Research Article

MECHANISM OF ANTIBACTERIAL ACTION OF HONEY ON PATHOGENIC WOUND BACTERIAL STRAINS: A PROTEOMIC ANALYSIS

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Article Received on: 11/09/15 Revised on: 12/10/15 Approved for publication: 31/10/15

DOI: 10.7897/2230-8407.0611151

ABSTRACT

Honey has been a product that has received a growing attention in wound care. This study aimed to determine the potential efficacy of honey as antibacterial agent, and its role in improving wound infection combined with different antibiotics against wound pathogens. The susceptibility of methicillin sensitive Staphylococcus aureus (MSSA), methicillin resistant Staphylococcus aureus (MRSA) and Pseudomonas aeruginosa (PA) isolates to medical types of honey collected in KSA and to their combination with various antimicrobial compounds was determined. The effect of honey on the morphology of the strains was studied by using Scanning Electron Microscope (SEM). The mechanism of action of honey on the bacterial pathogens was assessed using Nanodrop 2000C. Results revealed critical discrepancies between the antibacterial activities of Honeys tested on the different isolates. Southern (SSH) and Yemen Sidr honey (YSH) showed the most potent results followed by multi-flower mountain honey. Different combinations of honey with antibiotics showed potent synergistic effect. SEM images demonstrated a clear change in the morphology of the isolates upon treatment with honey and total destruction of bacterial cells in honey antibiotic combination. Cellular proteins were released due to disruption in cell wall and cytoplasmic membrane in the supernatants of both MRSA and PA by both YSH and SSH treatments. An increase in MRSA membrane protein release by 0.550 and 0.640 mg/ml than the control was demonstrated for SSH and YSH, respectively. The PA membrane proteins released after treatment with honey were higher compared to that of the MRSA strain.

KEY WORDS: Honey, wound infections, MRSA, Scanning electron microscope, Proteomic analysis.

INTRODUCTION

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. Although antibiotics, taken alone or combined are reliable sources of treatment of bacterial contamination of wounds, still the side effects they have on the body and the known drug-drug interactions for certain patients who are on drugs other than antibiotics leave them undesirable to patients. Not to mention the increased possibilities of their toxicity when used in combination. With the irrational and massive use of antibiotics in underdeveloped and developing countries, resistant pathogens develop and spread. As a result, the effectiveness of the antibiotics is diminished. Furthermore, there was an increased resistance to even newly discovered antibiotics.

Therefore, the need for novel alternative antimicrobial strategies has renewed interest in natural products exhibiting antibacterial properties. This situation has led to a re-evaluation of the therapeutic use of ancient remedies including honey. Honey is well known in many religions and cultures as a magic drug for almost all kinds of diseases, not to mention the fact that many people do depend more on folk medicine and natural remedies that have been known for their therapeutic effects over the past decades.

Honey is a natural, inexpensive, and nontoxic old remedy for the treatment of infected wounds and has been proved to have no adverse effects on the healing process of tissues. It has recently been ‘rediscovered’ and there are many published reports that describe its healing properties and its effectiveness in rapidly cleaning infection from wounds. These powers can be ascribed to the fact that it offers antibacterial activity, maintains a moist wound environment that promotes healing and has a high viscosity that helps to provide a protective barrier to prevent infection. Additionally, it was reported to be effective on antibiotic resistant strains of bacteria such as MRSA and PA. It was also reported that honey, not only increases the sensitivity of microorganisms to antibiotics but decreases the microbial resistance to antibiotics as well. Furthermore, microbial resistance to honey has never been reported, which makes it a very promising topical antimicrobial agent.

For honey to be approved as an official treatment for diseases, its exact mechanism of action should be known. Many studies have been conducted on honey for this purpose. Its inhibitory effect was initially attributed to inhibit, but later the enzymatic production of hydrogen peroxide was identified as the inhibitory agent. However, none of the previous studies assured the effect of honey's action on different pathogens.

Although the antibacterial activity of honey collected worldwide against many pathogens in vitro has been established but to our knowledge, the potential efficacy of different types of local honey collected and purchased in KSA were not studied before.

With all that in mind, it is definitely worthy to consider honey as a promising future local antibiotic and as a great subject to be tested and studied. The present study aims to evaluate the antimicrobial effect of representative bee honeys collected or purchased locally in KSA and produced under natural and farm environment on organisms isolated from infected wounds compared with that of certain antibiotics that are commonly used in the treatment of infected wounds. Furthermore, combination of different antibiotics with different honey types were assessed for their antibacterial activity and their comparison with the commercially available Effective
antibiotics. Finally, some assumptions and conclusions of the possible mechanism of action of honey were proposed.

