# INTERACTIVE EFFECT OF TRIGONA HONEY AND AMPICILLIN ON *Staphylococcus aureus* ISOLATES OF INFECTED WOUND

By

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## ABSTRACT

### INTERACTIVE EFFECT OF TRIGONA HONEY AND AMPICILLIN ON *Staphylococcus aureus* ISOLATES OF INFECTED WOUND

**Lye Ping Ying**

Honey is said to be the most longstanding medicine that has passed on and applied by people since ancient times. The significance of apitherapy has been recorded in the world's oldest medical archive as honey is well known for its medicinal value to treat variety of ailments such as bacterial infections. Misuse of antibiotics had led to the arisen of resistance bacteria. Hence, the combination therapy is always recommended to fight against antibiotic resistance bacteria with greater efficiency of treatment. This research was conducted to identify the synergistic effect of Trigona honey with ampicillin against strains of *Staphylococcus aureus* (ATCC25923, ATCC33591 and two clinical strains isolated from wound samples). Based on the outcome from well-diffusion assay, it showed both antibacterial activities of honey alone and ampicillin alone were lower than the combination effect of Trigona honey and ampicillin. The efficacy of synergistic effect was further proved with plate count method which showed all the tested bacterial isolates were killed with zero colony growth after 24-hour incubation period. Moreover, the scanning electron microscopic examination also revealed the combination action of Trigona honey with ampicillin exerted significant morphological changes on *S. aureus* including the antibiotic resistance strains. In conclusion, synergism of Trigona honey and

ampicillin exhibited greater antistaphylococcal effect when compared to Trigona honey alone and ampicillin alone.

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Special thanks must also go to Faculty of Science, UTAR for providing me suitable environment and laboratory facilities to complete my research project. Last but not least, I would like to apologize for any troublesome caused during my research period. I promised to improve myself in the future.

## DECLARATION

I hereby declare that this project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree or award at UTAR or other institutions.

Signature :

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## APPROVAL SHEET

This project report entitled "**INTERACTIVE EFFECT OF TRIGONA HONEY AND AMPICILLIN ON *Staphylococcus aureus* ISOLATES OF INFECTED WOUND**" was prepared by Lye Ping Ying and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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**PERMISSION SHEET**

It is hereby certified that **LYE PING YING** (ID No: 12ABD06536) has completed this final year project entitled "**INTERACTIVE EFFECT OF TRIGONA HONEY AND AMPICILLIN ON *Staphylococcus aureus* ISOLATES OF INFECTED WOUND**" under the supervision of Mr. Ng Wen Jie (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours sincerely,

.....

(LYE PING YING)

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| | |
|---|---|
| % | Percentage |
| ˚ | Degree |
| ˚C | Degree Celcius |
| µL | microliter |
| µg/mL | microgram per millimeter |
| L | liter |
| g | gram |
| mg/mL | milligram per milliliter |
| µg | microgram |
| ADH | Arginine dihydrohylase |
| API | Analytical Profile Index |
| AST | Antibiotic susceptibility test |
| B | Beta |
| CFU/ml | Colony-forming unit per milliliter |
| EDTA | Ethylenediaminetetraacetic acid |
| et al. | *Et alii* |
| FRU | Fructose |
| GLU | Glucose |
| LAC | Lactose |
| M | Molar |
| MAL | Maltose |
| MAN | Mannitol |

| | |
|---|---|
| MEL | Melibiose |
| MIC | Minimum inhibitory concentration |
| MBC | Minimum bactericidal concentrations |
| MDG | Methyl-α-D-glucopyranoside |
| MDR | Multiple drug resistant |
| MH | Mueller Hinton |
| mm | millimeter |
| MNE | Mannose |
| MRSA | Methicillin-resistant *Staphylococcus aureus* |
| MSA | Mannitol salt agar |
| MSSA | Methicillin-sensitive *Staphylococcus aureus* |
| NaCl | Sodium chloride |
| NAG | N-acetyl-glucosamine |
| NIT | Nitrate |
| nm | nanometer |
| PAL | Phosphatase |
| PBP | Penicillin-binding protein |
| PBP2a | Penicillin-binding protein 2a |
| PBS | Phosphate buffer saline |
| pH | Power of Hydrogen |
| PVL | Panton-Valentine leukocidin |
| RAF | Raffinose |
| SAC | Sucrose |
| SCC | Staphylococcal chromosome cassette |

| | |
|---|---|
| spp. | Species |
| SEM | Scanning electron microscope |
| TRE | Trehalose |
| μg/mL | microgram per millimeter |
| URE | Urea |
| VP | Sodium pyruvate |
| v/v | Volume per volume |
| w/v | Weight per volume |
| XLT | Xylitol |
| XYL | Xylose |

# CHAPTER 1

# INTRODUCTION

Bacteria-like organisms have been existed on earth for about 3.5 billion years and Antony van Leeuwenhoek was the first person to observe bacteria and protozoa using microscope in 1674 (Tortora, Funke and Case, 2007). Later on, Louis Pasteur showed that microbes can cause fermentation and food spoilage. He also demonstrated the Germ Theory together with Robert Koch, concluding that many diseases are caused by the presence of microbes in the body and not by sins (Uzarski, 2006).

Colonization of normal flora on the surfaces of human skin and mucosal tissue acts as an effective barrier in preventing the growth of pathogenic bacteria. However, any cut or breach on the skin such as burns, ulcers, surgical and traumatic wounds would promote the entry of bacteria (Baron, 1996). According to Bessa, et al. (2013), *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most common bacteria isolated from infected wounds. In laboratory, the two methods routinely used in identification of wound-isolated bacteria are culturing and molecular testing. Among these two bacteria, *S. aureus* is the most commonly identified bacteria via culture and molecular testing (Baker and Acharya, 2004).

On the other hand, honey has been documented in the world oldest medical literatures since ancient times for its wound healing properties and antibacterial effects (Mandal and Mandal, 2011). The ancient Greeks and Egyptians used it for more than a thousand years in wound care treatment by reducing pain and inflammation that subsequently promote wound healing (Simon, et al., 2009). A review done by Molan (1992) mentioned honey mixed with lemon was used to treat common cold and various infections in Middle East and China. Despite of its effective antibacterial effect, the use of honey as antibacterial agent was slowly vanished and eventually replaced by antibiotics.

Discovery of antibiotics in the 19$^{th}$ century has been the most leading and greatest advances in therapeutic cure for infections and diseases caused by bacteria. The discovery of penicillin by Alexander Fleming in United Kingdom marked an important turning point in the development of medicine in human history (American Chemical Society, 1999). Antibiotic was the most effective therapeutic agent in preventing the growth of bacteria and killing them, but indiscriminate use of antibiotic made the bacteria to emerge resistance against it (David and Daum, 2010). Levy and Marshall (2004) reported that worldwide increased frequencies of not only single drug resistance but also multiple antibiotic resistance due to overuse and misuse of antibiotics had become a very serious threat to the public health. The development of bacteria resistant gene has intrigued the researchers to search for other alternative antibacterial methods (Al-Jabri, 2005).

Until recently, honey was rediscovered as one of the effective wound treatment agents due to the antimicrobial properties against multi-resistant bacteria (Reza, Afshin and Ory, 2013). Studies have proven that honey exhibits anti-bacterial, anti-inflammatory and antioxidant properties (Vallianou, et al., 2014). A study by Al-Jabri, et al. (2005) has proved that the bactericidal rate of Omani honey and gentamicin combination was superior compare to bactericidal rate of Omani honey alone and gentamicin alone. Although honey is well-recognized as an effective antimicrobial agent, there is only limited research published about the interactive effect of honey and antibiotics.

This study was undertaken to evaluate the interactive action of Trigona honey and ampicillin against *S. aureus*, the commonly causative agent isolated from infected wounds and also known as a common nosocomial pathogen responsible for community-associated skin and soft tissue infections (SSTIs).

Hence, the objectives of this study were:

i. To isolate *Staphylococcus aureus* from clinical wound samples.
ii. To study the anti-Staphylococcal activity of Trigona honey.
iii. To investigate whether Trigona honey possesses synergistic effect with ampicillin in inhibiting the growth of *Staphylococcus aureus*.
iv. To observe the morphological change of *Staphylococcus aureus* by using scanning electron microscope.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Honey

### 2.1.1 Introduction

Long before the era of modern medicine, other than a sweetener and flavouring agent, honey is well known as traditional medicine to treat illness and it is said to be the most longstanding medicine that has passed on and applied by people (Dorai, 2012).

Honey is a natural product produced by bees using nectar from flowers. Conversion of the nectar into honey in the honeycomb requires enzyme invertase from bees to partially digest sucrose into simple sugars, glucose and fructose (Chepulis, 2008). Generally, honey is made up of 80-85% of carbohydrates, 15-17% of water content, 0.3% of proteins, 0.2% ashes, minor amounts of amino acids, vitamins and other components like antioxidants (Cantarelli, et al., 2008). Although the constituents of honey are nearly similar, but the differences in climate conditions and floral sources which the bees forage do affect the exact composition and properties in each honey (Kayode and Oyeyemi, 2014). The quality of honey is always associated with its color intensity and U.S. Department of Agriculture has classified honey into seven categories according to the Pfund scale shown in Figure 2.1 (Fell, 1978). Pontis,

et al. (2014) reported that darker honey contains higher phenolic and flavones content with greater antioxidant activity as compare to lighter honey.

| USDA Color Standards Designations | Color Range USDA Color Standards | Color Range Pfund Scales Millimeters | Optical Density 1/ |
|---|---|---|---|
| Water White . . . . . | Honey that is Water White or lighter in color. | 8 or less | 0.0945 |
| Extra White . . . . . | Honey that is darker than Water White, but not darker than Extra White in color. | Over 8 to and including 17. | .189 |
| White . . . . . | Honey that is darker than Extra White, but not darker than White in color. | Over 17 to and including 34. | .378 |
| Extra Light Amber . . . . | Honey that is darker than White, but not darker than Extra light Amber in color. | Over 34 to and including 50. | .595 |
| Light Amber . . . . . | Honey that is darker than Extra Light Amber, but not darker than light Amber in color. | Over 50 to and including 85. | 1.389 |
| Amber . . . . . | Honey that is darker than light Amber, but not darker than Amber in color. | Over 85 to and including 114. | 3.008 |
| Dark Amber . . . . . | Honey that is darker than Amber in color. | Over 114 . . . . . | . . . . . |

1/ Optical Density (absorbance) = $\log_{10}$ (100/percent transmittance), at 560 nm for 3.15 cm thickness for caramel - glycerin solutions measured versus an equal cell containing glycerin.

**Figure 2.1:** Colour designations of honey (United States Department of Agriculture, 1985).

In general, honey is highly saturated with sugar and has low water content which makes it highly viscous in nature and able to inhibit the growth of microbes (Simon, et al., 2009). White, Subers and Schepartz (1963) mentioned about the glucose oxidase enzyme produced in honey oxidizes the glucose to gluconic acid and hydrogen peroxide. The presence of gluconic acid is always correlated with the acidic pH of 3.2-4.5 and it is a part of the important factors in antimicrobial activity (Molan, 1992; Chepulis, 2008). Besides of its antimicrobial properties, honey has the potential to fight against infections by boosting the immune system, reducing inflammation, scavenging free radicals and stimulating cell growth in human system (Al-Jabri, 2005).

### 2.1.2 Antimicrobial properties of Honey

Plenty of researches have demonstrated honey as an effective therapeutic agent against wide range of microorganisms due to its antimicrobial properties (Molan, 1992; Simon, et al., 2009). A review of Kwakman and Zaat (2012) stated that the high sugar content, hydrogen peroxide and acidic pH are the key antibacterial factors of honey and recently, methylglyoxal and antimicrobial peptide bee defensin-1 have been identified as important antimicrobial components in honey as well.

Generally, honey is composed of a mixture of sugars such as fructose, glucose, sucrose and maltose with low proportion of water. It is interesting that different concentrations of honey have different antibacterial impacts as the higher concentrations have greater antibacterial activities (Badawy, et al., 2004). Super-saturated condition of honey can induce osmotic stress that prevents fermentation and spoilage of honey by microorganisms (Kwakman and Zaat, 2012). Bittmann, et al. (2010) concluded that the high viscosity nature of honey makes it capable of hastening the wound healing by retracting exudates from the wounds. Thus, the hygroscopicity of honey is largely depending on the concentration of fructose as it provides a moisture environment desirable for wound healing (Boukraa, 2013).

The high sugar content of honey alone is capable of inhibiting the growth of microbes. Yet, the application of honey as dressings on wound may dilute the

honey due to wound exudate and lower down its inhibitory ability (Molan, 2002). Hence, osmolarity is not the sole reason for the antimicrobial action of honey, other components like low pH, phenolic compounds and hydrogen peroxide also contribute in hindering the bacterial growth (Mandal and Mandal, 2011).

The average pH of honey can range from 3.2 to 4.5, which is acidic enough to inhibit the growth of most pathogens (Allen, Molan and Reid, 1991; Chepulis, 2008). Honey is believed as an effective agent in preventing the colonization and penetration of bacteria at wound surface due to its acidic nature (Dudhamal, Gupta and Bhuyan, 2010). The low acidity of honey is primarily due to the production of gluconic acid in honey. Gluconic acid is formed when bees incorporate enzyme glucose oxidase into the honey which then lower down the pH sufficiently to inhibit growth of most microbes (Garedew, Schmolz and Lamprecht, 2003). As the optimum pH for most microbes ranges from 7.2 to 7.4, this makes them unable to survive in the acidic environment.

On the other hand, hydrogen peroxide ($H_2O_2$) is also identified as the major antibacterial agent of honey (White, Subers and Schepartz, 1963). In honey, $H_2O_2$ is produced enzymatically from the conversion of glucose into gluconic acid catalyzed by glucose oxidase. However, the action of glucose oxidase is only activated when the honey is diluted or the sugar concentration falls between 25-30% (Crane, 1990). Taormina, Niemira and Beuchat (2001) also

reported that the level of hydrogen peroxide is directly proportional to the glucose oxidase level and inversely proportional to the catalase level in the honey. Molan (1992) stated that the concentration of $H_2O_2$ after dilution is 1000 times lesser than the commonly used aseptic 3% $H_2O_2$ aseptic solution, so it is safe to use without any harmful effect to human health. Although hydrogen peroxide is the major key factor to the antibacterial properties of honey, but it is highly susceptible to destruction when exposed to heat and light (White, Subers and Schepartz, 1963).

