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Short Communication
Synergistic Effect of Trigona Honey and Ampicillin on *Staphylococcus aureus* Isolated from Infected Wound

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Abstract

Objective: The synergistic effect between Trigona honey and ampicillin was studied for antibacterial properties against wound isolated *Staphylococcus aureus*. **Materials and Methods:** Clinical isolates of *S. aureus* were identified biochemically and antibiotic resistance was determined by antibiotic susceptibility test. The clinical isolates and reference strains were treated with Trigona honey, ampicillin and a mixture containing of Trigona honey and ampicillin individually using well diffusion and plate count assays. Scanning electron microscope was used to observe the morphological changes of bacteria tested. **Results:** A greater anti-staphylococcal activity was observed in the sample with a combination of honey and ampicillin compared to the honey or the ampicillin, with the largest inhibition zone (7.7-18.7 mm) and the highest bactericidal rate (100%). Lysed bacteria and significant morphological alteration on *S. aureus* were observed in electron micrograph after 24 h incubation with the combination sample. **Conclusion:** Synergism of Trigona honey and ampicillin exhibited a high degree of antibacterial activity against *S. aureus*, including antibiotic resistant strains which would be beneficial for the development of wound dressing to prevent and treat wound infection.

Key words: Trigona honey, *Staphylococcus aureus*, ampicillin, antibacterial properties, synergistic effect

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Combination antibacterial therapy is generally recommended for medical treatment. This may be due to its effectiveness in treatment and deterrence of antibiotic resistance emergence as well as other adverse effects. In recent years, the potential health benefits of honey, especially in wound treatment against multiple antibiotic resistant bacteria[1] have attracted increasing amount of attention. In Malaysia, the honey which is produced by stingless bee (*Trigona* spp.) is commonly known as Trigona honey or Kelulut honey. Trigona honey has been recognized to have economic potential due to its growing availability mainly through cultivation. It is less viscous, is darker in color and has stronger acid flavor compared to honeybee honey[2]. Trigona honey was suggested to have higher effectiveness in antibacterial activity with a wider spectrum than honeybee honey[2]. The synergistic activity of a combination of honey and antibiotic, which exhibited superior effect in inhibiting *Pseudomonas* spp.[3]. Antibacterial properties of honeybee honey have been studied extensively but very little information on antibacterial activity is available on Trigona honey. Therefore, the objectives of present study were to investigate the antibacterial properties of Trigona honey against *Staphylococcus aureus*, one of the most common antibiotic-resistant bacteria isolated from wounds[4] and to study the potential synergism of Trigona honey and ampicillin for antibacterial activity.

MATERIALS AND METHODS

Honey sample: Trigona honey sample was obtained from authorized bee farmers in Malaysia. The sample was kept in the dark at room temperature.

Bacterial isolates: Clinical wound samples were collected from a local hospital and were cultured on mannitol salt agar. Single colonies formed were further identified with biochemical tests. Standard strains of *Staphylococcus aureus* (ATCC 25923 and ATCC 33591) were also tested and adopted as the reference for the identification of clinical isolates.

Biochemical identification: Preliminary identification of clinical isolates was carried out with mannitol fermenting test and gram staining, followed with catalase and coagulase tests. Then, the isolates were further identified using the API® Staph identification system. The generated numerical profile was interpreted by using the api web™ identification database for the identity of each bacterial isolate.

Antibiotic susceptibility tests: The antibiotic susceptibility of each identified *S. aureus* isolate was then determined with Kirby Bauer test and was interpreted in accordance with the standard criteria suggested by CLSI[5]. Commercial antibiotic discs (Oxoid) used in this study include: methicillin, ampicillin, penicillin G, chloramphenicol, trimethoprim and tetracycline.

Well diffusion assay: A prepared 0.5 McFarland *S. aureus* suspension was streaked evenly on the surface of Muller Hinton agar by using a sterile cotton swab. After that, wells with 5 mm diameter were prepared on the agar with a sterile cock borer. Each well was then filled with 55 µL of the diluted Trigona honey (50% v/v), ampicillin (5 µg mL^{-1}), a mixture containing of Trigona honey (50% v/v) and ampicillin (5 µg mL^{-1}) individually. Following 24 h incubation at 37°C the diameters of the zone of inhibition for each sample were then recorded in millimeter (mm). Assays were completed in triplicate and an average value was obtained.

Plate count assay: A volume of 20 µL of 0.5 McFarland *S. aureus* suspension was added individually into each sample tube containing 2 mL of diluted Trigona honey (50% v/v), 2 mL of ampicillin (5 µg mL^{-1}) and 2 mL of a mixture containing of Trigona honey (50% v/v) and ampicillin (5 µg mL^{-1}). Each stock mixture was then diluted with saline solution (1:10 v/v). A volume of 10 µL from each dilution was plated evenly on mannitol salt agar, followed by incubation at 37°C for 24 h. All diluted mixtures were also incubated at the same condition. The plating procedure was repeated after 24 h incubation. The number of colonies formed on agar plates were counted for both 0 and 24 h incubations. This assay was performed in triplicate and the bactericidal rate, K was calculated as Eq. 1:

$$K = \frac{A - B}{A} \times 100 \quad (1)$$

where, A is the total number of colonies formed at 0 h incubation and B is total number of colonies formed at 24 h incubation.

