**Original Article**

**Effect of Non-hydrogen Peroxide on Antibacterial Activity of Malaysian Meliponini Honey against Staphylococcus aureus**

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**Introduction:** Stingless bee is an insect that belongs to the family Apidae. Its name is based on its disability of stinging. It has a high product of Meliponini honey and propolis which are commonly referred to as stingless bee honey and stingless bee propolis. Meliponini honey is one of the crucial natural sources and has the potential to kill infectious microorganisms. Previous studies have proved that the antibacterial activity of natural honey was an effect of hydrogen peroxide, a substance contained in the honey. However, these claims were contradicting with too many studies. **Objective:** Therefore, this study aimed to identify the antibacterial activity of Malaysian Meliponini honey which contained non-hydrogen peroxide against Staphylococcus aureus, an opportunistic microbial.

**Materials and Methods:** Meliponini honey was used as an antibacterial agent for the treatment of S. aureus in agar well diffusion assay. An amplex red hydrogen peroxide kit was used to identify the hydrogen peroxide in the honey sample. Meanwhile, non-hydrogen peroxide activity was performed by using honey-catalase treated.

**Results:** For the first time, we found that hydrogen peroxide was absent in all Meliponini honey samples. Meliponini honey has higher antibacterial activity (13.30 ± 0.56 mm) compared to Apis honey (9.03 ± 0.22 mm) in agar well diffusion assay.

**Discussion:** Non-hydrogen peroxide in Meliponini honey is a bioactive compound and beneficial to kill the microbial infection.

**Conclusion:** Antibacterial activity of Malaysian Meliponini honey is directly contributed by non-hydrogen peroxide.

**Keywords:** Antibacterial activity, Meliponini honey, non-hydrogen peroxide

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**INTRODUCTION**

Previous clinical studies have demonstrated the effectiveness of honey as an antibacterial therapeutic agent for human illness.\(^1\)\(^-\)\(^3\) Antibacterial activity of honey is achieved by the hydrogen peroxide, non-hydrogen peroxide, or their combined effect. Hydrogen peroxide in honey is produced by the enzymatic action of glucose oxidase, an enzyme which originates from hypopharyngeal glands of the bee\(^4\)\(^-\)\(^5\) and from the bacteria of genus Gluconobacter.\(^6\) It is an active enzyme in the nectar and can only be active in the diluted honey. Initially, scientists hypothesized that glucose oxidase had contributed to the antibacterial activity of honey because its role is to altering the glucose into gluconolactone before producing gluconic acid. Gluconic acid (also known as inhibine) is the primary acid in honey and hydrogen peroxide.\(^7\)\(^-\)\(^9\) Finally, researchers found out the antibacterial property of various honey samples was an effect of hydrogen peroxide.\(^10\)

A previous study demonstrated that Canadian honey, after being treated with catalase has lower antibacterial...
activity. Catalase is an efficient enzyme for hydrogen peroxide decomposition. They concluded that the antibacterial activity of Canadian honey is obviously dependent on the hydrogen peroxide. A higher concentration of hydrogen peroxide in the honey resulting in a higher antibacterial activity. However, characterization of non-hydrogen peroxide in the honey sample is now became more concern among researchers. It is because non-hydrogen peroxide antibacterial activity supporting a distinct or a novel bioactive compound in honey. Some honey samples contain only non-hydrogen peroxide but are able in demonstrating the antibacterial therapeutic activities. The reason is the antibacterial activities of those honey samples are achieved by non-hydrogen peroxide. Efficacy of non-hydrogen peroxide as an antibacterial property was first identified in the New Zealand Manuka honey sample. Botanical nomenclature for Manuka tree is *Leptospermum scoparium*. Unfortunately, the efficacy of hydrogen peroxide as an antibacterial property is inconsistent and will be affected by an indigenous catalase, a natural component of honey and is found in pollen or nectar of the plant. Exposure of honey to the light or heat might reduce the stability of hydrogen peroxide and leads to the impairment of antibacterial property. Therefore, honey that contains hydrogen peroxide composition only has less interest.

