THE ANTIMICROBIAL POTENTIAL EFFECTS OF MALAYSIAN HONEY ON SOME HUMAN PATHOGENS

By

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CERTIFICATE

This is to certify that the dissertation entitled

The Antimicrobial Potential Effects of Malaysian Honey

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is the bonafide record of research work done by

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ABSTRAK

Madu mempunyai kesan antimikrobial dan nilai perubatan yang tinggi sejak berabad yang lalu. Madu Malaysia dikenali oleh kualitinya. Oleh itu, kajian ini menggunakan madu Malaysia (Madu Liar™) untuk dikaji dengan beberapa jenis patogen manusia seperti *Staphylococcus aureus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Streptococcus bovis*, *Streptococcus pyogenes* Group A, *Listeria monocytogenes*, *Enterococcus faecalis*, *Acinobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnei*, *Klebsiella pneumoniae* dan *Candida albicans* dengan menggunakan 'honey broth media' yang berkepekatan (%, v/v): 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% dan 100%. Semua ujian setiap patogen diduplikasikan bagi validasi keputusan yang didapati. Daripada keputusan yang diperolehi, *S. pyogenes* adalah paling sensitif yang mana pertumbuhan direncat sepenuhnya dalam 'honey broth media' berkepekatan 30% dan diikuti oleh *S. bovis*, 40%; *P. aeruginosa* dan *A. baumannii*, 50%; *E. coli*, *K. pneumoniae* dan *S. sonnei*, 60%; *L. monocytogenes* dan yeast *C. albicans*, 70% manakala antara yang paling rentan adalah *E. faecalis*, *S. aurues* dan MRSA, 80%. LD_{50} ditentukan daripada graf 'dose-response' yang diperolehi, *S. bovis* dan *P. aeruginosa* adalah yang terendah, 4%; diikuti oleh *S. pyogenes*, *L. monocytogenes*, *A. baumannii* dan *K. pneumoniae*, 5%; *S. aureus* dan *E. coli*, 9%; *E. faecalis*, 14%; MRSA, 15% manakala LD_{50} yang tertinggi, 29% dicatatkan oleh *C. albicans*. Kesimpulannya, madu Malaysia (Madu Liar™) telah terbukti mempunyai nilai perubatan dan kesan antimikrobial terhadap pelbagai jenis patogen penyebab penyakit manusia.

ABSTRACT

Honey is a nutrient food nutrient. It was also reported to have potential antimicrobial effects and medication values since centuries ago. Malaysian honeys are well recognized because of their quality. Thus, in this study Malaysian honey (Madu Liar™) was tested against several strains of human pathogens including gram positives, gram negatives, antibiotic-resistant bacteria and fungi. *Staphylococcus aureus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Streptococcus bovis*, *Streptococcus pyogenes* Group A, *Listeria monocytogenes*, *Enterococcus faecalis*, *Acinobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnei*, *Klebsiella pneumoniae* and *Candida albicans* were tested against different concentrations of honey broth media (%, v/v): 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Duplicate were performed for each pathogen. The results showed *S. pyogenes* was the most sensitive in which totally inhibited in 30% concentration of honey broth media and followed by *S. bovis*, 40%; *P. aeruginosa* and *A. baumannii*, 50%; *E. coli*, *K. pneumoniae* and *S. sonnei*, 60%; *L. monocytogenes* and yeast *C. albicans*, 70% and the most resistant are *E. faecalis*, *S. aurues* and MRSA, 80%. LD_{50} (dose of the test chemical that is lethal to 50% of the bioassay organisms) for each pathogen was determined from each dose-response graph. *S. bovis* and *P. aeruginosa* have the lowest LD_{50}, 4%, and followed by *S. pyogenes*, *L. monocytogenes*, *A. baumannii* and *K. pneumoniae*, 5%; *S. aureus* and *E. coli*, 9%; *E. faecalis*, 14%; MRSA, 15% and *C. albicans* has the highest LD_{50}, 29%. This study showed that Malaysia's honey (Madu Liar™) prevented growth of a wide range of potential human pathogens in growth cultures and thus revealed its broad-spectrum antimicrobial activity.

1.0. Introduction

1.1. Honey

Honey, as defined by the Codex Alimentarius (1989) is the natural sweet substance produced by honeybees from the nectar of blossoms, from the secretion of plants, from excretions of plant-sucking insects, which honey bees collect, transform, store and leave in the honey comb to ripen and mature. Honey is a complex mixture and can be very great variations in characteristics due to its geographical and floral origin (Crane, 1975, 1980; Ramirez, 2000). The composition and quality of honey also depend on several environmental factors during production such as weather and humidity inside the hive, nectar conditions and treatment of honey during extraction and storage.

