the honey, and the presence of complex organic acids which may easily destroy the bacterial cell wall[12]. This study demonstrates that Malaysian stingless bee honey has broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria.

| Microorganisms | Honey Samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | D | |
| | a | b | a | b | a | b | a | b |
| ***S. aureus*** | 0.62 | 0.31 | 0.62 | 0.31 | 0.62 | 0.31 | 0.62 | 0.31 |
| ***S. epidermidis*** | 0.62 | 0.46 | 0.62 | 0.46 | 0.62 | 0.46 | 0.62 | 0.46 |
| ***S. pyogenes*** | 0.62 | 0.31 | 0.62 | 0.46 | 0.62 | 0.46 | 0.62 | 0.46 |
| ***E. coli*** | 5 | 2.5 | 5 | 2.5 | 5 | 3.7 | 5 | 3.7 |
| ***K. pneumoniae*** | 7.5 | 5 | 7.5 | 5 | 7.5 | 5 | 7.5 | 5 |
| ***S.* Typhi** | 7.5 | 5 | 7.5 | 5 | 7.5 | 5 | 7.5 | 5 |

**Table 3.** MIC values (%) by Microplate reader and Visual Observation

*a = microplate reader MIC ≥ 90% inhibition, b= visual*

## *Minimum Bactericidal Concentration*

MBC results indicate vital antibacterial activity of all stingless bee honey samples against Gram-positive and Gram-negative bacteria. MBC for all honey samples against Gram-positive organisms; *S. pyogenes*, *S. epidermidis*, and *S. aureus*, was 0.625%. Meanwhile, for *E. coli* it was 5%, and *K. pneumoniae* and *S.* Typhi were 7.5%, as shown in Table 4. The stingless bee honey samples have an MBC range between 0.625% and 7.5%. The different values are because of the different honey origins or bee species. The MBC value must be equal to or higher than the MIC. An antibacterial agent is declared bactericidal if the MBC value is not more than four times the MIC. This study demonstrated that the MIC and MBC are similar, which is concurrent with Zainol's team[9] in investigating stingless bee honey.


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| Microorganisms | Honey Samples | | | |
|---|---|---|---|---|
| | A | B | C | D |
| *S. aureus* | 0.62 | 0.62 | 0.62 | 0.62 |
| *S. epidermidis* | 0.62 | 0.62 | 0.62 | 0.62 |
| *S. pyogenes* | 0.62 | 0.62 | 0.62 | 0.62 |
| *E. coli* | 5 | 5 | 5 | 5 |
| *K. pneumoniae* | 7.5 | 7.5 | 7.5 | 7.5 |
| *S.* Typhi | 7.5 | 7.5 | 7.5 | 7.5 |

**Table 4.** MBC Values (%) of Stingless Bee Honey

## Conclusion

Our study demonstrates the broad-spectrum antibacterial activity of the Malaysian *Trigona itama* and *Trigona thoracica* honey against *S. aureus*, *S. epidermidis*, *S. pyogenes*, *E. coli*, *Salmonella* Typhi, and *K. pneumoniae*. Malaysian stingless bee honey has promising potential as an antibacterial agent against Gram-positive and Gram-negative bacteria.

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## Declarations

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