Other than hydrogen peroxide, the non-peroxide components such as phenolic acids and flavonoids are also known as the important contributors to the antimicrobial activity of honey. Manuka honey, for example, is a non-peroxide honey which its antibacterial action stems partly from phytochemical components (Molan, 1992). The phenolic acids and flavonoids are relatively stable than hydrogen peroxide as they do not breakdown easily when exposed to light, heat and dilution (Molan, 2001). Besides that, flavonoids with antioxidant capacity are also important in determining the color, flavor, floral source and origin of the honey (Anklam, 1998). Studies done by Gheldof and Engeseth (2002) and Beretta, et al. (2005) showed honey in darker color has higher content of phenolic compounds and thus, a higher antioxidant activity.

Recently, Kwakman and Zaat (2012) discovered the importance of methylglyoxal (MGO) and bee defensin-1 as bactericidal agents in honey. MGO

originates from the conversion of dihydroxyacetone presents in the nectar of Manuka trees (*Leptospermum scoparium*) (Adams, Manley-Harris and Molan, 2009). Kwakman et al. (2011) has proved that MGO is the major component in non-peroxide Manuka honey that is responsible for inhibiting the growth of *Staphylococcus aureus*. Another recent study by Bhandary, et al. (2012) demonstrated the perturbation of cell membrane by MGO indirectly disrupted the DNA synthesis machinery that eventually led to the degradation of bacterial DNA.

Moreover, coincide with the previous studies by Adams, Manley-Harris and Molan (2009), Kwakman, et al. (2010) and Lin, Molan and Cursons (2011), Jing, et al. (2013) found out that negligible level of MGO could still inhibit growth of bacteria even when the activity of hydrogen peroxide was neutralized by enzyme catalase. Therefore, Manuka honey with exceptionally high level of MGO is more preferable nowadays in medical and nutritional use. Study showed that the antibacterial activity of Revamil honey relies on the presence of $H_2O_2$ and antimicrobial peptide bee defensin-1 which is absent in Manuka honey (Kwakman, et al., 2010). Although there is only little investigation on the composition of bee defensin-1 in honey and how it works, it is believed that the antimicrobial peptide bee defensin-1 could be the answer for future medication to fight against antibiotic resistant infections (Federation of American Societies for Experimental Biology, 2010).

### 2.1.3 Antioxidant Properties of Honey

Besides antibacterial activity, honeys is also well known with its antioxidant capacity. Antioxidant capacity is part of the therapeutic values of honey as the ability of scavenging free radicals is a feature of the antioxidant properties in honey (Aljadi and Kamaruddin, 2004). Estevinho, et al. (2008) demonstrated that phenolic compounds are partially responsible for both antibacterial and antioxidant properties of honey.

Significant results have showed darker honey has stronger antioxidant activity than honey that is lighter in color. This is attributed to presence of phenolic components that can scavenge free radicals and suppress the inflammatory response indirectly (Molan, 2001; Tewari and Irudayaraj, 2004; Estevinho, et al., 2008). It is noteworthy that honey can either neutralize the free radicals or inhibit the free radicals formation directly (Al-Jabri, 2005).

Despite of color intensity, both geographical origin and botanical source are also responsible for the differences in phenolic concentration and antioxidant capacity of various honey samples (Baltrusaityte, Venskutonis and Ceksteryte, 2007). As shown by Yao, et al. (2005), the phenolic compounds are derived from plants so different botanical sources have a direct impact on the amounts and types of phenolic compounds in honey.

Likewise, flavonoids can also be found as natural antioxidants in honey other than phenolic compounds. Flavonoids play a vital role in reducing the formation of free radicals and also scavenging the free radicals. Such potent antioxidant abilities have intrigued the interest of researchers to investigate the antioxidant activities of flavonoids in both *in vitro* and *in vivo* studies (Pietta, 2000). On top of that, Ansari, et al. (2013) claimed that flavonoids possess antimicrobial properties as they can disrupt the biofilm establishment by repressing bacterial quorum sensing and inhibit bacterial growth factors (Lee, et al., 2011).

### 2.1.4 Other Medicinal Properties of Honey

Anti-carcinogenic properties of honey are found to be related to the killing of cancer cells by immune cells. A study by Al-Waili (2003) showed that honey increased the number of monocytes, lymphocytes and eosinophils in the human serum. Moreover, another *in vitro* study by Abuharfeil, Al-Oran and Abo-Shehada (1999) also indicated an increase in proliferation of B and T-lymphocytes and neutrophils after stimulated by honey. It is stated that honey concentration as low as 0.1% was sufficient to activate human immune system. This study revealed the importance of honey as a potential anti-carcinogenic agent as the formation of many malignant tumors is linked with immunodeficiency.

Although there was a hypothetical risk of raising blood glucose level in diabetic patients after glucose in honey is absorbed via wound beds but to date, no such

case is reported (Akhtar and Khan, 1989). On the other hand, Erejuwa, Sulaiman and Wahab (2012) hypothesized that honey reduces hyperglycemia in humans but the mechanism of how fructose exerts its hypoglycemic effect still remain unknown. However, several studies suggested that fructose could delay gastric emptying which then slow down the intestinal absorption of glucose. Besides prolonging intestinal absorption, other evidence suggested that consumption of fructose can limit the absorption of carbohydrates induced satiety by increasing the duration of contact between fructose and intestinal receptors, causes most of the macromolecules to pass into large intestine. This study confirmed the substitution of refined sugar by natural honey is beneficial and harmless for diabetic patients. The animal studies by Erejuwa, et al. (2011) also proved that combination of diabetic medications with honey not only improves glycemic control, it also provides additional metabolic benefits including lowering the triglycerides, VLDL, cholesterol levels and amount of creatinine and bilirubin in the blood.

Furthermore, a recent research by Khalil, et al. (2015) concluded that honey is cardio-protective and consumption of honey can restore antioxidant levels and inhibit lipid peroxidation in heart tissue, which in turn lower down the risk of cardiovascular disease. The protective effect of honey is evidenced by significant reduction in the concentration of malondialdehyde (MDA) with normal architecture of heart tissue.

Apart from that, honey has also been used as an effective remedy to treat infected wounds and accelerating the process of wound healing since ancient times (Simon, et al., 2009). But, in the present days, most of the medical practitioners are not well aware of the benefits of honey to treat infected wounds (Molan, 2001). However, many convincing evidences have cited that honey contains anti-inflammatory and soothing effects on wounds without any side effects (Molan, 2001; Al-Jabri, 2005). The study by Church (1954) demonstrated that honey was capable of receding the stiffness of inflamed wrist joints in guinea pigs and this healing capability was ascribed to the fact that honey was able to reduce edema and exudation, promoting the formation of granulation tissue and hastening wound epithelialization (Hejase, Bihrle and Coogan, 1996; Bittmann, et al., 2010; Iftikhar, et al., 2010).

## 2.2 Stingless Bees

### 2.2.1 Taxonomy, Morphology and Distribution

Stingless bees are one of the three eucosial bees other than honey bee and bumblebee (bombini). Stingless bees are taxonomically characterized into two major groups, Trigona and Melipona (Vit, Pedro and Roubik, 2013). The stingless bee, Meliponini, is a close relative to the honey bees, Apini, and both belong to the same family Apidae (Winston and Michener, 1977). Stingless bees are distinguished from other bees through three features: reduction in wing venation, vestigial and atrophy of sting and presence of penicillum on hind tibia as shown in Figure 2.2 and 2.3. Although their sting is atrophied in size, but

they still can attack by biting if their nest is disturbed (Wille, 1983; Temaru, et al. 2007).

**Figure 2.2:** Reduction of sting in stingless bee (Australian Native Bee Research Centre, 2008).

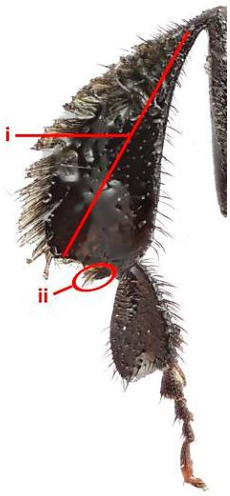


**Figure 2.3:** Presence of i) corbicula and ii) penicillum on the hind tibia (Chui, 2014).

Trigona is the most widely distributed and largest genus of stingless bees living in the tropical and subtropical regions of the world, including Australia, Africa, Southeast Asia and tropical America (Michener, 2007). The distribution of 11 subgenera of Trigona species is shown in Figure 2.4. By far, the number of known stingless bee species worldwide is estimated to be around 400 to 500. However, more new species of stingless bee are expected to be identified every year and to date, there are more than 30 species are found in Malaysia (Michener, 2013; Chien, 2015). Estimated 32 species of stingless bees are currently recorded in Malaysia as shown in Figure 2.5. Malaysian Agricultural Research and Development Institute (MARDI) stated that there are at least 11 species of

stingless bees are highly potential to be domesticated in Malaysia. Out of 11 species, four of them including *Heterotrigona itama*, *Geniotrigona thoracica*, *Tetragonilla atripes* and *Tetrigona peninsularis* are able to pollinate agricultural crops in Malaysia (Jalil, 2014). However, another research conducted by Kelly, et al. (2014) revealed only five Trigona bee species are found in bee farm at Kelantan and only *T. itama* and *T. thoracica* are commonly used in meliponiculture.

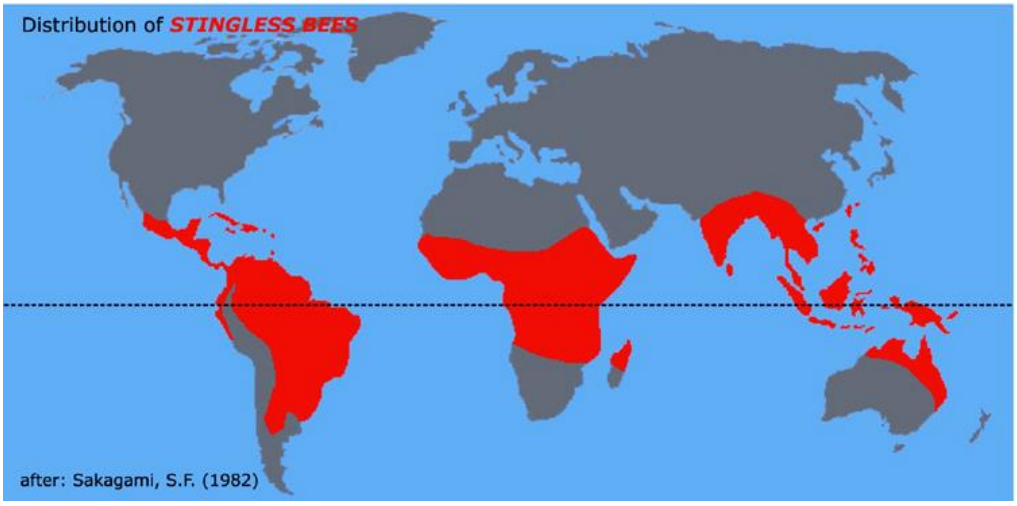


**Figure 2.4:** Distribution of stingless bee subgenera in the world (Sakagami, 1982).

| Number | Species | (Schwarz, 1939) | Specimens in MARDI insect Museum | (Osawa and Tsubaki, 2003) |
|---|---|---|---|---|
| 1 | *Trigona itama* | X | X | |
| 2 | *T. erythrogastra* | X | X | |
| 3 | *T. canifrons* | X | X | X |
| 4 | *T. fimbriata* | X | X | X |
| 5 | *T. thoracica* | X | X | |
| 6 | *T. fuscobalteata* | X | X | |
| 7 | *T. iridipennis* | X | X | |
| 8 | *T. geissleri* | X | X | X |
| 9 | *T. atripes* | X | X | X |
| 10 | *T. atripes var collina* | X | X | |
| 11 | *T. atripes var fuscibasis* | X | X | |
| 12 | *T. apicalis var smith* | X | X | |
| 13 | *T. apicalis var melanoleuca* | X | X | |
| 14 | *T. apicalis var peninsularis* | X | X | X |
| 15 | *T. scintillans* | X | X | |
| 16 | *T. pendleburyi* | X | X | |
| 17 | *T. nitiventris* | X | | |
| 18 | *T. ventralis* | X | X | |
| 19 | *T. terminata var smith* | X | X | |
| 20 | *T. terminata var latabalteata* | X | | |
| 21 | *T. minor sakagami* | X | X | |
| 22 | *T. rufibasalia* | X | X | |
| 23 | *T. moorei schwarz* | X | X | |
| 24 | *T. pagdeniformis* | | X | X |
| 25 | *T. minangkabau* | | X | X |
| 26 | *T. leeviceps* | | X | X |
| 27 | *T. nitidirentris* | | | X |
| 28 | *T. reepeni* | | X | |
| 29 | *T. pagdeni* | | X | |
| 30 | *T. melina* | | X | |
| 31 | *T. nitidiventris* | | X | |
| 32 | *T. klossi* | | X | |

**Figure 2.5:** Thirty-two species of stingless bees recorded in Malaysia (Norowi, et al., 2010).

### 2.2.2 Trigona Honey

Trigona honey or commonly known as "Kelulut" honey in Malaysia is produced by stingless bee (*Trigona* spp.). Shahjahan, et al. (2007) evaluated the hypothesis where "Kelulut" honey was more effective and performed better than honeybee honey with wider spectrum antibacterial activity. Generally, Trigona honey produced by stingless bee is less viscous, darker in color and has strong acid flavor (Garedew, Schmolz and Lamprecht, 2003).

In the past, majority of the researches were focused on honey produced by *Apis* spp. or commonly known as honeybee and lesser attention was paid to stingless bee honey (Boorn, et al., 2010). Till now, only limited information is available about the physico-chemical and antibacterial characteristics of Trigona honey. Most of the reports on antimicrobial properties of *Trigona* spp. were on propolis but not on honey (Surendra, Bhushanam and Ravikumar, 2012). In 2009, *Trigona laeviceps* stingless bee honey from Thailand was reported by Chanchao to exhibit antimicrobial activity attributed to its acidic nature, hyperosmotic effect and presence of hydrogen peroxide or antimicrobial peptides.

Recently, Andualem (2014) compared the composition of honeybee honey and stingless bee honey and concluded that Trigona honey showed much higher antimicrobial capacity than honeybee honey. This is because Trigona honey has very low pH and high sugar content which greatly increase its antimicrobial action in comparison with other honeys. Moreover, the minimum inhibitory

concentrations (MIC) and minimum bactericidal concentrations (MBC) of honey produced by stingless bee are also significantly lower than honey produced by honeybee (Ewnetu, Lemma and Birhane, 2013).