Honey is a drug more than a nutrient. Two millennia before bacteria were identified as the cause of disease and physicians at that time were aware that certain types of honey were the best for treating particular ailments and infections. Prophet Mohammed (S.A.W) had recommended honey for treatment of diarrhea. It was mentioned in the Holy Quran 1400 years ago. It was also mentioned in Holy Talmud and Holy Bible. Honey was used to treat infected wounds as long ago as 2000 years before bacteria were discovered to be the cause of infection. In 50 AD, Dioscorides described honey as being "good for all rotten and hollow ulcers" (Gunther, 1934), and Aristotle referred to pale honey as being “good as a salve for sore eyes and wounds" (Aristotle 350 BC). Besides, Hippocrates and Celsus used honey for wounds and ulcers.

The antimicrobial activity of honey has been demonstrated *in vitro* and *in vivo*. Laboratory studies and clinical trials have shown that honey is an effective broad-spectrum antimicrobial agent. More recently, honey has been reported to have an inhibitory effect to around 60 species of bacteria including aerobes and anaerobes, gram positives and gram negatives (Molan, 1992a). An antifungal action has also been observed for some yeasts and species of *Aspergillus* and *Penicillium* (Molan, 1992a), as well as all the common dermatophytes (Brady *et al.*, 1997). The current prevalence of antibiotic-resistant microbial species has led to a re-evaluation of the therapeutic use of ancient remedies, including honey.

1.2. Human Pathogens

Different pathogens are responsible for human wound colonization and clinical infection. Microorganisms such as *Staphylococcus aureus*, Methicillin-Resistant *S. aureus* (MRSA), *Streptococcus bovis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Shigella sonnei* and *Candida albicans* are frequently isolated from humans and animals (Todar, 2007).

Staphylococcus aureus can cause a range of illnesses from minor skin infections, such as boils, abscesses, skin lesions, impetigo and wound infection to life-threatening diseases, such as pneumonia and toxic shock syndrome (TSS) (Todar, 2007). It is still one of the four most common causes of nosocomial infections including *E. coli*, *Enterococcus* species and *Pseudomonas* species, often causing post surgical wound infections (Black, 2005). Methicillin-Resistant *S. aureus* (MRSA) is the main strain involved in difficult-to-

treat skin and underlying tissue infections associated with gram positive bacteria (Halco'n and Milkus, 2004). MRSA is a resistant variation of the common bacteria, *S. aureus*. It has evolved an ability to survive treatment with beta-lactam antibiotics, including penicillin and methicillin (Foster, 1996). MRSA is especially troublesome in hospital-acquired (nosocomial) infections. In hospitals, patients with open wounds and weakened immune systems are at greater risk for infection than general public (Todar, 2007). MRSA is often referred to in the press as a "superbug" by the mass media.

Streptococcus bovis is commonly found in the alimentary tract of cows, sheep and other ruminants (Ghali *et al.*, 2004). It is a known human pathogen that has been implicated in cases of human endocarditis (Ryan and Ray, 2004) and, less frequently, neonatal septicemia and meningitis (Headings *et al.*, 1978; Grant *et al.*, 2000). *Streptococcus pyogenes* is the cause of many important human diseases ranging from mild superficial skin infections such as, skin lesions, impetigo to life-threatening systemic diseases such as streptococcal toxic shock syndrome. Infections typically begin in the throat or skin (Todar, 2007).

Enterococcus faecalis is a commensal bacterium inhabiting the gastrointestinal tracts of humans and other mammals. It can cause endocarditis, as well as bladder, prostate and epididymal infections (Ryan and Ray, 2004). It can cause life-threatening infections in humans, especially in the nosocomial environment. *Listeria monocytogenes* is the agent of listeriosis, a serious infection caused by eating food contaminated with the bacteria. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems (Todar, 2007).

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen particularly causes urinary tract infection, skin lesions and septicemia in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. Infection with *P. aeruginosa* is the most serious complication in burns patients (Nasser *et al.*, 2003; Altoparlak *et al.*, 2005), followed by infections with *K. pneumoniae*, *E. coli*, *S. aureus* and other pathogen microorganisms (Nasser *et al.*, 2003). *Escherichia coli* usually are responsible for human infections such as, gastroenteritis, urinary tract infections, and neonatal meningitis. It is also one of the common causes of the nosocomial infections (Todar, 2007).

