The antibacterial activity of diluted Tualang honey

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Abstract

Tualang honey (TH) is a Malaysian jungle honey obtained from the wild. The honey is produced by the rock bee (*Apis dorsata*), which builds hives on branches of tall Tualang (*Koompassia excelsa (Becc.) Taub)* trees located mainly in the north-western region of Peninsular Malaysia. Limited information is available on the antibacterial mechanism of local honey. The present study was conducted to determine the antibacterial activity of diluted 'Tualang' honey against *Escherichia coli* (*E. coli*). Three different Tualang honey concentrations, namely, 20, 30 and 40% were used to examine the effect of diluting Tualang honey on its antibacterial effect towards *E. coli*. The 40% diluted honey was the most effective in inhibiting the growth of *E. coli*, followed by honey diluted at 20 and 30%. The high antibacterial activity of honey towards *E. Coli* was thought to arise from the production of hydrogen peroxide, but requires further study to validate the action.

Keywords: Tualang honey, *E. coli,* antibacterial, hydrogen peroxide

Introduction

Honey has been used for medicinal properties to treat a wide variety of ailments since ancient times. It has been used in wound dressings. Honey is known to protect against damage caused by bacteria. Some honey also stimulate production of special cells that can repair tissues damaged by infection. In addition, honey has an anti-inflammatory action that can quickly reduce pain and inflammation once it is applied. In general, all types of honey have high sugar content but a lower water content and acidity, which prevents microbial growth. Tualang honey (TH) is known to have antimicrobial effects, which are attributed to the osmotic effect of its sugar contents, pH, and particularly its peroxidase activity. The antimicrobial effects are also due to the presence of non-peroxidase substances such as phenolic acids, flavonoids, and lysozymes. Hydrogen peroxide is a component of honey. It gives most honey its antibiotic quality, but some types of honey, including Manuka honey, also have other components with antibacterial qualities. Most types of honey generate hydrogen peroxide when diluted because of the activation of the enzyme glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide. Hydrogen peroxide is the major contributor to the antimicrobial activity of honey and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (Hern *et al.*, 2009).

TH has both bactericidal and bacteriostatic properties against a range of bacteria, including common bacteria of the skin. At concentrations of 6.25–25%, TH inhibits the growth of several bacterial strains, such as *Streptococcus pyogenes*,

Salmonella typhi, Staphylococcus aureus, coagulase-negative *Streptococcus* spp., and *Escherichia coli*. Currently, no information is available on the effect of diluting TH on its antibacterial properties. Thus, the objective of this study was to examine the effect of dilution with peptone water on the antibacterial activity of Tualang honey by monitoring the growth of bacteria.

Materials and Methods

Honey

TH was collected from a bee farm in Kota Bharu, Kelantan, Malaysia. The honey was stored in the dark at room temperature before use. The pH of TH was measured using a pH meter (Hanna Instrument, Romania).

Bacteria Preparation

Strains of *E. coli* were obtained from the laboratory of the Faculty of Agro-Based Industry, Universiti Malaysia Kelantan and maintained on nutrient agar at 4^{o}C. The culture used for inoculation purposes was obtained from streak plate cultures and grown overnight in nutrient broth. Single colony bacteria was inoculated into 10 ml Tryptic soy broth (TSB) and incubated overnight at 30^{o}C. The broth containing the bacterium was centrifuged at 3000 rpm at 4^{o}C for 10 min to precipitate the bacterial mass. The supernatant was removed and the bacterial pellet was re-suspended in peptone broth and vortexed for 1 min. The bacterial suspension was diluted with peptone broth to 1 X 10^4 ml^{-1} before use as inoculants.

TH Preparation

Honey solutions were prepared by diluting TH with sterile peptone water (0.01%) to make 20% (v/v), 30% (v/v) and 40% (v/v) dilutions.

Antibacterial Assay

The honey solutions were inoculated with 0.1 µl of *E. coli* 1 X 10^4 ml^{-1} and incubated at room temperature, for 24 h. The spread plate method was used to observe bacterial growth at 0, 3, 6, 12 and 24 h. The samples were diluted accordingly, spread plated onto nutrient agar and incubated aerobically at room temperature for 24 h. The total bacteria number was calculated according to colony-forming units in one ml solution (CFU ml^{-1}). Samples were prepared in duplicates and plated onto triplicate plates.

Statistical Analysis

Two-way Analysis of Variance (ANOVA) was used to estimate the effects of diluted TH on the concentrations and observation times on the length of the bacterial growth lag phase. Differences among the means were estimated using Turkey test at α level of 0.05 to determine statistical significance (95% confidence interval). The statistical analysis was carried out using Minitab 16.1 software.