## 2.3 Wound Infection

### 2.3.1 Wound Isolated Bacteria

Skin is one of the major barriers for infection establishment and physical disruption of skin creates a wound that facilitate bacterial colonization (Janda, Abbott and Brenden, 1997). Bacteria isolated from the infected wounds can be divided into aerobic bacteria and anaerobic bacteria. According to the wound care practitioners, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* are commonly known to be the primary cause of delayed healing and infection in wounds (Lawrence, 1992; Bowler, Duerden and Armstrong, 2001). A study by Bowler, Duerden and Armstrong (2001) stated that environment, surrounding skin microflora and endogenous sources like mucous membrane are the three main sources where most wound contaminants are likely to originate from. The arisen of multiple drug resistance by nosocomial bacteria, *P. aeruginosa* and *S. aureus* has been a huge concern internationally and obstacle in pharmaceutical industry (Frieden, 2010).

### 2.3.2 *Staphylococcus aureus*

In 1880, surgeon sir Alexander Ogston discovered the presence of micrococcus in pus from surgical abscesses and named it as *Staphylococcus* (Alice, et al., 2011). *S. aureus* was first discovered in Aberdeen, Scotland that responsible for causing wound infection (Ogston, 1984). *S. aureus* are Gram-positive coccal bacteria which present either singly, in pairs or in grape like-clusters as shown in Figure 2.6 (Salton and Kim, 1996). *S. aureus* reproduce asexually by binary fission but the two daughter cells remain attached to each other in clusters like form and do not separate during reproduction (Yakubu, 2009).

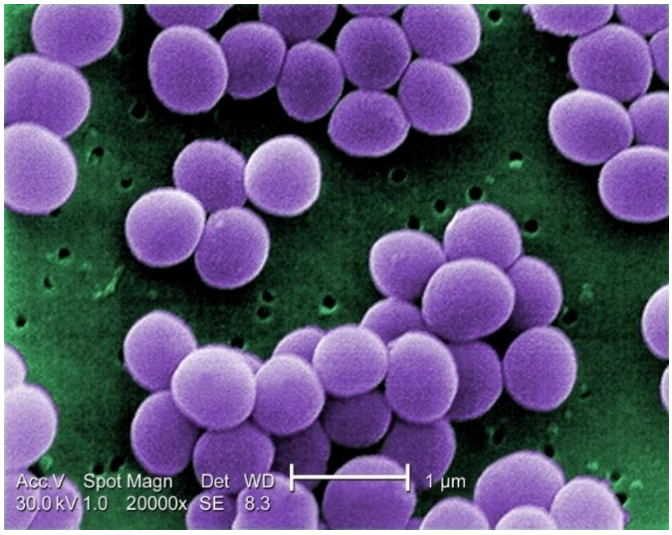


**Figure 2.6:** Scanning electron micrograph (SEM) of *S. aureus* (Centers for Disease Control and Prevention's Public Health Image Library, 2001).

*S. aureus* are non-motile and non-spore forming facultative anaerobes. Members of genus *Staphylococci* are oxidase negative and can synthesize enzyme catalase which breakdown hydrogen peroxide into water and oxygen

which differentiate them from genus *Streptococci* (Mandell, 1975; Wilkinson, 1997). Besides that, *S. aureus* are also known as high salt tolerance bacteria that can grow at about 85% of NaCl concentration (International Commission on Microbiological Specifications for Foods, 1996). In the view of Alebachew, et al. (2012), it is strictly important to consider proper antibiotic parameter in treating *S. aureus* infections to prevent occurrence of multi-drug resistant infection. *S. aureus* strains that are resistant to antibiotic methicillin are known as Methicillin-resistant *S. aureus* (MRSA).

### 2.3.3 Pathogenicity of *Staphylococcus aureus* in Wound

*S. aureus* are the most robust and virulent microbes among the *Staphylococci* family that infect humans. Its tendency to develop antibiotic resistance has been a huge concern to human health. Moreover, externally acquired *Staphylococci* bacteria can cause life threatening complications like pneumonia, endocarditis, meningitis and osteomyelitis when inoculated into open wounds (David and Daum, 2010). Vitko and Richardson (2013) once said that the ability of *S. aureus* to cause diseases is largely attributed to the presence of different virulence factors encoded in its genome and resistance to diversity of antibiotics. *S. aureus* consist of a variety of virulence factors that facilitate its pathogenesis of infection. Wertheim et al. (2005) believed that the basis of *S. aureus* colonization and establishment of infection is related to the adhesion ability to host cells and evasion from immune response as well.

Expression of surface protein, microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) by *S. aureus* facilitate the binding of *S. aureus* to host cells molecules such as collagen, fibronectin and fibrinogen (Gordon and Lowy, 2008). The interaction between fibrinogen and collagen promoted the attachment of *S. aureus* to damaged host cells (Foster, 1996). Besides that, the ability to produce coagulase by *S. aureus* also protects them from host defense systems. Enzyme coagulase converts fibrinogen to fibrin, forming blood clot to enclose the bacterial cells (Plata, Rosato and Wegrzyn, 2009). Such phenomenon explains the persistence of *S. aureus* in wound area.

In addition, a number of cytolytic toxins including alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin and Panton-Valentine leukocidin (PVL) produced by *S. aureus* also help in resisting the neutrophil killing and disruption of host cell membrane. Alpha-hemolysin is the most well understood protein among all the cytolytic toxins by forming pore on the host membrane and eventually lyse the cell (Plata, Rosato and Wegrzyn, 2009). Panton-Valentine Leukocidin is another potential cytotoxin produced by *S. aureus* with higher affinity toward leukocytes (Kaneko and Kamio, 2004). It forms pores in cell membrane and induces the release of lysosomal enzymes into cytoplasm which eventually leads to severe tissue necrosis and leukocyte destruction (Gladstone and VanHeyningen, 1957).

On top of that, *S. aureus* wound infection is also attributed to the formation of biofilms as shown in Figure 2.7. Staphylococcal biofilms delay the re-epithelialization process and prolong wound healing. Bacterial biofilms help the bacteria escape from phagocytosis and antimicrobial substances (Schierle, et al., 2009).

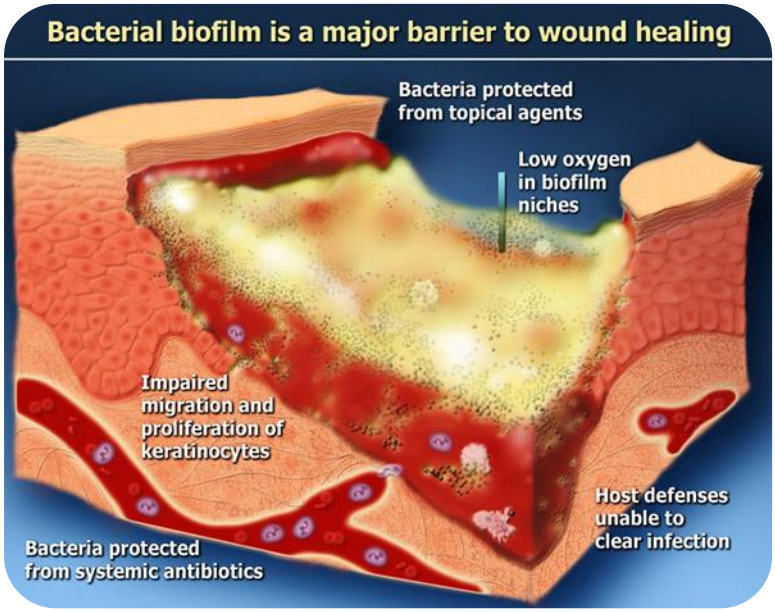


**Figure 2.7:** Staphylococcal biofilms impair wound healing (Schierle, et al., 2009).

## 2.4 Ampicillin

### 2.4.1 Beta-lactam Antibiotic

Due to its beta-lactam ring, ampicillin is categorized under the aminopenicillin family and this beta-lactam antibiotic has been used extensively since 1961 (Acred, et al., 1962). Ampicillin is a broad range antibiotic as it was the first semi-synthetic penicillin that is active against both Gram-positive and Gram-negative bacteria. However, it is not effective against *P. aeruginosa*,

*Enterobacter*, *Acinobacter*, *Serratia* and *Citrobacter* (Ash, Mauck and Morgan, 2002).

Beta-lactam antibiotic is one of the most commonly used antibiotics against many bacteria. The bactericidal activity of ampicillin is based on the action on several enzymes with peptidoglycan synthesis and lysis function. It acts in a way to disrupt the cell wall structure (Wivagg, Bhattacharyya and Hung, 2014). Ampicillin acts as an irreversible inhibitor of transpeptidase and carboxypeptidase that is essential for bacterial cell wall formation (Torimiro, Moshood and Eyiolawi, 2013). Absence of transpeptidase activity causes further degradation of bacterial cell wall with the release of autolytic enzymes, autolysins which eventually leads to cell lysis (Sears, Spear and Saenz, 2007). Nearly every pathogenic bacteria can be inhibited by targeting the peptidoglycan synthesis mechanism. Ampicillin targets the penicillin-binding proteins located in cytoplasmic membrane of bacteria that interferes the peptidoglycan biosysnthesis (Denyer, Hodges and Gorman, 2004). However, penicillins can be inactivated by beta-lactamase producing bacteria such as *S. aureus*.

### 2.4.2 Resistance to Ampicillin

The increasing incidence of antibiotic resistance due to excessive and uncontrolled use has been a huge concern in the treatment for infectious diseases. The commonest resistance mechanism to beta-lactam antibiotics is due to the

action of beta-lactamases encoded by blaZ gene, production of low affinity penicillin-binding protein 2a (PBP2a), modification of outer membrane permeability and inhibition of release of autolytic enzymes (Jacoby and Sutton, 1985; Fuda, Fisher and Mobahery, 2005; Santiago, et al., 2014; Wivagg, Bhattacharyya and Hung, 2014).

Resistance to ampicillin occurs by the action of bacterial enzymes, the beta-lactamases that produced by bacteria. In Staphylococcal species, the resistance to beta-lactam antibiotics is mediated through the hydrolysis of beta-lactam ring by enzymes and expression of PBP2a with low affinity (Torimiro, Moshood and Eyiolawi, 2013). Generally, there are four types of beta-lactamases with similar sequence but different substrate specificity have been discovered in *S. aureus* (Zygmunt, Stratton and Kernodle, 1992).

Apart from that, *S. aureus* also begin to develop resistance to specific antibiotics by synthesis of low affinity binding proteins. *S. aureus* or MRSA developed resistance to methicillin by acquiring *mec*A gene on Staphylococcal chromosome cassette (SCC*mec*) (Plata, Rosato and Wegrzyn, 2009). The gene *mec*A encodes low affinity PBP2a with beta-lactam insensitive domain called transglycosylase. PBP2a confers resistance by decreasing the affinity towards antibiotics (Pinho, et al., 2001).

Moreover, beta-lactam antibiotics can penetrate into Gram-negative bacteria through porins and hence, porins are also the potential targets for drug resistance in Gram-negative bacteria (Nitzan, Deutsch and Pechatnikov, 2002). In Gram-negative bacteria, beta-lactam antibiotic resistance is mediated through alteration in the number and structure of porin proteins that reduce its permeability to antibiotics (Heesemann, 1993). However, production of beta-lactamase by far is the most important key factor in conferring resistance to beta-lactam antibiotics including ampicillin (Pitout, Sanders and Sanders, 1997).

## 2.5 Synergistic Effect

### 2.5.1 Combination Therapy

Synergistic effect is defined as the increased intensity caused by the combination of two substances on an organism (Brittany, 2013). According to Kepner (2004), the synergistic effect can be said where the sum of combination effect is greater than sum of individual effects.

Combination therapy or polytherapy is the usage of multiple medications or therapies at the same time for treatment (OncoSec Medical, 2013). Most of the physicians and medical professionals recommend combination therapies as the choices for disease treatment. The concept of using combination therapy in treatment of infectious diseases is not new and has started to become more and more popular due to the emergence of multi-antibiotic resistance (Powers, 2004). After all, combination therapy is highly recommended due to its high

efficiency in treatment, lower risk of antibiotic resistance emergence, lesser adverse effect and shorter treatment period (Ilyushina, et al., 2006; Chanda, Rakholiya and Nair, 2011). For example, antibiotics can associate with plant extracts or other chemicals in combination therapy. Although the antimicrobial effects of plant extract alone may not be significant, but studies proved that consumption of plant extract together with therapeutic drug did enhance the effectiveness of drug treatment (Kamatou, et al., 2006).

### 2.5.2 Synergistic Effect between Honey and Antibiotics

Combination therapy of honey and antibiotic is believed to provide more advantages where lesser antimicrobial agent is needed that can reduce the development of antibiotic resistance and treatment cost (Jenkins and Cooper, 2012). The synergistic activity of honey and antibiotic against multiple drug resistant (MDR) bacteria was first reported in 1998. Karayil, Deshpande and Koppikar (1998) noted that combination of honey and antibiotic gave superior synergistic effect in inhibiting *Pseudomonas* spp., hence they suggested that it is possible to give honey along with antibiotic for MDR bacterial infection treatment.

In addition, Al-Jabri et al. (2005) also conducted an experiment to investigate the killing rate of Omani honey, gentamicin and combination of honey and gentamicin on *S. aureus*. They found out that the effect of combination of Omani honey and gentamicin was greater than gentamicin alone. For the same

duration, the killing rate of the combination of Omani honey and gentamicin was 92% at 30 minutes and 93% at one hour, which was superior than gentamicin alone which was only 70% and 88% respectively. The results of the study showed that Omani honey enhanced the activity of gentamicin by 22% in the early phase of interaction.

Besides that, in another study by Jenkins and Cooper (2012), the results showed that each combination of three antibiotics (tetracycline, imipenem and mupirocin) with Manuka honey respectively was synergistic against MRSA while another three antibiotics (rifampicin, tetracycline and colistin) showed addictive effect in combination with Manuka honey against *P. aeruginosa*. Synergism of Manuka honey and imipenem towards MRSA proved that it is conceivable that this combination could be applied as topical treatment for wound infections.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Honey Samples

Fresh Trigona honey samples were obtained from a bee farm located in Pedas, Negeri Sembilan. The honey samples obtained were stored in dark at room temperature. Exposure to light could destroy enzyme glucose oxidase and affect the production of hydrogen peroxide, thus it is important to keep honey in dark condition or amber glass to preserve its antimicrobial properties (Stanway, 2013).

### 3.1.2 Bacterial Samples

Standard reference strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923 and ATCC 33591) were obtained from Faculty of Science, UTAR. Another two *S. aureus* samples isolated from clinical wound samples were obtained from Metro Specialist Hospital located at Sungai Petani, Kedah. The bacteria samples were cultured and maintained with mannitol salt agar (MSA).

### 3.1.3 Chemicals and Media

Chemicals and media used in this study are summarized in Table 3.1.