Scanning Electron Microscope (SEM) examination: Treated 24 h cultures were centrifuged (3500 rpm, 10 min) and the pellets were primarily fixed with glutaraldehyde, 2.5% (v/v) in 0.01 M phosphate buffer solution for overnight, followed by centrifugation (3500 rpm, 10 min). The cultures were then washed with phosphate buffer solution and distilled water, underwent serial dehydration with ascending concentrations of ethanol and subjected to critical point drying. The sample

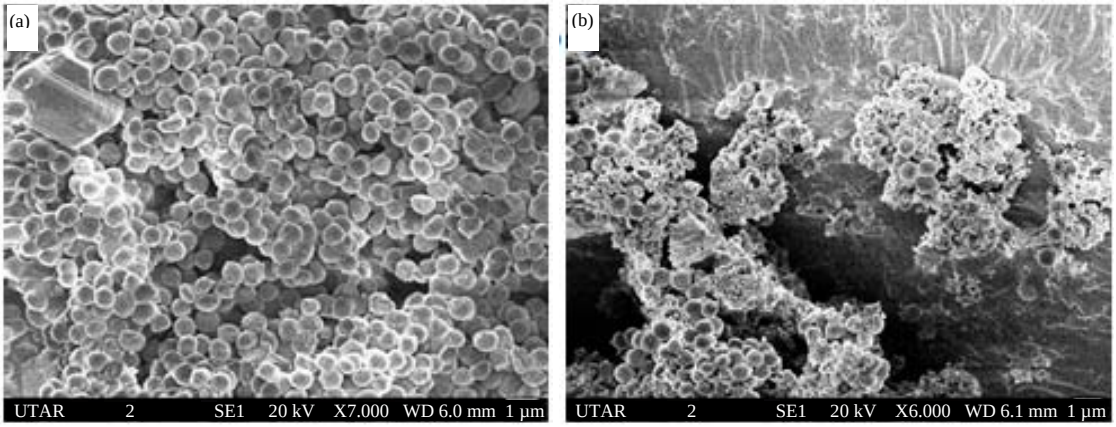

Fig. 1: Morphology of antibiotic-resistance *S. aureus* after treatment with (a) Ampicillin and (b) Combination of Trigona honey and ampicillin

Table 1: Antibiotic susceptibility profile of *S. aureus* isolates

		*Diameter of inhibition zone (mm)			
Antibiotic disc	Content (µg)	ATCC 25923	ATCC 33591	Clinical isolate 1	Clinical isolate 2
Methicillin	5	20 (S)	0 (R)	20 (S)	21 (S)
Ampicillin	10	45 (S)	10 (R)	15 (R)	15 (R)
Penicillin G	10	47 (S)	8 (R)	14 (R)	15 (R)
Chloramphenicol	30	24 (S)	11 (R)	23 (S)	23 (S)
Trimethoprim	25	25 (S)	15 (R)	23 (S)	27 (S)
Tetracycline	30	25 (S)	8 (I)	23 (S)	8 (R)

*R: Resistant, I: Intermediate and S: Susceptible

was then subsequently coated with platinum, placed onto the copper stage holder and examined by scanning electron microscope (JEOL JSM-7610F FEG).

RESULTS

Biochemical identification: Two clinical isolates (Isolate 1 and isolate 2) were found to be similar to the reference strains (ATCC 25923 and ATCC 33591). They were Gram-positive, appeared as cocci in cluster, tested positive for mannitol fermentation, catalase and coagulase activities with *S. aureus* identification percentage of 97.8, 97.8, 97.7 and 97.8%, respectively.

Antibiotic susceptibility test: Table 1 shows the antibiotic susceptibility interpretation for Kirby-Bauer test. ATCC 25923 was confirmed to be the only antibiotic sensitive *S. aureus* strain, whereas ATCC 33591, isolate 1 and isolate 2 were multi-antibiotic resistance strains.

Well diffusion assay: Table 2 shows the anti-staphylococcal effects of each sample in well diffusion assay. No inhibition zone was found for all cultures incubated with Trigona honey, only ATCC 25923 strain was inhibited by ampicillin. However, with the presence of the combination of Trigona honey and ampicillin, the largest zones of inhibition for all cultures were observed, ranged from 7-19 mm.

Plate count assay: Table 3 shows the bactericidal rate of each sample in plate count assay. Other than ATCC 25923 strain, the combination of Trigona honey and ampicillin did not eliminate ATCC 33591 strain, isolate 1 and isolate 2 completely. However, it was found that the incorporation of honey with ampicillin resulted in a greater bactericidal rate against all tested *S. aureus*, including antibiotic resistant strains.

Scanning Electron Microscope (SEM) examination: As verified by Kirby-Bauer test, ATCC 33591 strain and the two clinical isolates were resistant to ampicillin as the spherical shape of bacteria still remains intact after treated with ampicillin (Fig. 1a). With supplementary of Trigona honey in the presence of ampicillin, the morphology of *S. aureus* was disrupted noticeably and cell lysis was observed after 24 h incubation (Fig. 1b).