Results and Discussion

Physical Properties of Tualang Honey

Appearance of 'Tualang' honey was observed as brown to dark brown in colour similar to the description of Tumin *et al.* (2005), it was found that the 'Tualang' honey was dark brown. The colour of honey varies with the botanical origin, age and storage conditions, but the transparency or clarity of honey is dependent on the amount of suspended particles such as pollen (White, 1975). This is supported by Al-Waili *et al.* (2011) who found that the colour of honey was affected by species of bee, geographic location and botanical origin. It was highly probable because unifloral honeys from

different geographic origins might show different physicochemical properties (Al-Waili *et al.*, 2011). Honey could also become lighter in colour with increasing crystallization of glucose to a much whiter colour which Rodriguez (1985) also suggested to be the results from the formation of monohydrate glucose crystals, which varied in number, shape, dimension and quality with the honey composition and storage conditions.

pH of Tualang Honey

The pH of 'Tualang' honey was 3.76. The differences in the pH value among different types of honeys may arise from the concentration of gluconic acid. Gluconic acid is a by-product of glucose oxidation by glucose oxidase, thus a much aged honey is expected to have a much lower pH value. The variation in the amount of glucose oxidase between the different honeys may also give rise to differences in pH. Yet, the age or the available concentration of glucose oxidase in honey used in this study was not known, thus the reason behind the differences in the pH of the honey was not clear.

Effect of Dilution on Antibacterial Activity in Tualang Honey

From the study, the honey dilutions used was observed to have antibacterial activity against *E. coli* (Table 1). At 20 and 30% dilution no change ($P > 0.05$) in bacterial population in TH was observed during the 24-h storage period (Table 1). When diluted to 40% the bacterial population decreased ($P < 0.05$) by almost 1 log CFU/ml. A study by Bang *et al*. (2003) showed that the antibacterial activity of honey was increased at a dilution of 30%. The dilution of honey is expected to increase hydrogen peroxide production, leading to a higher antibacterial action (Al-Waili *et al.*, 2011). The higher honey concentration which decreased bacterial number in this study was as expected, since more substrate were made available resulting in a much higher concentration of antibacterial properties. A similar study of 8 honey samples showed that maximum accumulation of hydrogen peroxide occurred when honey was diluted from 30 to 40% in 75% of the samples with two honey samples showed maximum hydrogen peroxide concentration when honey was diluted from 40 to 50% (Bang *et al.*, 2003).

This situation has made the production of glucose oxidase to be low thus lowering the activity of hydrogen peroxide in inhibiting the bacterial growth. This may explain why honey diluted at 40% inhibited the bacterial growth. Another possible explanation is that the enzymes need a sufficiently high level of free water to be active, and in undiluted honey the water present is almost all bound with the sugar molecules (White and Subers, 1963).

Dustmann (1971) proposed that the antibacterial action from hydrogen peroxide can be reduced with increased light presence and heat. Meanwhile Roth *et al.* (1986) proposed that the antibacterial activity was non-peroxide. Russell *et al.* (1990) observed that this non-peroxide activity which remained unaltered even during long storage times, was dependent on the flower source of the nectar used and so not all honey possessed this activity.

Table 1: Effect of dilution on the total bacteria count (log CFU/ml) in Tualang honey inoculated with *E. coli* and incubated at room temperature

Dilution (%)	Incubation time (h)				
	0	3	6	12	24
20	$4.1 \pm 0.2^{ab}{}_{x}$	$4.0 \pm 0.2^{a}{}_{x}$	$4.7 \pm 0.3^{b}{}_{x}$	$4.3 \pm 0.1^{ab}{}_{x}$	$4.3 \pm 0.2^{b}{}_{x}$
30	$4.7 \pm 0.3^{b}{}_{x}$	$4.5 \pm 0.4^{a}{}_{x}$	$4.3 \pm 0.3^{ab}{}_{x}$	$5.0 \pm 0.1^{b}{}_{x}$	$5.0 \pm 0.1^{b}{}_{x}$
40	$3.8 \pm 0.1^{a}{}_{y}$	$3.8 \pm 0.5^{a}{}_{y}$	$3.8 \pm 0.2^{a}{}_{y}$	$3.7 \pm 0.2^{a}{}_{xy}$	$3.0 \pm 0.5^{a}{}_{x}$

^{ab}Means within rows with different superscripts differ at $p<0.05$
^{xy}Means within columns with different subscripts differ at $p<0.05$
Values represent the mean ± standard deviation.

Meanwhile, in another experiment, when no bacteria was inoculated to the diluted honey (20-40%), bacteria number increased with storage period. Although the initial bacterial numbers at 0 hour was below 2.0 log CFU/ml, the bacteria number increased to 5.4 log CFU/ml and 5.2 log CFU/ml for honey diluted to 20 and 30%, respectively. On the other hand, when honey was diluted to 40%, the bacteria number only reached 4.2 log CFU/ml after the 24-h storage period which was lower ($P < 0.05$) than the 20 and 30% honey dilutions. This might have arouse due to the presence of bacteria that could survive in honey thus was able to withstand the concentrated sugar, acidity and other antibacterial characteristics of honey. According to Snowdon and Cliver (1996), most microorganisms do not grow in honey because of its low water activity. However, honey sometimes contains yeast and spore-forming bacteria namely *C.botulinum*. Primary sources of microbial contamination are likely to include pollen, digestive tracts of honey bees, dust, air, earth and nectar - sources which are very difficult to control. The same secondary (after-harvest) sources that influence any food product are also sources of contamination for honey which include air, food handlers, cross-contamination, equipment and buildings (Snowdon and Cliver, 1996).