MATERIALS AND METHODS

Bacteria and culture conditions: The tested bacteria were obtained from the clinical microbiology laboratory of King Khaled Hospital, Riyadh, KSA. All bacterial isolates were subjected to the conventional identification tests 15. In total, eight isolates of methicillin-susceptible SA (MSSA), eight isolates of methicillin-resistant SA (MRSA) and eight isolates of Pseudomonas aeruginosa (PA) were studied. The isolates were obtained on sterile sheep blood agar plates (SPML, Riyadh, KSA). They were re-isolated on mannitol salt agar (Oxoid, UK) for the MRSA and MSSA and Cetrimide agar (Oxoid, UK) for the pseudomonal strains. Stock cultures were preserved on glycerol broth at -80 °C.

Honey and antimicrobials: Honey was purchased from honey collection centers in Riyadh, KSA. Ten medical types were examined namely: Clover Flower Honey (CLH), Cotton Flower Honey (CTH), Yemen Sdir Honey (YSH), Southern Sdir Honey (SSH), Nigella Sativa (Black Seed) Honey (NSH), Yemen Thorn Honey (YTH), South Date Honey (SDH), Al-Madinah Honey (AMH), Hungarian Honey (HH) and Kashmiri Sdir Honey (KSH). Only one commercial type was used for comparison namely Multi-Flower Mountain Honey (Nectaflor®) (MMH).

Amoxicillin clavulanate was used as Amoclan® 600 mg (Hikma Pharmaceuticals, Jordan), ceftriaxone sodium as Triaxone® 1 g (Tabuk Pharmaceutical Mfg. Co., KSA) and ciprofloxacin as Cipro® 200 mg (Bayer HealthCare Pharmaceuticals, Kansas). Ten antibiotic discs were used and all the antibiotic discs used were product of Oxoid, UK. Six were used for both gram-negative and gram positive bacteria, including Ampicillin (AMP 25), Ceftriaxone (CRO30), Ciprofloxacin (CIP5), Sulfamethoxazole/Trimethoprim (SXT 25), Amoxicillin/Clavulanic acid (AMC30) and Gentamicyn (CN 30). The discs used for gram-positive bacteria were Vancomycin (VA30), Oxacillin (OX1), Quinupristin/Dalfopristin (QD 15) and Linezolid (LZD 30). Antibiotic powders and discs were kept at -30 °C until used.

Determination of antibacterial activity of antimicrobials and honey: The sensitivity or resistance of pathogenic bacteria to various antimicrobial compounds and honey was determined by Kirby-Bauer disk diffusion susceptibility test described in CLSI 17, 18. The exponential phase cultures of the bacterial isolates under test were made in sterile normal saline and adjusted to the 0.5 McFarland standard approximately corresponding to 1-2 x10⁸ CFU/mL by adding more organism if the suspension is too weak or too heavy. The cultures were then swabbed on Muller Hinton Agar (MHA) plates (Oxoid, UK) uniformly by means of sterile swab dipped in the suspension and streaked on the agar plate surface and the plates left on the bench for excess fluid to be absorbed.

Antibiotic discs were then placed aseptically on the seeded MHA plates and Honey discs were prepared by using dry sterile filter papers (having the same thickness and size, 6 mm, as the antibiotic discs) immersed in the different undiluted bee honey. Each agar plate was divided by a marker pen into two halves. The antibiotic discs were plated in one-half and, on the opposite side; each antibiotic disc immersed in honey was plated opposite the same antibiotic disc. At the center of the agar, a sterile filter paper disc immersed in honey was applied (Fig. 2). Cultured plates were incubated at 37 °C for 24 h. Once all discs are in place, the plates were inverted and placed in a 35 °C air incubator for 16 to 18 hours. When testing staphylococcus against oxacillin or vancomycin, incubation for a full 24 h was done before reading 19. The plates were done in triplicates. After incubation, the zone diameters were measured to the nearest millimeter using a ruler and the mean diameter was calculated for each sample.

Scanning electron microscopy: Trials to assess the mode of action of honey were carried out. Scanning electron microscope photos were taken to monitor any change on the morphology of the bacteria after addition of honey or the antibiotic or their combination. Scanning electron microscopy (SEM) analysis was performed on both MRSA and PA under test.