Acinetobacter baumannii forms opportunistic infections. It usually infects those with compromised immune systems, such as the wounded, the elderly, children or those with immune diseases. It causes various other infections including skin and wound infections (Gerischer, 1999). *Klebsiella pneumoniae* is an opportunistic pathogen which can cause bacterial pneumonia, typically due to aspiration by alcoholics, though it is more commonly implicated in hospital-acquired urinary tract and wound infections, particularly in immunocompromised individuals (Ryan and Ray, 2004).

Shigella sonnei causes shigellosis or bacterial dysentery that result in the destruction of the epithelial cells of the intestinal mucosa in the cecum and rectum. Shigella species is implicated as one of the pathogenic causes of reactive arthritis worldwide (Hill Gaston *et al.*, 2003). *Candida albicans* is a causal agent of opportunistic oral and genital infections in humans. Candidiasis, the most common nosocomial fungal infection, is seen in patients with diseases such as tuberculosis, leukemia and AIDS. Candidiasis also may occur in the blood and in the genital tract (Black, 2005).

2.0. Objectives

Malaysian honeys are well recognized because of their quality. However, there is little scientific research published about antibacterial activity of Malaysian honeys. Besides, the susceptibility of normal human pathogens and bacteria isolated from human to honeys of known floral source and defined antibacterial activity has been reported. However, the inhibition of antibiotic-resistant bacteria by honey has not been fully explored.

Therefore, the objectives of this study are

1) To determine the antimicrobial potential effects of Malaysian honey against different species of human pathogens.
2) To optimize the affectivity of honey in preparation of honey broth media.
3) To determine the dose-response relationships between honey broth media and different human pathogens.
4) To introduce Malaysian honey as one of the potential remedies in clinical treatment.

3.0. Literature review

Honey is acceptable in the medical profession as an antibacterial agent for the treatment of some diseases and infections resulting from wounds and burns (Zumla and Lulat, 1989). In many cases, it is used with success on infections not responding to standard antibiotic and antiseptic therapy. Its effectiveness as an antibacterial agent is widely reported (Molan, 1992b).

Honey is the substance made when the nectar and sweet deposits from plants are gathered, modified and stored in the honeycomb by honey bees. The definition of honey depends upon who defines it. Most people think of honey as excellent food, but some others consider it an elixir, and still others as medicine (Zaghloul *et al.*, 2001). Essentially, honey is an invert sugar (a mixture of glucose and fructose) dissolved in 14 to 20% water with minor amounts of organic acids, along with traces of minerals and vitamins. Honey is derived from the nectar of flowering plants which the honey bee collects. Nectar consists primarily of 10 to 50% sucrose, glucose, and fructose and 50 to 90% water (Audrey *et al.*, 1995).

The source of honey determines many of the attributes of honey such as aroma, flavor, color and composition. Honey has had a valued place in traditional medicine for centuries. The ancient Egyptians, Assyrians, Chinese, Greeks and Romans employed honey for wounds and diseases of the gut. Honey was the most popular Egyptian drug being mentioned 500 times in 900 remedies (Zumla and Lulat, 1989). Whilst Hippocrates (3rd and 4th centuries BC) made little use of drugs in treatment, he prescribed a simple diet,

favoring honey given as oxymel (vinegar and honey) for pain, hydromel (water and honey) for thirst and a mixture of honey, water and various medicinal substances for acute fevers (Zumla and Lulat, 1989). During the biblical era honey received a religious endorsement by both Islam and Christianity.

More intensive studies did not commence until the year 1955 where the word 'inhibine' for the antibacterial activity of honey was introduced, a term which has been widely used since the beginning of literature on honey (White and Subers, 1963). Since then there have been many reports on the antimicrobial activity of honey. Some have been of simple testing that has shown honey to have antibacterial activities (Molan, 1992a). Most, however, have involved investigation of the activity spectrum of honey (i.e. determining which species of microorganisms are sensitive to the action of honey), or comparison of different types of honey for the potency of their action against one or more species of bacteria. Also, there have been many investigations of the antibacterial substances present in honey (Molan, 1992a).

Honey has a wide range of antibacterial activities. Research (Willix *et al.*, 1992; Cooper *et al.*, 2002) suggests that honey is effective in vitro against wound-infecting bacteria including *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica* enterica *Ser. Typhimurium*. In earlier studies have shown that conjunctival application of honey can eradicate acute bacterial conjunctivitis due to *Staphylococcus aureus* and *Psedomonas aeruginosa* (Al-Waili *et al.*, 2001b; Al-Waili and Jafari, 2001). Pure honey is bactericidal for many pathogenic organisms, including enteropathogens such as *Salmonella* spp, *Shigella* spp, enteropathogenic *Escherichia coli*, and other gram negative organisms

(Cavanah *et al.*, 1968; Ibrahim, 1981; Jeddar *et al.*, 1985). *In vitro* studies have shown that a 30–50% dilution of honey in liquid broth inhibits the growth of various human pathogenic bacteria, as well as *Candida albicans* (Al-Waili *et al.*, 2001a).