Olaitan *et al.* (2007) stated that microbes found in honeycombs were principally bacteria and yeast which came from bees, nectar and other external sources. However, mostly the spore forming microorganisms can survive in honey at low temperature. It has been suggested that aerobic spore forming *Bacillus* are the most frequently encountered microbes on the external surface, crop and intestine of the honey bees. The intestine of bees has been found to contain 1% yeast, 27% Gram-positive bacteria including *Bacillus, Bacteridium, Streptococcus* and *Clostridium* spp., 70% Gram negative or Gram variable bacteria including *Achromobacter, Citrobacter, Enterobacter, Erwinia, E. coli, Flavobacterium, Klebsiella, Proteus* and *Pseudomonas* (Olaitan *et al.*, 2007). Another factor that leads to the increased number of bacteria over time in the honey dilution is competition to grow with any other bacteria, i.e. *E.coli* which was inoculated in the first set of experiment was the primary bacteria available, and its sheer initial number might explain why bacterial number grew.

Table 2 showed that the growth of bacteria over time in 20 and 30% honey dilution was basically the same; having the highest total bacteria count compared to honey dilution at 40%. The growth of bacteria at 40% honey dilution was slightly slower than the other two honey dilutions

where the bacteria count at 0 and 3 h were below log 2, i.e. the number of bacteria grown in the honey dilution was too small. The number of bacteria started to increase from 6 h but they were still lower than the number of bacteria in 20 and 30% honey dilution This condition happened for several reasons. The antibacterial properties of honey as reported by Abdullah *et al.* (2007) showed that honey had a greater inhibitory effect on isolated Gram-negative bacteria (*P. aeruginosa, E. coli, Enterobacter* spp. and *Klebsiella*). El-Sukhon *et al.* (1994) showed Gram-negative bacteria to be more sensitive to action of honey than Gram-positive bacteria. Taormina *et al.* (2001) studied the antimicrobial effect of honey on Gram-negative bacteria and attributed it to the presence of factors such as high content of tetracycline derivatives, hydrogen peroxide, powerful antioxidants, and also to a naturally low pH, which is unsuitable for bacterial growth.The presence of biochemical components such as phenolic acids, lysozyme and flavonoids might have also led to the inhibition of bacteria.

Hamouda and Marzouk (2011) added that water concentration lower than 30% was considered too low to have any bacteriostatic effect. Water concentration of at least 10 mg/l was required to inhibit bacterial growth. However, Willix *et al.* (1992) found that honey had sufficient antibacterial potency to stop the bacterial growth if diluted at least nine times and up to 56 times in the presence of bacteria. The present result was as expected since dilution might affect the expression of hydrogen peroxide where the production of hydrogen peroxide would increase as the honey is further diluted (Al-Waili *et al.,* 2011).

Water content in honey is critical at 0.6 % which is the characteristic lower moisture content inhibiting the growth of bacteria (Olaitan *et al.,* 2007). In the present study
the honey dilutions at 20 and 30% had higher water content compared to the honey dilution at 40%. As honey was further diluted with water, the moisture content of honey increased and therefore providing a better environment for bacterial growth. In normal honey condition, bacteria would burst its cell wall allowing the increase of moisture in honey to create an environment that is sufficient for growth (Snow andManley-Harris,2004).

Table 2: Effect of dilution on the total bacteria count (log CFU/ml) in Tualang honey incubated at room temperature.

Dilution (%)	Incubation time (h)				
	0	3	6	12	24
20	*	$2.2 \pm 0.3^{a}_{x}$	$2.8 \pm 0.2^{a}_{y}$	$5.3 \pm 0.1^{b}_{z}$	$5.4 \pm 0.2^{b}_{z}$
30	*	$2.1 \pm 0.1^{a}_{x}$	$3.0 \pm 0.1^{a}_{y}$	$4.9 \pm 0.1^{b}_{z}$	$5.2 \pm 0.1^{b}_{z}$
40	*	*	$3.0 \pm 0.6^{a}_{x}$	$3.7 \pm 0.2^{y}_{a}$	$4.2 \pm 0.2^{a}_{y}$

*Below 2.0 log CFU/ml

[ab]Means within rows with different superscripts differ at $p<0.05$

[xy]Means within columns with different subscripts differ at $p<0.05$

Values represent the mean ± standard deviation.

Conclusion

It can be concluded that the honey dilutions at 20 and 30% were able to inhibit the growth of *E. coli*, while the honey dilution at 40% gave higher inhibition value. It is suggested that the availability of hydrogen peroxide would result in increased inhibition capabilities, such that dilution of 40% honey inhibits the growth of *E. coli* because more substrate is involved in producing the enzyme glucose oxidase leading to higher production of hydrogen peroxide.

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