**Table 3.1** Chemicals and media used and their respective manufacturers.

| **Chemicals/Media** | **Manufacturers** |
|---|---|
| Absolute ethanol | |
| Ampicillin sodium salt | Bio Basic Inc., USA |
| Glutaraldehyde | Sigma-Aldrich, USA |
| Mannitol salt agar | Laboratorious CONDA, Madrid |
| Muller Hinton agar | HiMedia Laboratory, India |
| Methicillin (5 µg), ampicillin (10 µg), penicillin G (10 µg), ciprofloxacin (5 µg), gentamicin (10 µg), chloramphenicol (30 µg), trimethoprim (5 µg) and tetracycline (30 µg). | Oxoid Ltd, England |
| Nutrient broth | Merck, Germany |
| Phosphate buffer saline (0.01M) | Takara, Japan |
| Sodium chloride | Merck, Germany |

### 3.1.4 Equipments and Labwares

The equipments and labwares used in this study are listed in Table 3.2.

**Table 3.2** Equipments and labwares used and their respective manufacturers.

| **Equipments/Labwares** | **Manufacturers** |
|---|---|
| Cotton swab | Premier Diagnostics, Malaysia |
| Cuvette | Greiner Bio-one, Malaysia |
| Eppendorf tubes | AXYGEN, INC., USA |
| Falcon tubes | Becton Drive, USA |
| Nylon filter (0.45 µm) | MS®, USA |
| Incubator | Memmert, Germany |
| Laminar flow cabinet | Camfil Farr, Malaysia |
| Microscopic slides | Biomedia Laboratories, Malaysia |
| Micropipette tips | AXYGEN, INC., USA |
| Petri dish (90 x 15 mm) | Brandon™, Canada |
| Portable Bunsen | CAMPINGANZ® LABOGAZ 206, Netherlands |
| Schott bottle | DURAN, Germany |
| Shaking incubator | Straits Scientific, Malaysia |
| Spectrophotometer | Thermo Scientific Genesys 20, Malaysia |
| Sterile syringe | TERUMO, Philippines |
| Vortex mixer | VELP® SCIENTICA, Europe |

## 3.2 Methodology

### 3.2.1 Experimental Design

The overview of this study was designed as shown in Figure 3.1.

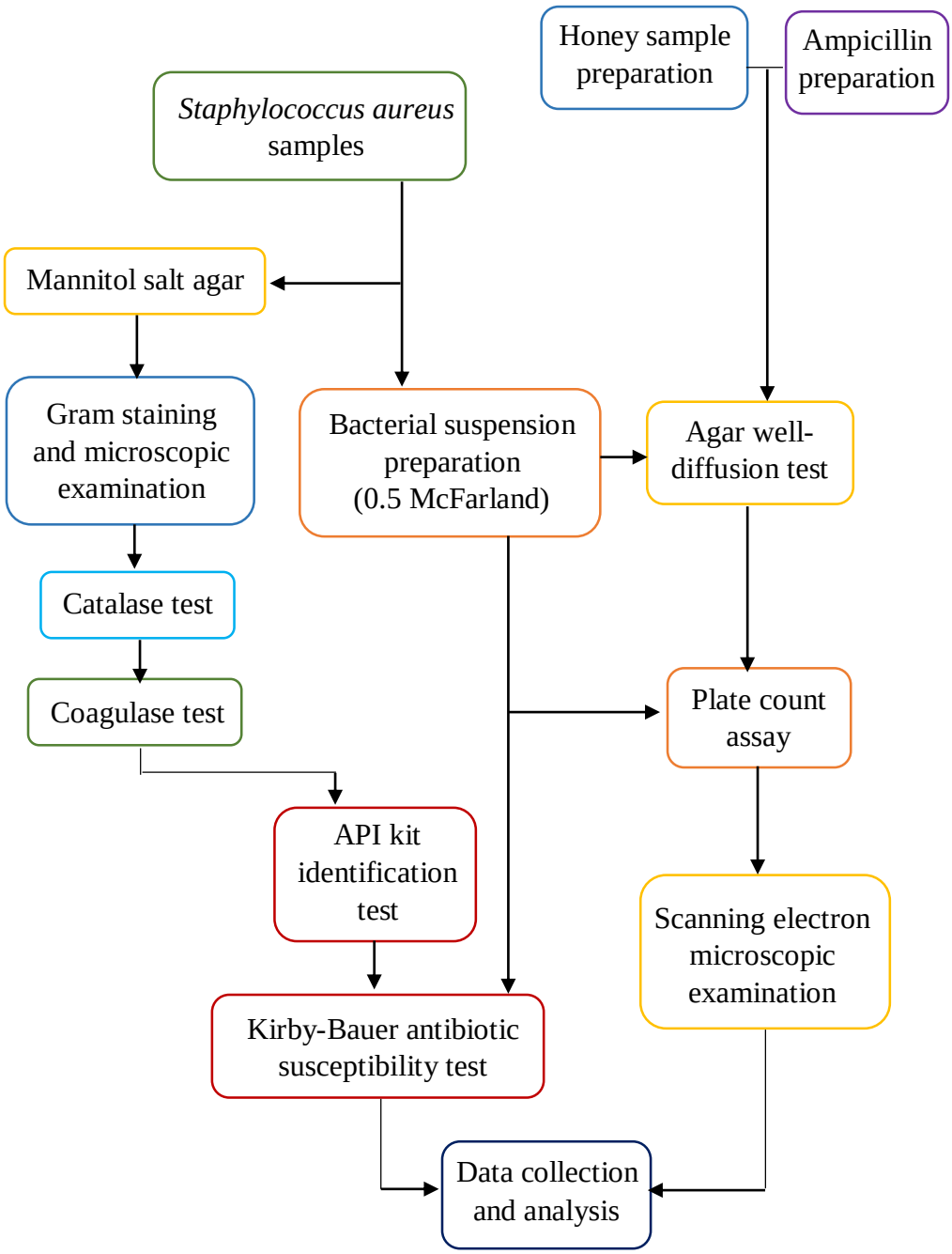


**Figure 3.1**: Experimental design of this study.

### 3.2.2 Preparation of Media and Solution

#### 3.2.2.1 Ampicillin solution

A stock solution of ampicillin with concentration of 10 mg/mL was prepared by dissolving 0.01 g of ampicillin sodium powder in 1 mL of distilled water. Then, 1 µl of ampicillin stock solution was then added into 1 mL of distilled water to make a final concentration of 10 µg/mL. The prepared solution was stored at 4˚C until use.

#### 3.2.2.2 Mannitol Salt Agar

Mannitol salt agar (MSA) is both selective and differential medium specifically used for isolation of *Staphylococcus* species. MSA consists of 7.5% of sodium chloride that selectivity distinguish most of the bacteria that cannot tolerate high salt concentrations and allow growth of *Staphylococcus* species (Shields and Tsang, 2006). The presence of mannitol and phenol red pH indicator differentiates mannitol-fermenting Staphylococci from non-fermenting bacteria. Appearance of yellow color is due to the fermentation of mannitol that produces acid and hence lower the pH of agar (Anderson, 2013).

MSA was prepared by suspending 111 g of MSA powder in 1 L of distilled water. The medium was mixed well and autoclaved at 121˚C for 15 minutes. After that, 20 mL of medium was dispensed into each sterile petri dish and allowed to cool and solidify at room temperature (Laboratorios Conda S.A., 2010).

### 3.2.2.3 Mueller Hinton Agar

Bauer et al. (1966) suggested the usage of Mueller Hinton (MH) agar in antibiotic susceptibility tests (AST) due to its capability to support the growth of non-fastidious bacteria and low amount of sulfonamide, trimethoprim and tetracycline inhibitors which provide better diffusion for antibiotics (Clinical and Laboratory Standards Institute, 2006).

In this study, 38 g of MH agar powder was suspended in 1 L of distilled water and autoclaved at 121˚C for 15 minutes. After that, 20 mL of medium was poured into each sterile petri dish and allowed to solidify at room temperature (HiMedia Laboratories, 2011).

### 3.2.2.4 Nutrient Broth

Nutrient broth is a general purpose medium used for cultivation of wide variety of microorganisms. Nutrient broth is suitable for the enumeration and cultivation of fastidious bacteria (Neogen, 2010). Nutrient broth was prepared by adding 13 g of nutrient broth powder into 1 L of distilled water. The medium was mixed well and autoclaved at 121˚C for 15 minutes (Oxoid, 2013).

### 3.2.2.5 Saline Solution

Saline solution was prepared by dissolving 8.5 g of sodium chloride in distilled water to achieve 0.85% (w/v). The solution was mixed well and autoclaved at

121˚C for 15 minutes (U.S. Food and Drug Administration, 2001). The saline solution was then used to prepare bacterial suspension equivalent to 0.5 McFarland for Kirby-Bauer test, agar well-diffusion assay, plate count assay and sample preparation for scanning electron microscopic examination.

### 3.2.3 Identification and Antibiotic Susceptibility Determination of Staphylococcal Isolates

#### 3.2.3.1 Mannitol Fermenting Test

Clinical wound samples together with reference strains (*S. aureus* ATCC 25923 and ATCC 33591) were cultured on mannitol salt agar (MSA) to detect the presence of *Staphylococcus* species. After overnight incubation at 37 ˚C, each yellow color bacterial colony was further subjected to Gram staining, catalase and coagulase tests.

#### 3.2.3.2 Gram Staining

Gram staining was performed to confirm the Gram type and microscopic appearance of bacterial isolates. The bacterial smear was stained with crystal violet (1 minute), followed by addition of mordant iodine (30 seconds) to fix the dye. Next, the bacterial sample was decolorized with alcohol (10 seconds) and counter stained again with safranin (10 seconds).

#### 3.2.3.3 Catalase Test

Catalase is an enzyme that catalyzes breakdown of hydrogen peroxide into water and oxygen. The ability of *S. aureus* to produce catalase differentiate them from *Enterococci* and *Streptococci* species (Seal and Pleyer, 2007). In this study, identified Gram-positive Staphylococcal bacteria isolates were smeared on a sterile glass slide with the addition of a few drops of 3% hydrogen peroxide. The formation of bubble due to oxygen generation in catalase test was used to identify the Staphylococcal isolates as catalase-positive.

#### 3.2.3.4 Coagulase Test

Majority of *S. aureus* can produce coagulase that converts fibrinogen in blood plasma into fibrin and clot the blood. This test was also used to differentiate *S. aureus* from coagulase-negative Staphylococci (Ryan, Ray and Sherris, 2004). In this assay, a tube containing 1 mL of EDTA blood plasma was inoculated with a few colonies of catalase-positive Staphylococcal isolate. The presence of plasma coagulation was checked after overnight incubation at 37 ˚C.

#### 3.2.3.5 API Identification Test

Next, identified catalase-positive and coagulase-positive Staphylococcal isolates were then subjected to API Staph Identification test as shown in Figure 3.2. API Staph identification system manufactured by bioMerieux of Raleigh was employed for rapid identification of *S. aureus* from other Staphylococcal species. The API Staph strip consists of 20 microampules containing dehydrated

substrates or nutrient media. A homogenous bacterial suspension was prepared by inoculating few Staphylococcal colonies into the API Staph Medium and the turbidity was adjusted to 4.0 McFarland ($1 \times 10^8$ CFU/mL) based on the standard given. An incubation box was prepared and about 5 mL of distilled water was distributed into the honeycomb wells of the tray to create a humid atmosphere. The strip was removed from the packaging and placed in the incubation box. By using a pipette, only the tube portion of microampules was filled with the inoculated API Staph Medium. Few drops of mineral oil was added into the ADH and URE cupules to ensure anaerobiosis. The incubation box was then closed with lid and incubated at 37˚C for 18-24 hour ().

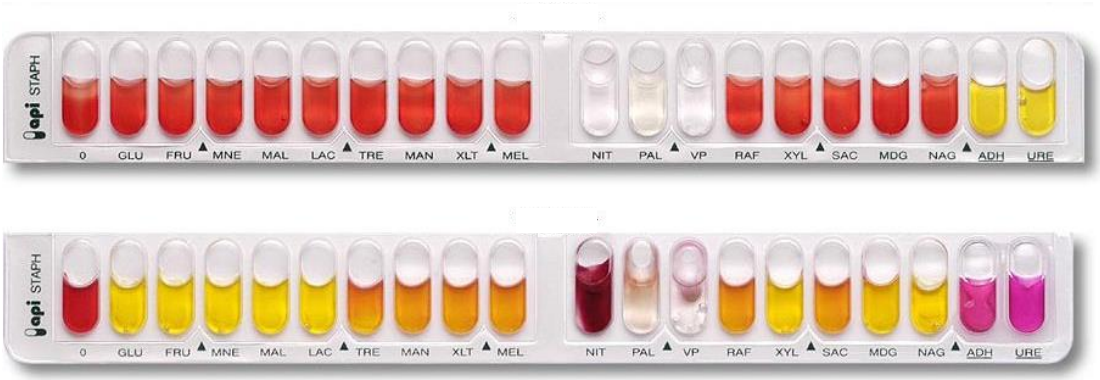


**Figure 3.2:** API Staph Identification Test (API® Staph, 2002).

### 3.2.3.6 Kirby Bauer Test

Kirby Bauer test is also known as disk diffusion test, the most commonly used method in clinical laboratory to determine the resistance or sensitivity of bacteria towards different types of antibiotic in order to assist the physician in selecting antibiotics for infection treatment (Bauer et al., 1966). Kirby Bauer test relies on the diameter of zone of inhibition formed around the antibiotic

impregnated disks on the agar plate. The measurement is then interpreted in accordance to the standard criteria (Clinical and Laboratory Standard Institute, 2011).

In this test, a few single colonies of each identified *S aureus* isolates were aseptically picked from the fresh culture plate using sterile cotton swab and suspended into 10 mL of saline solution. The inoculum density was standardized to 0.5 McFarland which is equivalent to $1 \times 10^8$ CFU/mL by adjusting the absorbance value within range from 0.08 to 0.10 at 625nm (Hudzicki, 2009). A sterile cotton swab was then dipped into the bacterial suspension and rotated against side of the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of plate for three times and each time the plate was rotated approximately 90 $^{o}$ to ensure even distribution of bacterial suspension onto the agar surface (Clinical and Laboratory Standards Institute, 2006). Next, the antibiotic impregnated discs including methicillin (5 µg), ampicillin (10 µg), penicillin (10 µg), gentamicin (10 µg), chloramphenicol (30 µg), trimethoprim (25 µg) and tetracycline (30 µg) were placed onto the surface of MH agar using a sterile forceps. The plates were then incubated overnight at 37˚C.

After incubation, the diameter of zone of inhibition formed around each disc was measured to the nearest millimeter (mm). The antibiotic susceptibility was

determined according to the guidelines published by Clinical and Laboratory Standards Institute (2011).