Other inhibition effects of honey include its use in the treatment of fungal infections (Obaseiki-Ebor *et al.*, 1984; Ceyhan and Ugur, 2001). Besides, there is an *in vitro* study confirms the potent anti-mycobacterial effect of honey (Asadi-Pooya *et al.*, 2003). Also, honey has been reported to have anti-leishmanial effects, as well as anti-rubella virus activity (Zeina *et al.*, 1996; Zeina *et al.*, 1997).

Honey has been used since ancient times for the treatment of some respiratory diseases and for the healing of skin wounds. It has been proposed that the healing effect of honey could be due to various physical and chemical properties (Russell *et al.*, 1990; Snow and Manley-Harris, 2004).

Honey is a saturated or super-saturated solution of sugars, 84% being a mixture of fructose and glucose. The water content is usually only 15-21% by weight. The strong interaction of these sugar molecules with water molecules leaves very few of the water molecules available for microorganisms. This "free" water is what is measured as the water activity (aw): mean values for honey have been reported from 0.562 to 0.62. Although some yeasts can live in honeys that have a high water content, causing spoilage of the honey, the aw of ripened honey is too low to support the growth of any species, no fermentation occurring if the water content is below 17.1%. Many species of bacteria have their growth completely inhibited if the aw is in the range 0.94-0.99. These values

correspond to solutions of a typical honey (aw of 0.6 undiluted) of concentrations from 12% down to 2% (v/v). On the other hand, some species have their maximum rate of growth when the aw is 0.99, so inhibition by the osmotic (water-withdrawing) effect of dilute solutions of honey obviously depends on the species of bacteria (Waikato Honey Research Unit, 2006).

The low pH of honey is inhibitory to many animal pathogens. The pH of honey is being between 3.2 and 4.5, which is low enough to be inhibitory to many animal pathogens. The optimum pH for growth of these species normally falls between 7.2 and 7.4. The minimum pH values for growth of some common wound-infecting species is: *Escherichia coli*, 4.3; *Salmonella* sp., 4.0; *Pseudomonas aeruginosa*, 4.4; *Streptococcus pyogenes*, 4.5. Thus in undiluted honey the acidity is a significant antibacterial factor (Waikato Honey Research Unit, 2006). Under experimental conditions, especially with heavily diluted honeys, the growth medium tends to neutralize the acidity of the honey so that it does not cause inhibition but when honey is used as a dressing on a wound or ulcer, bacteria may be in contact with honey that is much less diluted and the acidity could well be of importance. The fairly strong buffering capacity of body fluids may likely neutralize the acidity of honey in other situations where there is greater dilution of honey (Al-Jabri, 2005).

The major antibacterial activity in honey has been found to be due to hydrogen peroxide produced enzymically in the honey. The glucose oxidase enzyme is secreted from the hypopharyngeal gland of the bee into the nectar to assist in the formation of honey from the nectar. The hydrogen peroxide and acidity produced by the reaction:

Glucose + H_2O+ O_2 ---▶ Gluconic acid + H_2O_2 (Hydrogen peroxide)

The hydrogen peroxide produced would be of effect as a sterilizing and preservative agent only during the ripening of honey. Full-strength honey has a negligible level of hydrogen peroxide because this substance is short-lived in the presence of the transition metal ions and ascorbic acid in honey which catalyses its decomposition to oxygen and water. The enzyme has been found to be practically inactive in full-strength honey, it giving rise to hydrogen peroxide only when the honey is diluted. This is because the acidity produced in the action of the enzyme drops the pH to a point which is too low for the enzyme to work any more. On dilution of honey the activity increases by a factor of 2,500 - 50,000, thus giving a "slow release" antiseptic at a level which is antibacterial but not tissue-damaging (Waikato Honey Research Unit, 2006).

Hydrogen peroxide has been out of favor with the medical profession since it first came into use in the late 19th century (Molan, 2001). It has been suggested that it readily decomposes in solutions containing traces of catalytic metals such as iron or copper. This may be the reason why hydrogen peroxide went out of favor as an antiseptic after initially being hailed as an antibacterial and cleansing agent when first introduced (Turner, 1983). There was an upsurge of interest in its use later when stabilized preparations became available, with good germicidal activity being reported (Turner, 1983). But in recent times, it has again gone out of favor as awareness has developed of its inflammatory properties and damage caused to tissue by its oxygen free radicals (Salahudeen *et al.*, 1991; Halliwell and Cross, 1994; Saissy *et al.*, 1995).