Overnight bacterial cultures of MRSA or PA (diluted to obtain 10⁶ cfu/mL) were further incubated for 24 h with either vancomycin alone or vancomycin with SS for MRSA isolate and either ciprofloxacin alone or ciprofloxacin with MMH for PA isolate. Primary fixation of samples was done by buffered Guinealdehyde 2.5 % over night in refrigerator, washed by phosphate buffer (pH = 7) and centrifuged (3000 g, 15 min, 4 °C). Secondary fixation was done by buffered Osmium Tetroxide 1 % for one hour, then dehydation by serial concentration of ethanol, embedding by resin mixture from SPI (SPI-Pom™- Araldite® Epoxy Embedding Kit). The bacterial pellets were mounted on membrane filters (Anodisc; Whatman International Ltd, Maidstone, UK). Before examination under a scanning electron microscope (JEOL, JSM-6060 LV), specimens were coated with 100 Å of a gold–palladium mix in an ion sputter (JEOL JFC 1100) 20.

Crystal violet assay: Alteration in cell wall permeability was further detected by crystal violet assay 21. Suspensions of MRSA and PA were prepared in Brain Heart Infusion broth (BHI) (Oxoid, UK). Cells were harvested at 4500 g for 5 min at 4 °C. The cells were washed twice and resuspended in PBS (pH 7.4). SSS and YSH was added to the cell suspension and incubated at 37 °C for 30 min. Control samples were prepared similarly without treatment. The cells were harvested at 9300 g for 5 min. After that, the cells were resuspended in PBS (pH 7.4) containing 10 µg/mL of crystal violet. The cell suspension was then incubated for 10 min at 37 °C. The suspension was then centrifuged at 13,400 g for 15 min and the OD 590 of the supernatant was measured in HITACHI UV–VIS spectrophotometer.

The OD value of the crystal violet solution, which was originally used in the assay, was taken and it was considered as 100%. The percentage of crystal violet uptake of all the samples was calculated using the following equation:

\[
\text{OD value of the sample} = \text{OD value of crystal violet solution × 100%}
\]

Loss of 260nm absorbing material: Concentrations of the released UV-absorbing material were measured by UV–VIS spectrophotometer 22. Overnight broth cultures of MRSA and PA in Brain Heart Infusion medium were adjusted to OD 600 of 2.0. Cells were harvested by centrifugation at 400 xg for 15 min, the supernatant was discarded and pellet was washed twice and then resuspended in PBS (pH 7.4). SSS or YSH were added to the bacterial cell suspension. The experiment was done in triplicates. Cells without honey treatment were used as control. All the samples were incubated at 37 °C for 60 min. After treatment, the cell suspension was centrifuged at 13,400 xg for 15 min and OD 260 value of the
supernatant was taken as a percentage of the extracellular UV-absorbing materials released by cells. All the measurements were done using Nano drop 2000c.

**Analysis of cell wall and cytoplasmic membrane associated proteins:** To verify the cell wall damage, a subcellular fractionation method was followed to determine the amount of proteins released due to honey treatment that were present in cell wall or associated with cytoplasmic membranes. MRSA and PA bacterial culture were grown to OD 600 of 2.0 and 1mL of culture was taken in a series of centrifuge tubes. SSH and YSH honey was added to the cell suspensions and the samples were incubated at 37°C for 60 min. After treatment, bacteria were then spun down at (15000 g, 15 min, 4°C), washed and suspended in PBS pH 7.4. The cells were then disrupted by sonication. Unbroken cells were removed by low-speed centrifugation (5000 g, 5 min, 4°C). The supernatant was centrifuged at high speed (200000 g, 20 min, 4°C) to obtain a pellet containing the membrane material, which was then treated with 0.4% Triton X-100 (3 h, 4°C) and centrifuged at 15000 g, 30 min, 4°C in a microtube. The 0.4% Triton X-100 soluble material corresponded to the fraction enriched in cytoplasmic membrane proteins. The insoluble pellet was suspended in PBS pH 7.4. It corresponded to the fraction enriched in cell wall proteins. Control samples were prepared similarly without treatment. The readings were done using Nano drop 2000c by A205 Custom Method for Protein and Peptide Quantification.

**RESULTS**

Table 1 shows the mean inhibition zone diameters of different types of honey locally available in Saudi Arabia against different pathogenic bacteria isolated from wounds. It is clear from the table that Sidr honey, either from Yemen source or from Southern source showed effective inhibition to all types of bacterial isolates. In contrast, Kashmir Sidr honey had almost no activity against any of the strains tested. The Southern Sidr honey showed the most potent effect against both Gram-positive and Gram-negative bacteria. Noticeably, multiflower honey obtained commercially showed potent effect against MRSA and PA but less effect against MSSA. Only clover flower honey, cotton flower honey and southern date honey showed no effect on any of the strains tested either Gram-positive or Gram-negative.