### 3.2.4 Determination of Interactive Effects between Honey and Ampicillin

For ampicillin preparation, 1 µL of ampicillin stock solution (10 mg/mL) was mixed with 1 mL of distilled water to obtain final concentration of 5 µg/mL. For 50% (v/v) honey preparation, 1 mL of Trigona honey was diluted with 1 mL of distilled water. The mixture was then vortexed to achieve homogenous state. For mixture test sample preparation, 1 mL of Trigona honey was added with an equal volume of 10 µg/mL of ampicillin solution. The mixture was then vortexed to acquire a homogenous mixture of ampicillin of 5 µg/mL and honey in 50% (v/v) concentration.

#### 3.2.4.1 Agar Well-diffusion Test

Agar well-diffusion test was carried out to identify the effect of honey, antibiotic and combination of both honey and antibiotic against the growth of bacteria. In this test, 0.5 McFarland bacterial suspension was prepared and then, a sterile cotton swab was dipped into the bacterial suspension and rotated against side of the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of MH agar for three times and each time the plate was rotated approximately 90 °to ensure even distribution of bacterial suspension onto the agar surface (Clinical and Laboratory Standards Institute, 2006). After that, four wells with 5 mm diameter was cut into the agar with a sterile cock borer. Each

of the well was filled with 54 µL of the honey sample (50% v/v), ampicillin (5 µg/mL) and mixture of honey (50% v/v) and ampicillin (5 µg/mL) respectively. The fourth well was loaded with the same volume of distilled water and served as negative control. The plates were then incubated at 37˚C for 18-24 hour. The diameter of zone of inhibition formed was then measured to the nearest millimeter (mm). This assay was carried out in triplicate and average value was obtained.

#### 3.2.4.2 Plate Count Assay

This modified assay was conducted with the reference of Al-Jabri et al. (2005). Three tubes containing 2 mL of 50% (v/v) of honey, 5 µg/mL of ampicillin and mixture of honey (50% v/v) and ampicillin (5 µg/mL) respectively were used in this assay. Next, 20 µL of 0.5 McFarland bacterial suspension was added into each tube and mixed well. Then, 20 µL of each test sample was aspirated out and added into a microcentrifuge tube containing 180 µl of saline solution to reach a dilution ratio of 1:10. A volume of 10 µL from each dilution was evenly plated on the MSA agar and incubated at 37˚C for 24 hours. The three tubes containing test samples were also incubated at 37˚C for 24 hours. Using the same samples after incubation, the procedures were repeated. The number of colonies formed on MSA plates was counted for both 0-hour and 24-hour incubation.

This assay was performed in triplicate to ensure the accuracy of result and the bactericidal rate K was calculated using the following formula:

$$\text{Bactericidal rate, } K = [(A - B)/A] \times 100\%$$

where A represents the total number of colonies formed at 0 hour, B represents total number of colonies formed at 24 hours.

### 3.2.5 Scanning Electron Microscopic Examination

The effects of honey and ampicillin on the morphology of bacteria was examined using scanning electron microscope (SEM). The general protocol for SEM specimen preparation is as below.

Three tubes containing 5 mL of 50% (v/v) of honey, 5 μg/mL of ampicillin and mixture of honey (50% v/v) and ampicillin (5 μg/mL) were prepared, then 5 mL of 0.5 McFarland bacterial suspension was inoculated into each tube and incubated at 37˚C for 24 hours. All of the test samples were then subjected to centrifugation at 3500 rpm for 5 minutes to pellet down the cells and primary fixation was carried out with 2.5% (v/v) glutaraldehdye in 0.01M phosphate buffer solution (PBS) for overnight. Then, the samples were washed for three times with 0.01M PBS for 10 minutes followed by deionized water for 10 minutes.

Next, the samples were subjected to dehydration with ascending concentrations of ethanol:

| | |
|---|---|
| I | 25% (v/v) ethanol, 5 minutes |
| II | 50% (v/v) ethanol, 10 minutes |
| III | 75% (v/v) ethanol, 10 minutes |
| IV | 95% (v/v) ethanol, 10 minutes |
| V | 100% (v/v) absolute ethanol, 10 minutes (three changes) |

After dehydration, the samples were subjected to critical point drying and sufficient amount of sample was transferred to the carbon tape on copper stage and graduated to obtain an even and thin layer of sample. The sample was subsequently coated with platinum for 1 minute before placed onto the copper stage holder and viewed by SEM (JSM-7610F) as shown in Figure 3.3 and 3.4.

**Figure 3.3:** Coating of samples with platinum.


**Figure 3.4:** SEM (JSM-7610F) in Faculty of Science, UTAR.

# CHAPTER 4

# RESULTS

## 4.1 Isolation and Identification of *Staphylococcus aureus*

### 4.1.1 Biochemical Identification Tests

Table 4.1 summarizes the results of Gram staining, shape and arrangement, mannitol fermentation, catalase and coagulase tests of two reference strains (*Staphylococcus aureus* ATCC 25923 and ATCC 33591) and two clinical isolates from wounds. As shown in the table, all tested isolates are Gram-positive cocci, in clusters (Figure 4.1) and positive in mannitol fermentation (Figure 4.2). Positive reactions were seen in catalase (Figure 4.3) and coagulase tests (Figure 4.4). Together with reference strains, both clinical isolates are identified as *Staphylococcus* spp. and further tested with API Staph kit identification test.

**Table 4.1:** Results of biochemical identification tests for all tested bacterial isolates.

| Strains | Gram staining | Shape and arrangement | Mannitol fermentation | Catalase test | Coagulase test |
|---|---|---|---|---|---|
| ATCC 25923 | Gram-positive | Cocci, in clusters | Positive | Positive | **Positive** |
| ATCC 33591 | Gram-positive | Cocci, in clusters | Positive | Positive | **Positive** |
| Clinical isolate 1 | Gram-positive | Cocci, in clusters | Positive | Positive | **Positive** |
| Clinical isolate 2 | Gram-positive | Cocci, in clusters | Positive | Positive | **Positive** |

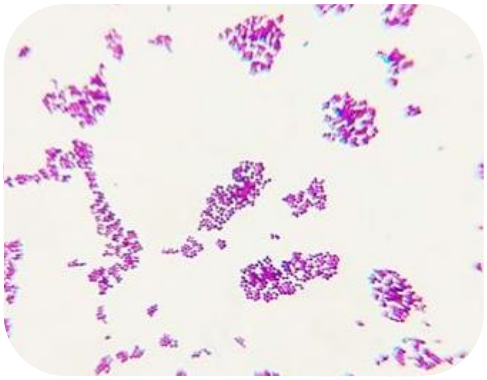

**Figure 4.1:** Photomicrograph of spherical (cocci) in cluster Gram-positive of clinical isolate 1 under oil immersion (100X magnification).


**Figure 4.2:** Yellow colonies due to fermentation of mannitol by clinical isolate 1.

**Figure 4.3:** Visible bubbles on the slide indicate catalase-positive for *S. aureus* ATCC 25923.

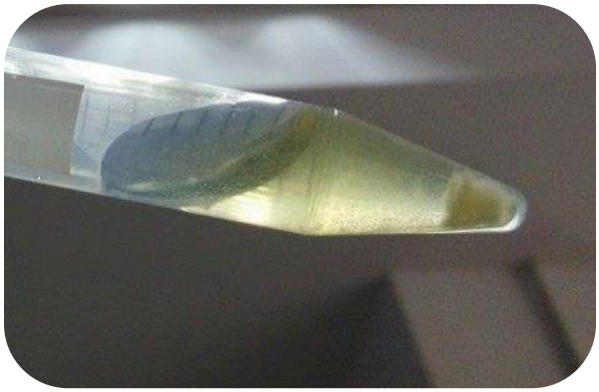

**Figure 4.4:** Clotted plasma is seen in tube coagulase test for *S. aureus* ATCC 25923.

### 4.1.2 API Staph Kit Identification Test

Table 4.2 shows the results of each individual test in the API Staph Kit and the respective numerical profile for each Staphylococcal isolate. The 7-digit numerical profile was checked via api*web*$^{TM}$ identification software database for the identity of the clinical isolates. Two reference strains (ATCC 25923 and ATCC 33591) and clinical isolate 2 showed resemblance of 97.8% to *S. aureus*. Clinical isolate 1 showed resemblance of 97.7% to *S. aureus*. After that, these confirmed *S. aureus* isolates were then tested in Kirby-Bauer assay.

**Table 4.2**: The API Staph kit tests and the respective numerical profile of each Staphylococcal isolate.

| **Test** | **Results** | | | |
|---|---|---|---|---|
| | **ATCC 25923** | **ATCC 33591** | **Clinical Isolate 1** | **Clinical Isolate 2** |
| 0 | - | - | - | - |
| GLU | + | + | + | + |
| FRU | + | + | + | + |
| MNE | + | + | + | + |
| MAL | + | + | + | + |
| LAC | + | + | + | + |
| TRE | + | + | - | + |
| MAN | + | + | + | + |
| XLT | - | - | - | - |
| MEL | - | - | - | - |

| | | | | |
|---|---|---|---|---|
| NIT | + | + | + | + |
| PAL | + | + | + | + |
| VP | + | + | + | + |
| RAF | - | - | - | - |
| XYL | - | - | - | - |
| SAC | + | + | + | + |
| MDG | - | - | - | - |
| NAG | + | + | + | + |
| ADH | + | + | + | + |
| URE | + | + | + | + |
| 7-digit numerical profile | 6736153 | 6736153 | 6726153 | 6736153 |
| Identification Percentage | 97.8% | 97.8% | 97.7% | 97.8% |

* + = Positive, - = Negative

### 4.1.3 Kirby-Bauer Antibiotic Susceptibility Assay

Kirby-Bauer antibiotic susceptibility test was performed to determine the antibiotic susceptibility profile of the identified *S. aureus* strains. The zone of inhibition obtained with different antibiotics is as presented in Table 4.3 as interpreted with the guidelines published by Clinical and Laboratory Standards Institute (2011). The control strains, only *S. aureus* ATCC 25923 was susceptible to all the antibiotics tested while *S. aureus* ATCC 33591 was only susceptible to gentamicin and showed intermediate sensitivity to tetracycline. This indicated that *S. aureus* ATCC 33591 is belong to methicillin-resistant *S. aureus* (MRSA) with multi-antibiotic resistance. Both clinical isolates 1 and 2 were resistance to ampicillin and penicillin although both clinical isolates were interpreted as methicillin-susceptible *S. aureus* (MSSA).

**Table 4.3:** Antibiotic susceptibility profile of *S. aureus* isolates.

| **Antibiotic Disc** | **Concentration (µg)** | **Zone of inhibition (mm)** | | | |
|---|---|---|---|---|---|
| | | **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 | 10 | 47 (S) | 8 (R) | 14 (R) | 15 (R) |
| Gentamicin | 10 | 22 (S) | 19 (S) | 22 (S) | 22 (S) |
| 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

## 4.2 Synergistic Assay

### 4.2.1 Agar Well-diffusion Test

The result of agar well-diffusion test is summarized in Table 4.4. The data show no zone of inhibition for all the *S. aureus* isolates after tested with Trigona honey alone. For the effect of ampicillin alone, only *S. aureus* ATCC 25923 showed zone of inhibition, which was 16.7 mm in diameter. As in the case of combination effect of honey and ampicillin, *S. aureus* ATCC 25923 recorded the largest zone of inhibition, which was 18.7 mm in diameter, followed by clinical isolate 2 (9.3 mm), clinical isolate 1 (9.0 mm) and antibiotic-resistant ATCC 33591 (7.7 mm). In other words, the combination of Trigona honey and ampicillin exhibited greater inhibitory effect than when Trigona honey or ampicillin was used alone.

**Table 4.4:** The anti-staphylococcal effects of Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin in agar well-diffusion test.

| | **Diameter of zone of inhibition (mm)** | | | |
|---|---|---|---|---|
| **Test Sample** | **ATCC 25923** | **ATCC 33591** | **Clinical Isolate 1** | **Clinical Isolate 2** |
| Trigona honey | NIL | NIL | NIL | NIL |
| Ampicillin | 16.7 ± 0.06 | NIL | NIL | NIL |
| Trigona honey + Ampicillin | 18.7 ± 0.06 | 7.7 ± 0.06 | 9.0 ± 0.00 | 9.3 ± 0.06 |

*Mean ± standard deviation, n = 3; NIL = No zone of inhibition

### 4.2.2 Plate Count Assay

The results of plate count test for all tested strains as presented in Table 4.5, showing the percentage of bactericidal rate of three test samples including Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin.

Other than ATCC 25923, Trigona honey alone and ampicillin alone were not able to eradicate the other three tested Staphylococcal isolates completely. However, it was observed that combination of Trigona honey and ampicillin was more potent in killing bacteria within 24 hours as compared to Trigona honey alone and ampicillin alone. In short, it shows that the incorporation of honey with ampicillin resulted in greater bactericidal rate against all tested *S. aureus*, including antibiotic-resistant strains.

**Table 4.5:** Bactericidal rate of Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin against *S. aureus* in plate count assay.

| | Bactericidal rate (%) | | | |
|---|---|---|---|---|
| **Test Sample** | **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 |

### 4.2.3 Scanning Electron Microscopic Examination

As verified by Kirby-Bauer test, *S. aureus* ATCC 33591 and the two clinical isolates are resistance to ampicillin action. Thus, the bacterial cell wall still remain intact after the treatment with ampicillin as shown in Figure 4.5(a). Moreover, this study also showed that honey changed the shape of cells and disrupted the cell wall integrity. The altered morphology of *S. aureus* after exposure to honey is shown in Figures 4.5(b) and which revealed the structural integrity of cell wall was disrupted due to action of honey. The micrographs also revealed that the addition of honey enhanced the antibacterial activity of ampicillin leading to cell lysis. Figure 4.6 (a) and (b) show more lysed cells were found due to the combination effects of ampicillin and honey.