Table 2: Anti-staphylococcal activities in well diffusion assay

	*Diameter of inhibition zone (mm)			
Samples	ATCC 25923	ATCC 33591	Clinical isolate 1	Clinical isolate 2
Trigona honey	NIL	NIL	NIL	NIL
Ampicillin	16.70±0.06	NIL	NIL	NIL
Trigona honey+ampicillin	18.70±0.06	7.70±0.06	9.00±0.00	9.30±0.06

*Mean±Standard Deviation, n = 3, NIL: No zone of inhibition

Table 3: Anti-staphylococcal activities in plate count assay

	Bactericidal rate (%)			
Samples	ATCC 25923	ATCC 33591	Clinical isolate 1	Clinical isolate 2
Trigona honey	100	98	95	94
Ampicillin	100	90	96	98
Trigona honey+ampicillin	100	100	100	100

DISCUSSION

It is well established that antimicrobial properties of honey are generally attributed to the hydrogen peroxide produced in honey and the natural phytochemicals[6]. To maintain a consistent quality of Trigona honey, it was stored in dark to prevent photo-oxidation of glucose oxidase. This is because the reduction content of glucose oxidase could affect the production of hydrogen peroxide and it might lead to decreasing of antibacterial properties[7]. It has been reported that low concentration ($\approx$1 mmol L^{-1}) of hydrogen peroxide activated by dilution in honey was still effective as an antibacterial agent. With glucose oxidase in honey, continuous generation of hydrogen peroxide escalates the effectiveness of antibacterial activities[7,8].

Our results (Table 2, 3) showed that ampicillin exhibited higher level of antibacterial activity against *S. aureus* in the presence of Trigona honey compared to ampicillin alone. Both well diffusion and plate count assays showed synergistic effect of Trigona honey and ampicillin against *S. aureus*. This is in agreement with other similar studies, such as the synergistic action of honey with gentamicin, amikacin and ceftazidime against *Pseudomonas* spp.[3] and the synergism between Medihoney and rifampicin against methicillin-resistant *Staphylococcus aureus* (MRSA)[9]. In comparison to honey or antibiotic alone, corporation of honey with antibiotic has shown the effectiveness in reducing the number of colonies formed[9,10]. The increase in the antibacterial activity of ampicillin with the combination of Trigona honey has the potential to improve the potency in inhibiting the growth of *S. aureus*.

The damaging effects on *S. aureus* (Fig. 1b) are believed to be attributed to the synergism of Trigona honey antibacterial components and ampicillin. Hydrogen peroxide can diffuse through the cell membrane of bacteria easily and generates hydroxyl free radicals in the cell without being excluded. They oxidize cellular essential components and various metabolic control molecules (possibly thiol groups), such as lipids proteins and DNA[8]. Oxidative stress that caused by hydroxyl free radicals encourages lipid peroxidation which would disrupt the integrity of cell membrane[8]. This may enhance the binding of ampicillin to the penicillin-binding proteins and kill *S. aureus* by inhibiting the terminal stages of peptidoglycan metabolism.

Similar study on Thailand stingless bee honey has shown that the formation of hydroxyl free radicals exhibited destructive effects of DNA and oxidation of thiol-groups in proteins and lipids showed significant peroxide-mediated antibacterial activity[11]. It is believed that the damaging of DNA inhibits the formation of enzyme β-lactamase which could greatly enhance the susceptibility of *S. aureus* towards the action of ampicillin. This further enhances the therapeutic reactions of ampicillin.

In addition, natural phytochemicals in Trigona honey, such as phenolic compounds have membrane-active properties that might cause membrane damage to bacteria and leakage of cellular contents[12]. It has been reported that the presence of plant flavonoids could disrupt the integrity of cell membrane by limiting the lipid and protein synthesis in *S. aureus*[13]. Our study showed that antibacterial effects of ampicillin exhibited synergistically with Trigona honey in destructing the structure integrity of *S. aureus* cell wall.

CONCLUSION

The present study has shown the synergism of Trigona honey and ampicillin in possessing greater antibacterial activity against *S. aureus*, isolated from infected wound, both antibiotic sensitive and resistant strains. Application of Trigona honey in combination therapy for wound infection

could be considered to prevent toxicity from prolonged antibiotic therapy and to reduce the emergence of antibiotic resistance.

SIGNIFICANCE STATEMENTS

The emergence of antibiotic resistant bacteria is a major global concern. In this study, the idea of combination therapy was adopted by using the oldest antibacterial agent in human history, honey mixed with an antibiotic, ampicillin to test against multiple-antibiotics-resistant *Staphylococcus aureus* which isolated from clinical wound samples. The outcomes showed the combination of honey and ampicillin exhibited the greatest anti-staphylococcal effect than either honey or ampicillin was given alone. The morphology of bacteria was found to be distorted in scanning electron micrograph as well. Hence, the synergism between these two agents would be beneficial for the development of wound dressing to prevent and treat wound infection, especially antibiotic resistant bacteria.

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