The antibacterial hydrogen peroxide generation from *Ziziphus Spina Christi* and *Acacia* spp. honey

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**ABSTRACT**

Honey and hydrogen peroxide are effective antibacterial agents. The antibacterial activity of honey is mainly attributed to hydrogen peroxide generation that is produced in the honey by the activity of the glucose oxidase (GOX) enzyme. *Ziziphus Spina Christi* and *Acacia* spp. honey samples were collected for the study. Hydrogen peroxide was measured using a sensitive enzymatic/colorimetric assay. The method was then used to measure peroxide in various degrees of honey dilutions. The kinetics of hydrogen peroxide production over time in honey solutions were also tested. There were differences between the two samples with respect to hydrogen peroxide levels. *Z. christi* produced up to 4.5 mM of peroxide compared with 1.2 mM generated by *Acacia* spp. (p=0.02). The maximum concentration of peroxide produced in the experiment was at 60% dilution. This degree of dilution was used to assess the generation of peroxide over time. The results showed that the GOX reached the maximum generation of hydrogen peroxide within the first 20 min of incubation and then gradually decreased over the next 100 min. In conclusion, honey collected for this study exhibited efficient hydrogen peroxide generation, which may have antibacterial activity. The production of peroxide was shown to be dependent on the honey dilution factor.

**Key words:** Hydrogen peroxide, honey, glucose oxidase, *Ziziphus Spina Christi*, *Acacia* spp.

**Abbreviations:** GOX: Glucose Oxidase; ROS: Reactive Oxygen Species.

**INTRODUCTION**

The major constituents of honey are fructose, glucose and enzymes. Honey also contains various quantities of minerals with potassium being the most abundant element, comprising approximately one-third of the total mineral content (Alqarni et al., 2014; Samarghandian et al., 2017). However, the composition of honey is influenced by both biotic and abiotic factors pertaining to the bee colony such as floral sources, climate conditions, soil, and beekeeper practices. In this study, *Ziziphus Spina Christi* and *Acacia* spp. honey were used to investigate hydrogen peroxide generation. In general, the main honey plants in Saudi Arabia are *Acacia* spp. and *Z. christi*. These two trees are found in the wild in all regions of Saudi Arabia, particularly in the mountainous areas of the southwest. Their flowering seasons start during June and August of each year, depending on the rainfall (Alqarni et al., 2011).

Honey has long been known to have wound healing properties (Cooper et al., 1999). Several studies have shown that some types of honey can effectively treat several wound infections such as abscesses, surgical wounds (Goharshenasan et al., 2016), burns (Molan, 2001) and ulcers (Majtan, 2014). Honey has an inhibitory effect on approximately 60 species of bacteria, including aerobes and anaerobes, both gram-positive and gram-negative...
The bactericidal effect of honey is attributed to its osmolality, acidity and viscosity. However, more recent studies attributed the antibacterial activity of most honey to the hydrogen peroxide generated enzymatically by the action of honey glucose oxidase (GOX) (Brudzynski et al., 2012; Oelschlaegel et al., 2012; Anthimidou et al., 2013), an enzyme that is secreted from the bee’s pharyngeal gland (White et al., 1963). In honey, the GOX enzyme is activated by dilution and the peroxide produced is too mild to cause tissue injury, but it still has antimicrobial activity (Brudzynski et al., 2012).

Hydrogen peroxide is also a well-known antimicrobial agent, initially studied for its antibacterial and cleansing properties when it was first introduced to clinical practice. Interestingly, the mechanism that honey uses to generate peroxide and fight pathogens is also present in human cells when they are exposed to pathogens. Phagocytic white blood cells present in localized inflammation within human tissues produce various amounts of reactive oxygen species (ROS), particularly hydrogen peroxide, as a part of the first line defense against microbial invasion (Alfadda et al., 2012). Recently, it was suggested that cryptotanshinone (a compound isolated from some medicinal plants) exhibits its antimicrobial activity against S. aureus strains by generating ROS (Feng et al., 2009). Importantly, this molecule targets S. aureus isolates in which more than 60% of the isolates are resistant to methicillin. Research in this area of utilizing ROS activity is promising and aims to discover novel oxidative molecules that target antibiotic resistant bacteria, a serious clinical problem that is increasingly encountered.

The aim of the present study was to demonstrate and compare the rate of hydrogen peroxide generation with several degrees of dilution in Z. christi and Acacia spp., popular types of honey in Saudi Arabia. The study also investigated the rate of peroxide production and consumption over a period of time using a sensitive colorimetric assay.