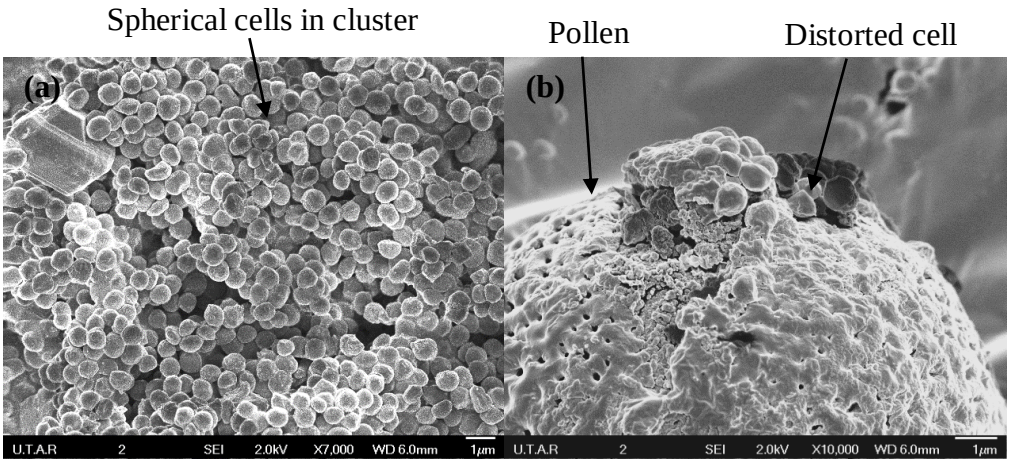


**Figure 4.5:** SEM of ampicillin-resistant ATCC 33591 after treated with (a) ampicillin and (b) Trigona honey.

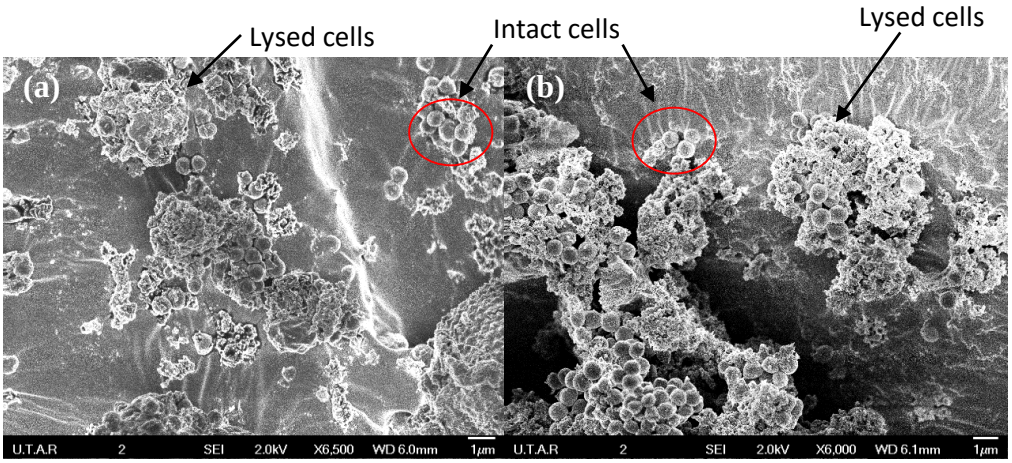


**Figure 4.6:** Morphological changes in (a) ampicillin-resistant ATCC 33591 and (b) clinical isolate 1 after treated with the combination of ampicillin and Trigona honey.

# CHAPTER 5

# DISCUSSION

## 5.1 Isolation of *Staphylococcus aureus* from Infected Wound

Highly predominance of aerobic pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are associated with wound infections over the past two decades (Halbert, et al., 1992; Danielsen, et al., 1998). This has become the primary concern among wound care practitioners as the bacteria delay the healing progress in both acute and chronic wounds (Bowler, Duerden and Armstrong, 2001).

According to Diekema et al. (2001), *S. aureus* is the preponderance cause of bloodstream, skin and soft tissue infections (SSTIs) globally. Similar study conducted by Moet, et al. (2007) revealed *S. aureus* as the most predominant bacteria associated with SSTIs in hospitalized patients as recorded in SENTRY Antimicrobial Surveillance Program database. Besides, *S. aureus* is commonly detected in cutaneous abscesses and has been recognized as the most frequent isolate in superficial infections seen in Accident and Emergency (A&E) department in hospitals (Meislin, et al., 1977; Brook and Finegold, 1981; Page and Beattie, 1992). Meanwhile, *S. aureus* was also reported as the most prevalent colonizer found in the chronic venous leg ulcer, either alone or associated with *P. aeruginosa* (Gilliland, et al., 1988) and *S. aureus* was found

to be localized in the superficial layer while *P. aeruginosa* was usually detected in the deeper region of wound bed.

## 5.2 Anti-staphylococcal Activity of Ampicillin

The results of both agar well-diffusion and plate count tests showed that ampicillin alone did not inhibit the growth of *S. aureus* effectively, especially antibiotic-resistant *S. aureus*. The results are consistent with a study done by Opere, et al. (2013) which recorded that *S. aureus* is relatively high resistant (73.0%) towards ampicillin. This finding corroborates that of Uwaezuoke and Aririatu (2004) which showed low sensitivity of *S. aureus* towards ampicillin. Also all of the *S. aureus* strains tested by Onanuga and Awhowho (2012) were shown to be resistant to ampicillin as well. The ability of *S. aureus* to develop resistance towards ampicillin is mainly due to the production of beta-lactamase that hydrolyze beta-lactam ring of aminopenicillin family. Beta-lactam ring is responsible for the antibacterial activity of ampicillin, hence hydrolysis of the beta-lactam ring rendering the antibiotic inactive (Kernodle, 2000).

As far as we know, apart from production of enzyme beta-lactamase, the expression of low affinity penicillin-binding protein (PBP2a) by methicillin-resistant *S. aureus* (MRSA) also capable of inducing resistance towards beta-lactam antibiotics (Fuda, Fisher and Mobashery, 2005). Lim and Strynadka (2002) reported that PBP2a inhibits the synthesis of normal PBP produced by *S. aureus*. This indicates that loss of PBP interferes the formation of cell wall.

However, study by Pinho, et al. (2001) demonstrated that PBP2a contains transpeptidase activity that can replace the function of PBP in cell wall synthesis, but the PBP2a expressed has relatively lower affinity towards beta-lactam antibiotics and it only serves as the surrogate of enzyme transpeptidase.

In this study, only *S. aureus* ATCC 25923 was sensitive to the action of ampicillin. Ampicillin inhibits the cell wall synthesis by irreversibly binding to the peptidoglycan-synthesizing enzyme and subsequently blocks the cross-linking of peptidoglycan and murein assembly (Korsak, Liebscher and Vollmer, 2005; Fry, 2013). Treatment with ampicillin disrupt the rigidity of cell wall and causes the lysis of bacteria (Maddison, Page and Church, 2008; Skidmore-Roth, 2014). The mechanisms corresponded to the micrographs obtained from SEM, where the cell wall of *S. aureus* ATCC 25923 treated with ampicillin was disrupted.

### 5.3 Anti-staphylococcal Activity of Honey

The results from the present study indicated that Trigona honey alone was less potent in inhibiting the growth of *S. aureus* including the antibiotic-resistant strains. The agar well-diffusion test showed no zone of inhibition was formed by Trigona honey alone. Meanwhile, the plate count method recorded that only *S. aureus* ATCC 25923, which is the antibiotic sensitive strain was completely killed by honey after 24 hours of treatment. However, *S. aureus* ATCC 33591

and two clinical isolates, which are the antibiotic resistant strains were not eradicated completely after 24 hours.

In a study by Boorn, et al. (2010), the largest zone of inhibition after treatment was observed when *S. aureus* was treated with different Trigona honeys. Another similar study by Temaru, et al. (2007) stated the tested *S. aureus* were more susceptible to the action of Trigona honey. Furthermore, several studies also showed that 50% concentration of Trigona honey exhibited greatest antibacterial action than other concentrations (Ewnetu, Lemma and Birhane, 2013; Eswaran, Priya and Bhargava, 2015). However, the results obtained from the present study did not correspond with other previous studies. The divergence of results might be due to several reasons such as different botanical origin and processing method.

Yet, a study done by Shahjahan, et al. (2007) showed that neat honey exhibited better antibacterial outcome than diluted honey. The efficacy of undiluted honey was superior against bacteria tested with greater inhibitory effect than diluted honey. Another study also reported that 100% concentration of honey formed largest zone of inhibition than 50% honey concentration and among all the bacteria investigated, all honey concentrations showed inhibitory effect and only *S. aureus* showed no zone of inhibition (Al-Naama, 2009). The divergence of results might be due to several reasons such the different botanical origin and processing method. Different botanical sources of honey contain different level

of active compounds including phenolic acids, flavonoids and hydrogen peroxide production (Alzahrani, et al., 2012; Moussa, et al., 2012).

Besides that, several limitations of agar well-diffusion test were discovered including the insensitivity in detecting low level of antimicrobial activity, variation in the experimental conditions and permeability of nonpolar components. Thus, agar well-diffusion test may not be the most appropriate method to evaluate the antibacterial activity of honey (Irish, et al., 2008; Kimoto-Nira and Amano, 2008).

### 5.4 Synergistic Effect of Ampicillin and Trigona Honey

Synergy refers to the interaction of two or more medicinal agents to produce a combined effect that is greater than the sum of their separated effect (American Heritage® Dictionary of the English Language, 2011). In order to increase the bactericidal activity of ampicillin towards *S. aureus*, combination therapy is highly recommended to broaden the spectrum of antibacterial action (Eliopoulos and Eliopoulos, 1988; Dawis et al., 2003). Several studies have shown that many natural compounds can enhance the effect of antibiotic and at the same time inhibit the development of antibiotic resistance (Gibbons, 2008; Ejim, et al., 2011).

As shown in this study, ampicillin exhibited greater activity against *S. aureus* in the presence of Trigona honey. Agar well-diffusion test confirmed the synergy effects, as combination of ampicillin and Trigona honey against strains of *S. aureus* recorded the largest zone of diameter than ampicillin or Trigona honey separately. Therefore, it is conceivable that synergistic combination between ampicillin and Trigona honey did convey a greater potency to inhibit the growth of *S. aureus*. Such interpretation is proven by Muller, et al. (2013) who revealed that there was a markedly increased in the zone of inhibition when honey was incorporated with antibiotic. Incorporation of honey with antibiotic decreased the number of colonies formed compared to the effects of honey or antibiotic alone. In addition, combination of honey with antibiotic also helps in preventing the emergence of rifampicin-resistant *S. aureus* and thus maintaining the sensitivity of *S. aureus* toward rifampicin.

Other than that, the results from plate count method also showed that combination of ampicillin and Trigona honey achieved 100% bactericidal rate after 24 hours incubation. The incorporation of Trigona honey augmented the bactericidal activity of ampicillin against the tested *S. aureus* strains. Such outcome is also supported by a similar study done by Cooper and Jenkins (2012) which showed that Manuka honey restored the susceptibility of MRSA towards oxacillin. Thus, Manuka honey interacted synergistically with oxacillin to inhibit growth of MRSA.

To the best of our understanding, the reasons behind the synergism of honey and ampicillin are believed to be attributed by the antibacterial components of honey which are hydrogen peroxide and phytochemical compounds. The damaging effects on bacterial cell wall constitute the underlying mechanism of antibacterial effects of both ampicillin and honey. Hydrogen peroxide can diffuse through the cell membrane of bacteria easily and generate hydroxyl free radicals inside without being excluded out from the cell. Hydrogen peroxide acts by attacking the cellular essential components which are lipids, proteins and DNA (McDonell and Russell, 1999; Brudzynski, et al., 2011). Moreover, the formation of hydroxyl free radicals causes oxidative stress that stimulates lipid peroxidation which disrupts the integrity of cell membrane (Casbiscol, et al., 2000). The altered membrane integrity may enhance the binding of ampicillin to the penicillin binding proteins and further disrupting the structure of cell wall. A study by Shin, Hayasawa and Lonnerdal (2001) indicated that hydrogen peroxide together with several compounds were involved in the inhibition of respiratory enzyme in *E. coli*.

Phytochemical compound like phenolic compounds have membrane active-properties by disrupting the integrity of cell membrane and cause leakage of cellular contents including proteins, nucleic acids and minerals (Johnston, et al., 2003). Mori and colleagues (1987) also suggested that presence of phytochemical flavonoids disrupted the integrity of bacterial membrane by limiting the lipid and protein synthesis in *S. aureus*. It is hypothesized that the damaging of DNA inhibits the formation of enzyme beta-lactamase and

disruption of proteins structure cause alteration in conformation of enzyme beta lactamase which greatly improve the sensitivity of *S. aureus* towards the action of ampicillin.

### 5.5 Scanning Electron Microscope Examination

As observed in electron microscope, *S. aureus* treated with ampicillin alone, Trigona honey alone and combination of ampicillin and Trigona honey for 24 hours experienced morphological changes that is attributed to various mechanisms of action by the honey or ampicillin.

Generally, the antibacterial activities of honey are explained with the effects of osmotic pressure, acidity, hydrogen peroxide generation, and phytochemical components (Mandal and Mandal, 2011). The osmotic effect of honey results in water withdrawal from the bacteria which subsequently lead to cell death. A study by Mori and colleagues (1987) suggested the presence of flavonoids disrupted the integrity of bacterial membrane by limiting the lipid and protein synthesis in *S. aureus*. Another study by Jantakee and Tragoolpua (2015) also stated that Thailand stingless bee honey exhibited peroxide-mediated antibacterial activity whereby the formation of free OH- radicals broke DNA and oxidized thiol-groups of proteins and lipids that damage the bacterial cells. In addition, the presence of abundant protons also can create an acidic environment which can further inhibit the production of ATP by blocking H+-

ATPase enzyme. Without the energy production, the bacteria die (Epand and Vogel, 1999).

Recently, a study conducted by Packer and his colleagues (2012) demonstrated that exposure of *S. aureus* and MRSA to Manuka honey disrupted the cell division process and whereby the daughter cells did not separate completely at the end of the cell cycle. Therefore, the bacteria did not form any colony without completing cell division (Packer, et al., 2012). Moreover, Manuka honey was observed to possess distinct cellular effects in Gram-positive bacteria than in Gram-negative bacteria (Majtan, et al., 2011). Proteomic analysis showed that Manuka honey successfully downregulated the expression of dihydrolipoamide dehydrogenase (DLD) and elongation factor Tu (EF-Tu) and upregulated cold shock protein C (CspC) (Packer, et al., 2012). Therefore, we believe that Trigona honey also exhibited the same action as Manuka honey in limiting the growth of *S. aureus*. Deficiency of expression of dihydrolipoamide dehydrogenase (DLD) reduced the inhibition of pyruvate dehydrogenase (PDH) by nicotinamide adenine dinucleotide (NADH), which eventually cause growth inhibition (Kim, Ingram and Shanmugam, 2008). Down-regulation of Elongation factor Tu results in the inhibition of elongation process in protein synthesis (Chen, et al., 2010). Cold shock protein C is important for bacteria to adapt to the stress imposed by surrounding environment temperature as CsP mutant strains have relative slow growth than wild-type strains (Anderson, et al., 2006; Michaux, et al., 2012).

It is an undeniable fact that ampicillin inhibited the bacterial cell wall synthesis and disrupted the integrity of cell wall. Significant morphological changes to *S. aureus* are indicated by changes in shape of cells (Skidmore-Roth, 2014). Ampicillin acts by inhibiting the enzymes involved in the biosynthesis of cell wall and covalently binds to penicillin-binding proteins (PBP) thus the cross-linking of peptidoglycan is interfered (Fry, 2013). As far as we can tell, honey does share similar antibacterial effect with ampicillin in destructing the structural integrity of cell wall. Thus, even though antibacterial action of ampicillin was inhibited by enzyme beta-lactamase synthesized in some of the antibiotic-resistant *S. aureus*, the *S. aureus* still remained sensitive to the action of honey (Brudzynski and Sjaardaa, 2014).