However, the hydrogen peroxide concentration produced in honey activated by dilution is typically around 1 mmol/l, about one thousand times the level in the 3% solution

that is commonly used as an antiseptic (Molan and Russel 1988; Molan, 1992b). So the mild acidity and low-level hydrogen peroxide release assists both tissue repair and contributes to the antibacterial activity of honey. This antibacterial activity is a major factor in promoting wound healing where infection is present (Dunford *et al.*, 2000; Lusby *et al.*, 2002).

Honey maintains a moist wound environment that promotes healing, and its high viscosity helps to provide a protective barrier to prevent infection (Lusby *et al.*, 2005). Besides, other two important classes of inhibitors found naturally in honey are flavonoids and phenolic acid (caffeic acid and ferulic acid) (Wahdan, 1998). Since phenolic acids are known to exert an antibacterial effect, their presence in honey explains its antibacterial activity (Weston, 1999; Aljadi and Yusoff, 2002).

Aristotle, in 350 BC, and Discorides, in AD 50, recommended that honey collected in specific regions and seasons could be used for the treatment of different ailments (Molan, 1992b). In modern clinical practice, however, these views have gone unnoticed, though laboratory findings have found large differences in the antibacterial potency of honey from different floral sources (Al-Jabri, 2005).

The sugar composition of honeys from different floral sources has inhibitory potential to the growth of various intestinal bacteria (Shin and Ustunol, 2005). In almost all studies in which more than one type of honey has been used, differences in the antibacterial activity of honey have been observed (Molan, 1992b; Al-Jabri *et al.*, 2003). For example, Manuka honey from New Zealand is recognized for its therapeutic properties (Molan,

2002a). As mentioned earlier, honey has long been known to possess antibacterial properties and has an established usage as wound dressing (Molan, 1999; Cooper *et al.*, 2002), although not all honeys are equally effective for wound healing (Molan, 2002a).

There is report of sugar being used with good effect as dressings for wounds and ulcers (Molan, 1992b). The good control of infection with both sugar and honey is attributed to the high osmolarity, but honey can have additional antibacterial activity because of its content of hydrogen peroxide and unidentified substances from certain floral sources (Molan, 1992b).

The antibacterial activity of honey was first recognized in 1892, by van Ketel (Dustmann, 1979). It has been found that mostly the activity is due to the hydrogen peroxide produced enzymically in honey, but there have been some reports of minor additional antibacterial components (Molan 1992a; Molan, 1992b). A survey of 345 samples of New Zealand honeys from 26 different floral sources carried out by researchers (Allen *et al.*, 1991) found that when catalase was added to destroy hydrogen peroxide the honey from only one of the floral sources, Manuka (*Leptospermum scoparium*), had any significant amount of antibacterial activity remaining. This was unique amongst the many reports on other honeys around the world in that this non-peroxide component was a major contributor to the antibacterial activity, although a subsequent survey of 340 samples of Australian honeys from 78 different floral sources (C. Davis, Queensland Department of Primary Industries: personal communication) made a similar finding for honey from jellybush (*Leptospermum polygalifolium*).

This novel antibacterial activity has been subsequently studied to determine the potential usefulness of Manuka honey as a therapeutic agent. In this research it has been compared with honey that has the usual type of antibacterial activity due to hydrogen peroxide (Allen *et al.*, 1991). In the survey, a large number of the samples of honey from the different floral sources were found to be of low activity (36% of the samples had activity near or below the level of detection in an agar diffusion assay), the rest having almost a Gaussian distribution over a thirty-fold range of activity. The non-peroxide activity in the samples of Manuka honey was found to be similarly distributed (Allen *et al.*, 1991). Consequently in the studies on the effectiveness of the antibacterial activity a representative Manuka honey and a honey with activity due to hydrogen peroxide were selected to be each near the median level of their respective type of activity (Allen *et al.*, 1991). The Manuka honey was also selected to have a low level of activity due to hydrogen peroxide, and in some of the studies catalase was added to break down any hydrogen peroxide that may have been formed (Allen *et al.*, 1991).

Besides, there was a study has shown the ability of honey to inhibit growth of food spoilage organisms (Mundo *et al.*, 2004). Specific honey samples were shown to be capable of inhibiting the growth of both spoilage microorganisms and foodborne pathogens and could be served as a natural food preservative under appropriate conditions. Previous research has demonstrated preservative power of honey by reducing enzymatic browning of fruits (Oszmianski and Lee, 1990; Chen *et al.*, 2000) and preventing lipid oxidation in meat (McKibben and Engeseth, 2002).