MATERIALS AND METHODS

Honey samples

Honey samples were obtained from local markets in the southern part of Saudi Arabia. For a comparative study, two unprocessed and unpasteurized honey samples, Z. christi and Acacia spp., were used for this study and stored undiluted at 8°C in the dark until analyzed.

Reagent preparation

Hydrogen peroxide (Merck Pty Ltd), 30% v/v, 9 M, was used as a calibration standard by dilution to approximately 9 mM in 100 mM Tris-HCl pH 7.5 (Tris buffer).

Concentrations of hydrogen peroxide were accurately determined spectrophotometrically in duplicate at 240 nm, assuming a molar absorptivity of 43.6 M⁻¹ cm⁻¹.

The peroxidase reagent contained 100 µM of Ampliflu Red (Sigma-Aldrich Pty) and 0.8 U/mL horseradish peroxidase (Sigma-Aldrich Pty), in 100 mM Tris-HCl, pH 7.5 (Tris buffer). Buffers and aqueous reagents for all experiments were prepared from MilliQ distilled water. In the presence of horseradish peroxidase, peroxide converts Ampliflu Red (10-acetyl-3,7-dihydroxyphenoxazine) to the red-fluorescent oxidation product, resorufin. This product has a maximum absorbance peak wavelength at 560 nm.

Honey solutions preparation

Honey solutions were prepared immediately prior to testing by diluting the honey to the appropriate concentrations (10, 20, 30, 40, 50, 60, 70 and 80% v/v), respectively using 100 mM Tris-HCl pH 7.5 (Tris buffer). All samples were then incubated for 30 min at 37°C in a shaking dry incubator.

The solutions were protected from light because both hydrogen peroxide and glucose oxidase are light sensitive. To test the activity of peroxide generation over time, samples of Z. christi and Acacia spp. were diluted to 60% in tris buffer, adjusted to pH 7.5 and incubated at 37°C for up to 100 min. Concentrations of accumulated hydrogen peroxide were measured after 5, 10, 20, 40, 60, 80 and 100 min, respectively to determine the levels reached after a period of incubation.

Measurement of hydrogen peroxide generation

The hydrogen peroxide generated was then measured using coupled enzymatic assays of GOX and horseradish peroxidase-Ampliflu Red reagent. Briefly, oxidation of honey glucose by GOX results in the generation of hydrogen peroxide coupled to the conversion of the Ampliflu Red reagent to the colored resorufin by the horseradish peroxidase. Equal volumes of honey solution and peroxidase reagent were mixed in micro-cuvettes (Becton Dickinson). The cuvette was then incubated at 37°C for 15 min in a shaking dry incubator. The absorbance of the formed product, resorufin, was measured spectrophotometrically at 560 nm. Each experiment was performed in duplicate. For each honey solution, a corresponding assay substrate blank (that is, excluding honey) was subtracted from each data point.

The hydrogen peroxide produced from the honey solutions was calculated from a standard curve generated by adding equal volumes of hydrogen peroxide (0.5, 1, 2, 4, 6, 8, 10 µM) and peroxidase reagent, respectively. The reaction mixture was incubated at 37°C for 30 min, and the absorbance was measured as earlier described.
Statistical analysis

The linear regression curve method was used to generate the study’s standard curve and the appropriate equation. Mean values were compared between the two different groups using an unpaired, two-tailed t-test. A p-value of 0.05 was the cut-off for statistical significance. All analyses were performed using GraphPad Prism 7 software.

RESULTS

Figure 1 shows the standard curve for the absorbance generated by the reaction of Ampliflu Red with horseradish peroxidase and various hydrogen peroxide concentrations displayed acceptable linearity ($r^2 = 0.997$) up to 10 μM. This standard curve was subsequently used for measuring the peroxide concentrations produced in the honey solutions.

The generation of hydrogen peroxide in both honey samples after 30 min of incubation shows that maximum accumulation occurred in 60% dilution. In the Z. christi honey sample, the highest accumulation of peroxide was 4.5 mM compared to only 1.5 mM accumulated by Acacia spp., $p=0.02$ (Figure 2).

To assess the consumption of hydrogen peroxide with time, 60% honey solutions were prepared from Z. christi and Acacia spp. Prolonged incubation of these solutions showed a significant difference between the two honey samples. Interestingly, the hydrogen peroxide concentration suddenly increased in the first 20 min to 3.5 mM for Z. christi and 1.3 mM for Acacia spp. (Figure 3). The hydrogen peroxide concentration then gradually decreased with time for Z. christi until it reached approximately 1.5 mM after 100 min of incubation. In comparison, the Acacia spp. honey showed a steady state of hydrogen peroxide production throughout the prolonged incubation.