Different origin of honey shows different efficacy on antibacterial activity. *Meliponini* honey, for example, has significant clarification on human diseases such as cough, throat infection, fever, skin bruises, ulcers, wounds, eye, or ear infections. All these illnesses are associated with various microbial infections from opportunistic or pathogenic bacteria. The antibacterial property of *Meliponini* honey may rely on hydrogen peroxide, non-hydrogen peroxide, or both compositions. Although the Malaysian *Meliponini* honey is of significant nutritional and therapeutic values, its mechanism regarding antibacterial activity is unclear. Therefore, this study aimed to identify the non-hydrogen peroxide antibacterial activity of Malaysian *Meliponini* honey against *Staphylococcus aureus*.

**Materials and Methods**

**Honey samples**

Five fresh honey samples were used for the experiment. Four *Meliponini* honey samples of *Trigona sp.* were collected from the bee farm in Kelantan and a research center in Terengganu. A sample of *Apis* honey from Tualang tree (*Koompassia excels*) was used for comparison and as a corresponding positive control for hydrogen peroxide assay. *Apis* honey was obtained from Agropolis, Universiti Sultan Zainal Abidin, Besut Campus.

**Hydrogen peroxide (H₂O₂) assay**

Amplex Red Hydrogen Peroxide/Peroxidase kit (Invitrogen Thermo Fisher Scientific, Eugene, OR, USA) was used for analysis. Master mixture of Amplex red reagent stock solution, one-time reaction buffer solution, 10 U/mL horseradish peroxidase stock solution, 20 Mm hydrogen peroxide working solution and amplex red working solution were prepared according to manufacturer’s instruction. The negative control of 50 μM reaction buffer without hydrogen peroxide working solution was used. Honey samples were prepared in a 96-well plate by diluting into one-time buffer solution to achieve 50 μL according to the manufacturer’s instruction. The plates were incubated at room temperature in the dark for 30 min before changes of color were observed. The presence of hydrogen peroxide was determined by red color.

**Catalase treatment**

The antibacterial activity from non-hydrogen peroxide of honey was determined by the addition of catalase. Hydrogen peroxide in the honey was decomposed into water and oxygen by catalase. Firstly, the catalase solution was prepared by adding the 20mg catalase (Sigma, USA) into 10-mL sterile deionized water, and then the solution was properly mixed. Stock solution of 50% honey sample was prepared by weighing 5g of honey and was added into 10mL of sterile distilled water, and then the solution was properly mixed. Stock solution of 50% honey sample was prepared by weighing 5g of honey and was added into 10mL of sterile distilled water. Two clean test tubes were labeled as A for catalase-treated honey and B for catalase-untreated honey. Subsequently, 1mL of 50% honey and 1mL of catalase solution were added into tube A to produce 25% catalase-treated honey. Meanwhile, 1mL of sterile distilled water was added into tube B to produce 25% catalase-untreated honey.

**Agar well-diffusion**

*Staphylococcus aureus* (ATCC 9144) was cultured in Mueller Hinton Agar (MHA) and was used for the analysis. MHA was prepared according to the manufacturer’s instructions. The MHA was autoclaved and was allowed to cool at room temperature. A lesser thermometer was used to check the temperature. Agar with the temperature of 45ºC-50ºC was immediately added with 100 μL of *S. aureus* after the inoculum was adjusted to 0.5 McFarland standard. The inoculum was gently swirled to ensure the proper mixture between the test organisms and was immediately aseptically poured into the petri dish before the agar plate was kept at 4ºC overnight. The wells of agar plate were created by using a sterile 7mm cork borer. The wells were labeled as A for
catalase-treated honey and B for catalase-untreated honey before were added with 200 µL of inoculum. The agar plate was then incubated at 37°C for 24 h. Digital venire caliper was used to measure the zone of inhibitions. The test was repeated in triplicate for each of honey sample.