### 5.6 Future Studies

Scientific investigation about the combination effect of honey and antibiotic in inhibiting the growth of bacteria is very limited. Therefore, the synergistic effect of Trigona honey and ampicillin can be further studied for better understanding in the bactericidal mechanism. Honey consists of multiple bioactive compounds which appeared to be targeting different cellular sites of the bacteria. The relationship between the components of honey and its antibacterial effect can be identified via proteomic analysis. Proteomic analysis utilizes the Omic technologies to detect changes occurred in the proteins targeted by components of honey such as damaging effects of hydroxyl radical on the structural integrity of cell wall. Furthermore, high performance liquid chromatography (HPLC) analysis also can be performed to identify the non-peroxide compounds of

honey such as methylglyoxal, phenols and flavonoids, in enhancing the antibacterial activity of ampicillin. One of the advantages of combination therapy is the effectiveness in killing antibiotic resistant bacteria and to date, no cases of bacterial resistance to combination therapy has been reported. Besides that, the combination therapy has proven to increase the efficacy of antibiotics at the same time.

# CHAPTER 6

# CONCLUSIONS

The present study has successfully proven that combination of Trigona honey and ampicillin showed greater bactericidal activity against *S. aureus*, including antibiotic resistant strains. Incorporation of Trigona honey with ampicillin exhibited higher inhibitory effect than when Trigona honey and ampicillin were used separately. Undoubtedly, this study showed significant additive effects of Trigona honey to ampicillin against *S. aureus*. Therefore, enhancement of antibiotic action by honey can be considered in therapeutic purpose to prevent toxicity from prolonged antibiotic therapy and reduce the emergence of antibiotic resistance.

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## Appendix A


Figure: Example of API Identification assay for clinical isolate 1 and clinical isolate 2.

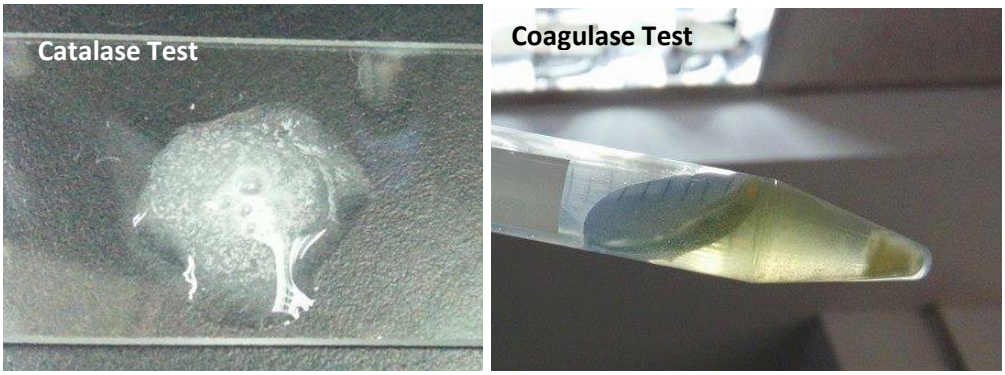



Figure: Example of catalase and coagulase tests positive results for clinical isolate 1.

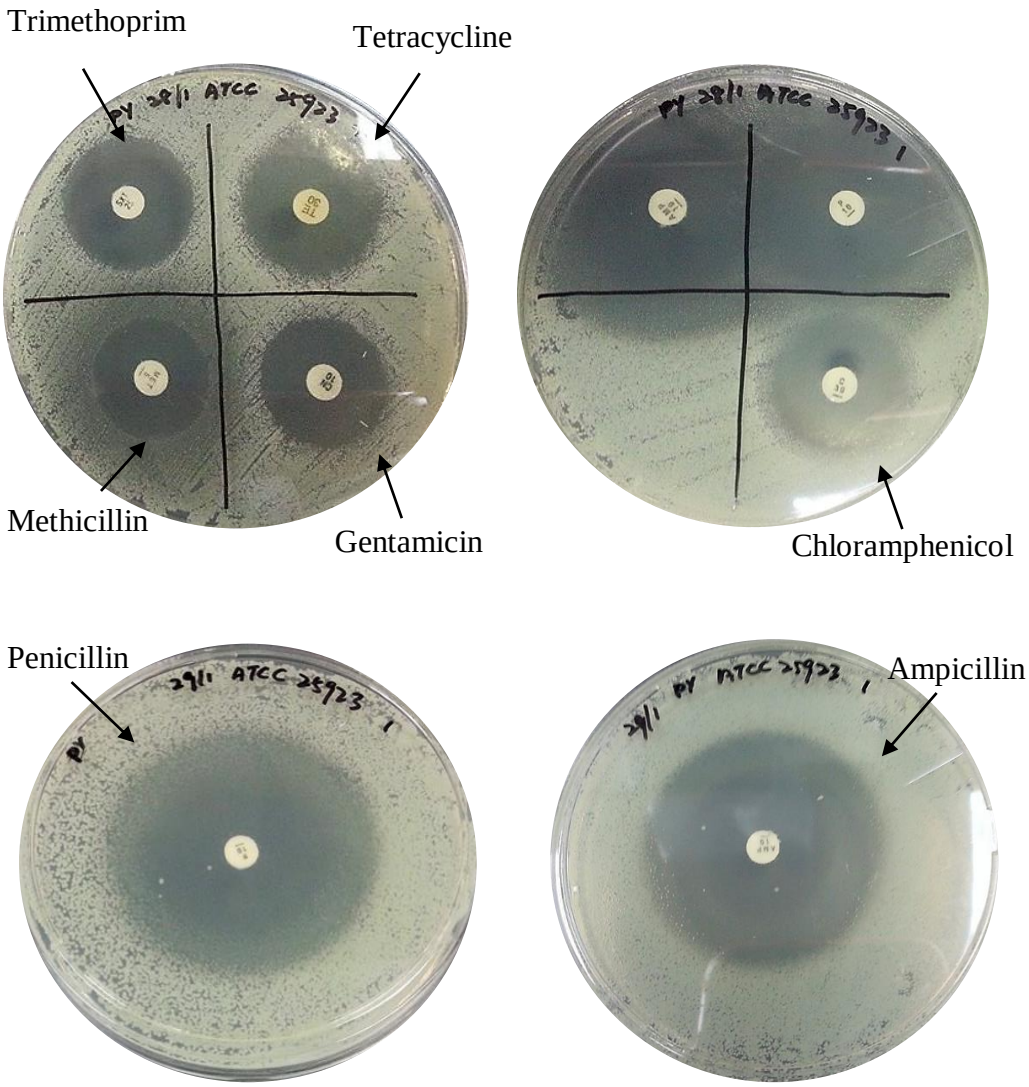



Figure: Example of antibiotic susceptibility test of *Staphylococcus aureus* ATCC 25923.

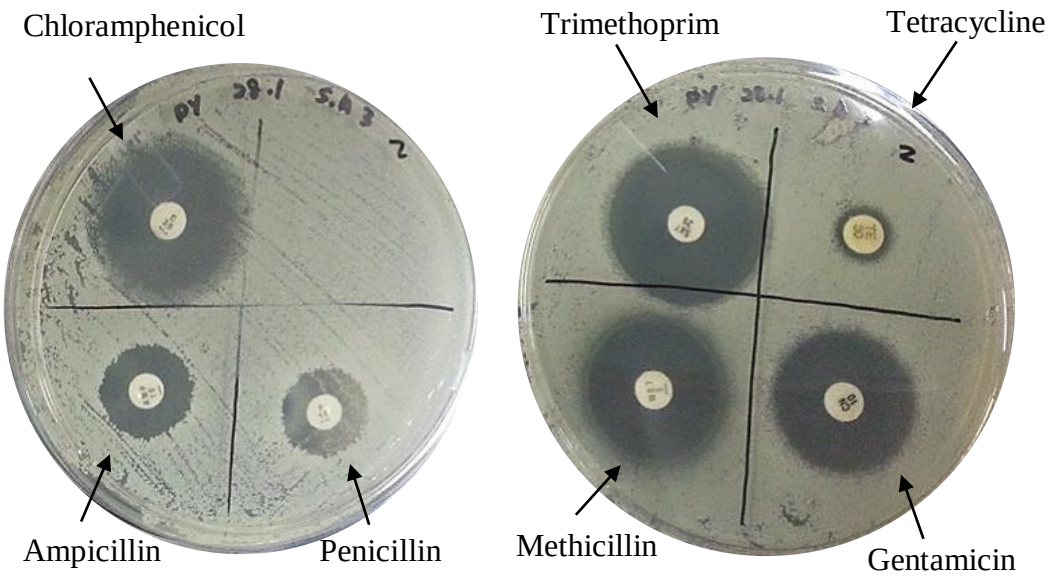



Figure: Example of antibiotic susceptibility test of *Staphylococcus aureus* clinical isolate 2.

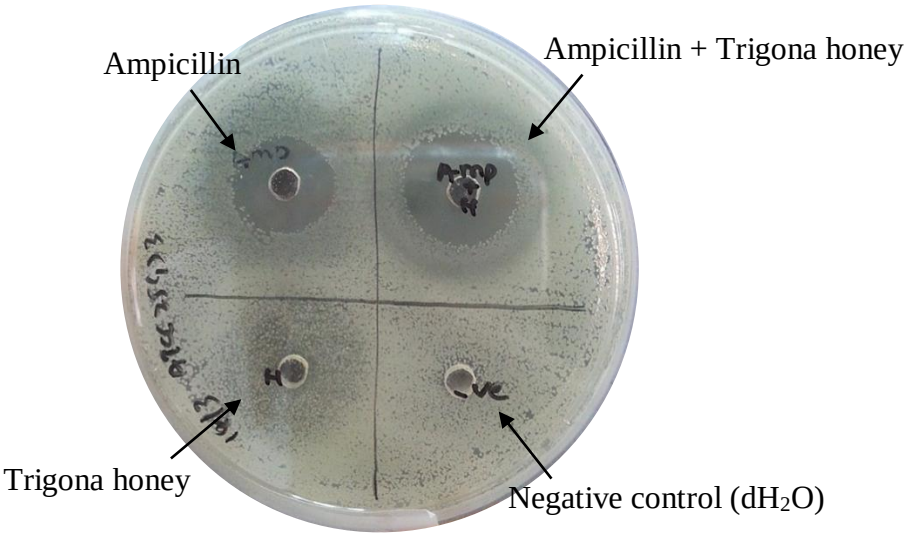



Figure: Example of plates of *Staphylococcus aureus* ATCC 25923 for Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin.

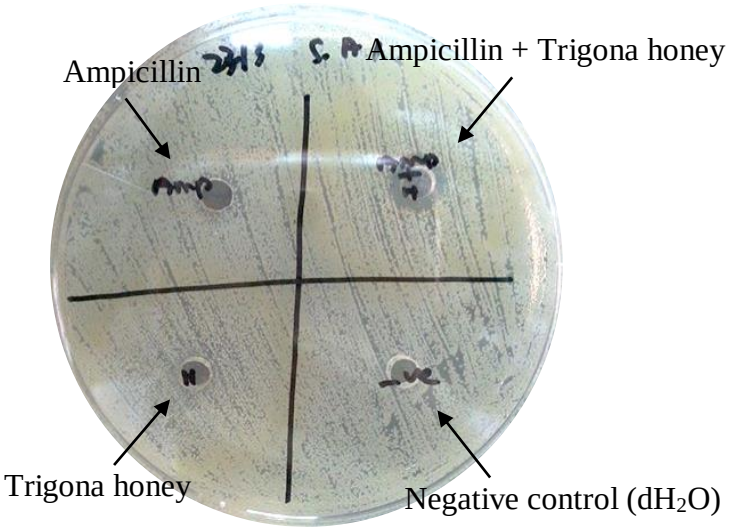
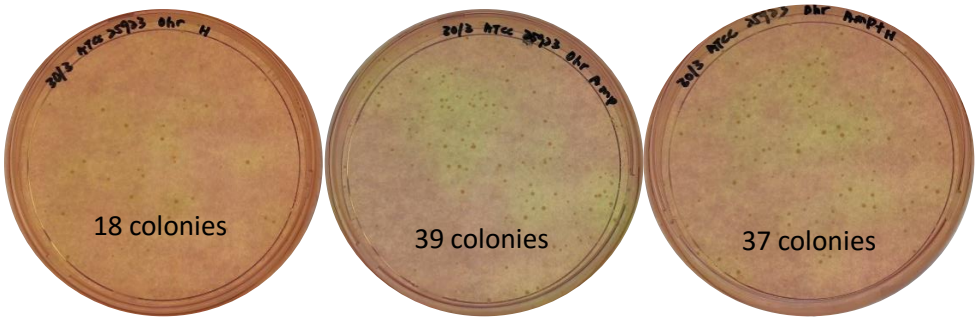


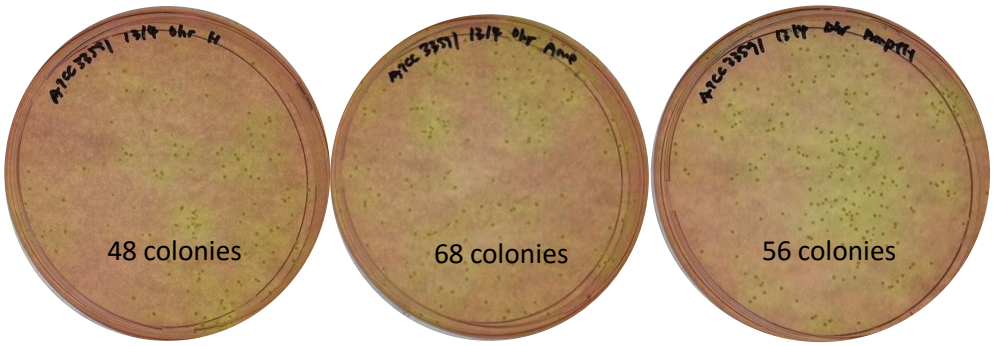

Figure: Example of plates of *Staphylococcus aureus* clinical isolate 1 for Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin.




(0 hour)


(24 hours)

Figure: Example of plates of *Staphylococcus aureus* ATCC 25923 and ATCC 33591 in 0-hour and 24-hour in plate count method for Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin.




(0 hour)


(24 hours)

Figure: Example of plates of *Staphylococcus aureus* ATCC 33591 in 0-hour and 24-hour in plate count test for Trigona honey alone, ampicillin alone and combination of Trigona honey and ampicillin.