DISCUSSION

Honey is not only used as a nutritional product but is also described in traditional medicine as an alternative treatment. Honey and hydrogen peroxide are well-known antibacterial agents, in which the bactericidal activity of honey is attributed to several factors including viscosity, osmolality and the generation of hydrogen peroxide (Oelschlægel et al., 2012). We selected these two honey samples because they represent the most common and
Figure 2: Hydrogen peroxide generation by honey GOX at various percentages of dilution (mean±SD, n=2). The concentration of produced peroxide is expressed as mM per 30 min.

Figure 3: Assessment of hydrogen peroxide production (mM) with respect to time (min) in 60% dilution for the *Ziziphus Spina Christi* sample and 60% dilution for the *Acacia* spp. The sample was incubated for up to 100 min. Values represent the mean ±SD, n=2.
popular types of honey produced from two different species of trees in Saudi Arabia (Alqarni et al., 2014).

In this study, it was shown that honey from *Z. christi* and *Acacia* spp. exhibits a powerful production of hydrogen peroxide by the action of GOX. Interestingly, the production of peroxide was significantly influenced by the degree of dilution. The finding that the GOX activity and its subsequent hydrogen peroxide generation depend on the honey's dilution factor was previously suggested by Brudzynski et al. (2012). The reason for the inactivity of GOX in concentrated honey in still not known. However, previous studies suggested that the reason is not due to the GOX enzyme itself, but due to other factors such as low pH and high substrate concentrations in the concentrated honey (White et al., 1963, 1964).

However, in the current study, the optimum pH was adjusted to 7.5 for all honey dilutions. In this study, there were differences in the levels of peroxide generation between the honey samples ranging from 1 to 4.5 mM at an optimum dilution of 60%, respectively. The concentration of peroxide measured in this study was higher than those obtained for several types of honey from different botanical and geographical regions, in which the peroxide levels ranged from 0.03 to 0.63 mM, respectively. The variation in peroxide concentrations among the honey samples is likely due to the presence of a catalase (Kwakman et al., 2011). This may suggest that the bactericidal potency of honey depends on its type and origin. It was found that this level of hydrogen peroxide is effective against several strains of pathogenic bacteria (Cooper et al., 1999; Brudzynski et al., 2012; Anthimidou et al., 2013). The inhibitory and potential killing ability of bacteria by hydrogen peroxide is attributed to the oxidation of DNA and RNA. The oxidative injury to these molecules impairs the permeability of the cell membranes and cell proliferation and ultimately leads to bacterial cell death (Imlay et al., 1988; Brudzynski et al., 2012).

In addition to the concentrations of peroxide measured in this study, another recent study showed that neonatal saliva contains high levels of the metabolites xanthine and hypoxanthine. These are substrates of the enzyme xanthine oxidase, which is highly abundant in breast milk (Al-Shehri et al., 2013, 2015). During breast-feeding, the mixing of neonatal saliva with breast milk generates micromolar levels of hydrogen peroxide, a necessary substrate for activation of the 'lactoperoxidase system' to produce additional bactericidal ROS. These metabolites provide a unique antibacterial activity within the neonatal mouth at a time when other immune mechanisms are not fully developed. This concentration of peroxide significantly inhibited some pathogenic bacteria, including *S. aureus*, by its production of ROS during *in vitro* experiments (Al-Shehri et al., 2015). In addition, an *in vivo* study of oral microbiome diversity in neonates using 16S rRNA sequencing demonstrated significant differences between formula-fed and breastfed infants (Al-Shehri et al., 2016). It was suggested that hydrogen peroxide generated by this mechanism may not only contribute to the innate immunity of neonates but may also provide the appropriate concentrations for rapid cell signaling and growth. However, the peroxide produced by honey in the current study was higher than that produced by the action of neonatal saliva and breast milk.

A high concentration of hydrogen peroxide is very effective against bacteria; however, the micromolar level found in this study might be cytotoxic, which could limit it to external use only, such as in the treatment of wound infections. The cellular physiologic concentration of peroxide is usually within micromolar levels and has several essential biological roles. Interestingly, it was observed that hydrogen peroxide and other ROS play important roles as small-molecule second messengers. Pan et al. (2011) suggested that hydrogen peroxide at low levels, particularly at approximately 20 μM stimulates cell viability and facilitates adhesion and wound healing in corneal cells. Furthermore, in activated T-cells, the superoxide anion at low micromolar concentrations of hydrogen peroxide increased the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein (Roth et al., 1987). This suggests that honey can stimulate wound healing not only by its bactericidal efficacy but also by its cellular growth enhancement through hydrogen peroxide generation.

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**REFERENCES**


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