**Results**

Based on the Amplex red hydrogen peroxide protocol, the presence of hydrogen peroxide is confirmed by red color. Meanwhile, hydrogen peroxide-free or non-hydrogen peroxide is demonstrated by colorless [Figure 1].

Table 1 shows the antibacterial activity of *S. aureus* following exposure to catalase-treated honey and catalase-untreated honey. Meliponini honey demonstrated greater well diffusion compared to *Apis* honey.

**Discussion**

Malaysian *Apis* honey or widely known as Madu Tualang is collected from Tualang tree, the highest tree in tropical rainforest. Based on its hydrogen peroxide antibacterial property,[26] *Apis* honey was used as a corresponding positive control and was compared to our *Meliponini* honey. Horseradish peroxidase in Amplex red hydrogen peroxide reacts with hydrogen peroxide to produce resorufin, a red oxidation substance. Although hydrogen peroxide is a mild oxidizing agent with low toxicity, it is vital for cellular activity and molecular function.[27] Hydrogen peroxide acts as a preservative for honey by offering protection from invading organism. Its toxicity is increased by potentiators.[28]

Biomarker in immunoassay is commonly identified by wavelength absorbance and is measured by an automated reader. Opportunely, direct chromatic observation in an immunoassay is an independent assay to reduce the experimenting cost and lessen the massive operation of a microplate reader. This kind of approach offers a simple rapid diagnostic test to be used at health-care center or hospital.[29]

Honey that retains its antibacterial activity after being treated with catalase is categorized as a non-hydrogen peroxide antibacterial activity and has more therapeutic values.[7,30] After exposed to catalase-treated *Meliponini* honey and catalase-untreated *Meliponini* honey, *S. aureus* was inhibited in both solutions. This is because *Meliponini* honey is composed of non-hydrogen peroxide. Therefore, decomposition of hydrogen peroxide does not occur in catalase-treated *Meliponini* honey. The antibacterial property of both catalase-treated and catalase-untreated *Meliponini* honey is similar.

*Staphylococcus aureus* was inhibited in catalase-treated *Apis* honey and catalase-untreated *Apis* honey. We hypothesized that *Apis* honey has both hydrogen peroxide and non-hydrogen peroxide antibacterial properties. It is because hydrogen peroxide was neutralized after *Apis* honey being treated with catalase.

**Table 1: Inhibition zone (mm) of agar well diffusion**

| Sample | Catalase-treated | Catalase-untreated |
|--------|-----------------|--------------------|
| A      | 10.75 ± 0.32    | 10.70 ± 0.34       |
| B      | 13.30 ± 0.56    | 13.24 ± 0.65       |
| C      | 11.05 ± 0.20    | 10.78 ± 0.31       |
| D      | 10.14 ± 0.11    | 10.05 ± 0.20       |
| E      | 9.03 ± 0.22     | 8.75 ± 0.35        |

mm = millimeter

Antibacterial activity determined by agar well diffusion assay

*Staphylococcus aureus* was cultured in catalase-treated honey and catalase-untreated honey

Data in mean ± standard deviation

*Meliponini* honey (samples A–D)

Sample E is *Apis* honey
The antibacterial activity of catalase-treated *Apis* honey was due to non-hydrogen peroxide. *Staphylococcus aureus* was chosen for this assay because it is commonly used for antimicrobial activity testing for a variety of new drugs or antibiotics.[7,31]

Malaysian *Apis* honey has a similar antibacterial activity to Britain Surgi honey in terms of hydrogen peroxide composition.[32] Nevertheless, Malaysian *Meliponini* honey for the first time has demonstrated its non-hydrogen peroxide antibacterial activity against *S. aureus*.

**CONCLUSION**

This study signifies and provides novel information on the crucial role of non-hydrogen peroxide of Malaysian stingless bee honey as an antibacterial agent. Our study demonstrated that Malaysian stingless bee honey has promising antibacterial activity against opportunistic bacteria and may be benefited against pathogenic bacteria.

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**Conflicts of interest**

There are no conflicts of interest.

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