## Appendix B

### Scanning Electron Microscopic Examination

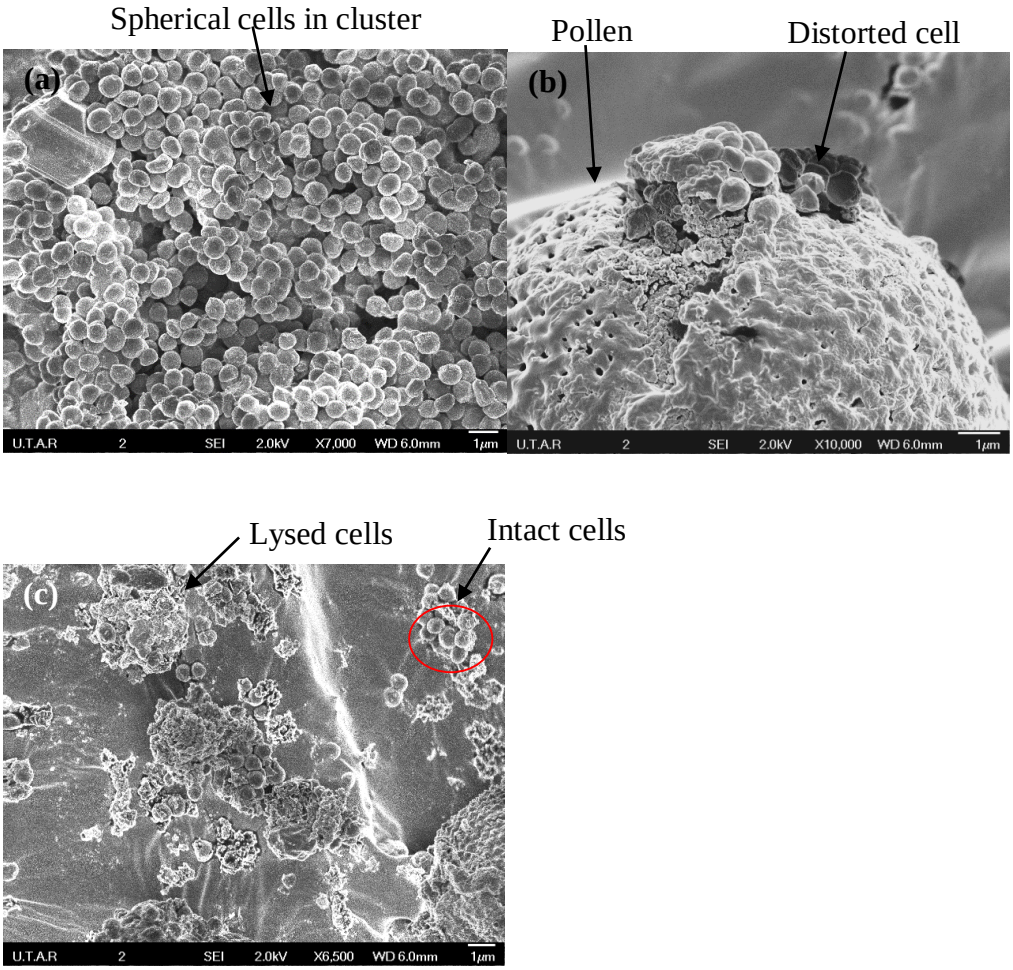



Figure: Electron Micrographs of ampicillin-resistant ATCC 33591 after treated with (a) ampicillin, (b) Trigona honey and (c) combination of Trigona honey and ampicillin.

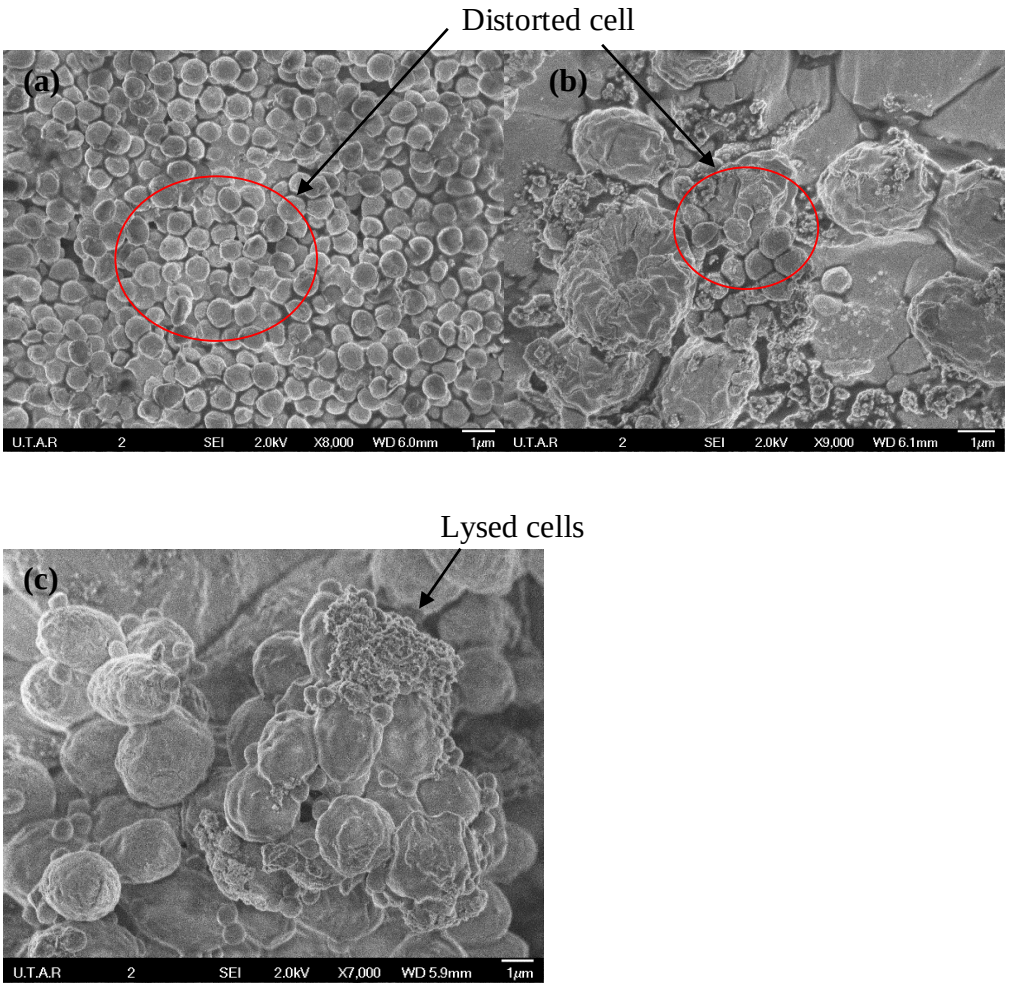



Figure: Electron Micrographs of ampicillin-sensitive ATCC 25923 after treated with (a) ampicillin, (b) Trigona honey and (c) combination of Trigona honey and ampicillin.

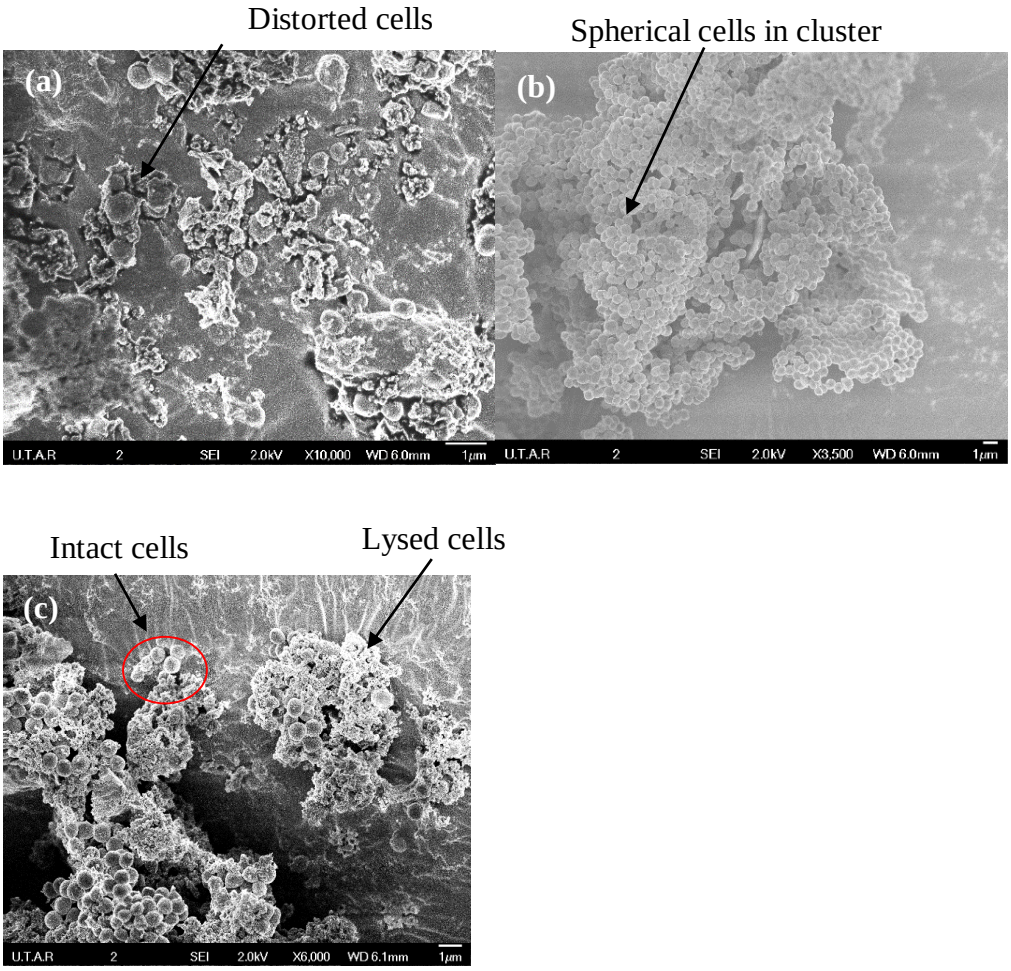



Figure: Electron Micrographs of ampicillin-resistant clinical isolate 1 after treated with (a) Trigona honey, (b) ampicillin and (c) combination of Trigona honey and ampicillin.

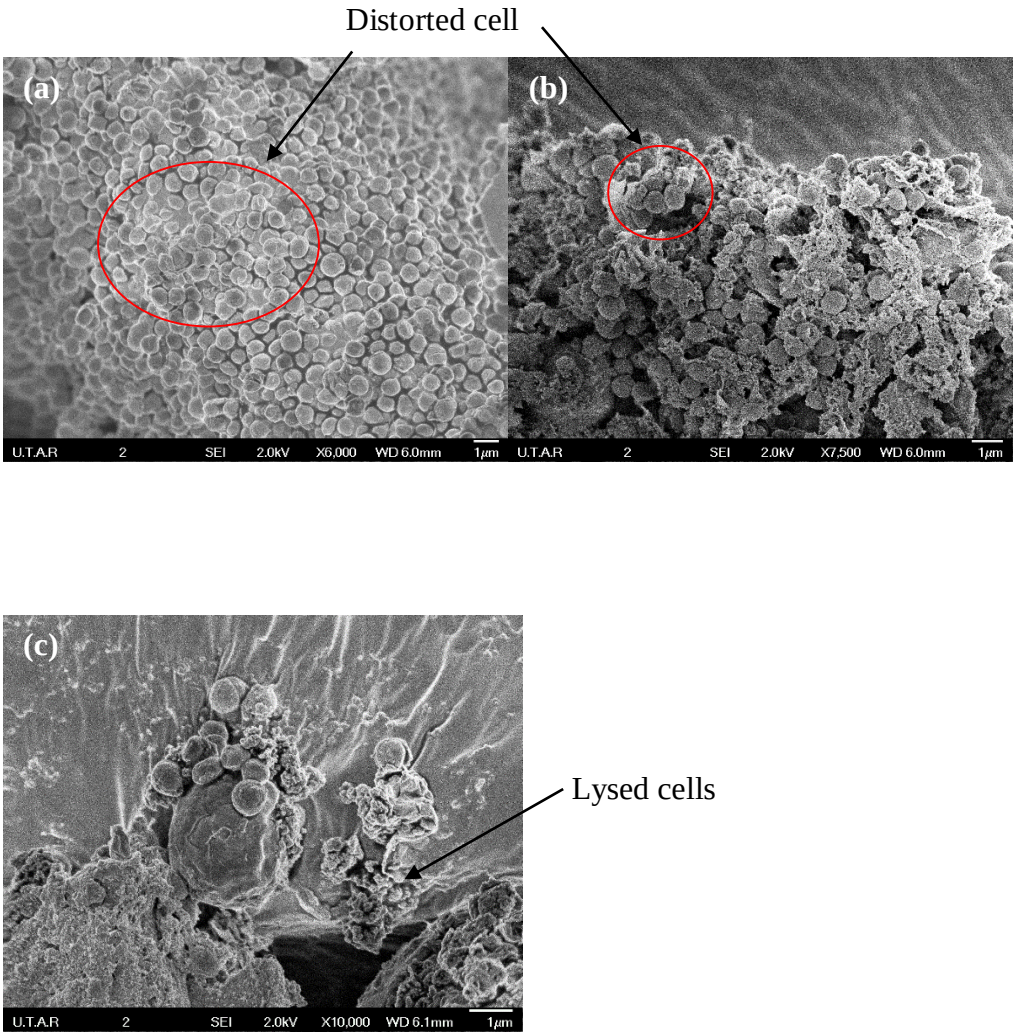



Figure: Electron Micrographs of ampicillin-resistant clinical isolate 2 after treated with (a) ampicillin, (b) Trigona honey and (c) combination of Trigona honey and ampicillin.

## Appendix C

Table: Interpretation of Inhibition Zones in Kirby-Bauer Assay.

| Antibiotic Disc | Concentration (µg) | Zone of Diameter, (To nearest mm) | | |
|---|---|---|---|---|
| | | **R** | **I** | **S** |
| Methicillin | 5 | ≤ 9 | 10-13 | ≥ 14 |
| Ampicillin | 10 | ≤ 28 | - | ≥ 29 |
| Penicillin | 10 | ≤ 28 | - | ≥ 29 |
| Gentamicin | 10 | ≤ 12 | 13-14 | ≥ 15 |
| Chloramphenicol | 30 | ≤ 12 | 13-17 | ≥ 18 |
| Trimethoprim | 25 | ≤ 10 | 11-15 | ≥ 16 |
| Tetracycline | 30 | ≤ 14 | 15-18 | ≥ 19 |

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

(Clinical and Laboratory Standards Institute, 2011)