The mean diameter of inhibition zones of the antibiotics tested either alone or in combination with different types of honey against MSSA, MRSA and PA are demonstrated in figure 1. The choice of the antibiotic and its combination with a honey type was done based on the combination that gave the largest inhibition zone.

Figure 1A clearly demonstrates that almost all combinations tested against MSSA were synergistic, though to different degrees. Both vancomycin and amoxicillin/clavulanic acid combinations exhibited the most prominent results giving a zone diameter difference of about 7 mm in both antibiotics compared to the effect of the antibiotic alone. Gentamycin combination with the selected types of honey showed slightly better results than linezolid combinations. MSSA isolates were slightly affected by gentamycin in combination with Southern Sidr honey and Al-Madinah honey, but this effect was further decreased with Cotton Flower honey, Yemen Sidr honey and Hungarian honey. Moreover, linezolid had very little effect with all types of honey and no synergistic effect with Yemen Sidr honey.

MRSA isolates gave different results as shown in Figure 1B. The isolates were not much affected by the antibiotic honey combination. Although vancomycin showed the best combination with honey against the strains tested, it only resulted in 2-3 mm difference in zone diameter with respect to all types of tested honey compared to applying vancomycin discs alone. No change in zone diameter was noticed with cotton flower honey with gentamycin against MRSA. On the other hand, using amoxicillin/clavulanic, gentamycin or linezolid showed little antagonism if combined with honey, whatever its type is, against MRSA.

Figure 1C shows the effect of different antibiotics on Pseudomonas isolates. Ciprofloxacin had a great effect on PA isolates when combined with Al-Madinah honey giving about 5 mm difference in inhibition zone diameter compared to applying ciprofloxacin discs alone. Although ciprofloxacin was less effective with Yemen Sidr honey, Yemen Thorn honey and Multi-flower Mountain honey, still its combination with the respective honey kind was considered synergistic. However, ciprofloxacin seemed to have negligible effect when added to Southern Sidr honey.

Furthermore, sulfamethoxazole/trimethoprim had considerable synergistic killing effect when combined with Southern Sidr honey and Multi-flower Mountain honey, while it was less effective with Yemen Sidr honey and Al-Madinah honey. Only ceftriaxone showed antagonistic effect with all the tested honey types.

Figure 2a and 2b are representative examples of the work done through this study where Fig. 2a shows the effect of Hungarian honey on MSSA where there was absolutely no zone of inhibition formed. By combining vancomycin with the Hungarian honey, the diameter of inhibition zone increased by 7 mm. Figure 2b showed the same previous techniques but against PA where the combination of ciprofloxacin and multi-flower mountain honey gave a zone of inhibition of 39 mm compared to 12 and 34 mm for MMH and ciprofloxacin, respectively.

MRSA strains treated with SSH alone, treated with vancomycin or their combination were photographed by electron microscopy to compare morphological alterations (Figure 3). For each combination, the most representative photograph was chosen even if morphologically normal organisms were also observed. The scanning electron microscope appearance of untreated MRSA is shown in Figure 3a. The bacteria were roughly spherical and smooth with grape like arrangement. Upon overnight treatment of MRSA cells with SSH the cells were deformed, the cells became enlarged and swollen. Moreover, cell destruction was observed and abnormal forms were visible (Figure 3b). Vancomycin had a profound effect on the morphological structure of bacteria. The bacterial lysis was observed for most of the cells probably because of alterations of the cell wall and inhibition of cell division. This is due to its inhibitory effect of cell wall synthesis by blocking glycopeptides polymerization through binding tightly to D-alanyl-D-alanine portion of cell wall precursor. (Figure 3c). The combination of SSH with vancomycin showed abnormal forms with separation of the central septum and bacterial lysis was also observed (Figure 3d).

SEM photos of PA strains untreated and treated with MMH was shown in figure 4. The images reveal that the normal bacterial morphology was thin short bacilli (Figure 4a). Upon treatment with MMH, some alterations in the morphology of the bacilli is clear in Figure 4b where the cells seemed to clump together before they start to swell making some of the cells appear semicircular and other still retaining their bacilli morphology while others appear as swollen cocci (Figure 4b). Ciprofloxacin inhibits bacterial growth by acting internally and promotes breakage of double-stranded DNA. So, bacterial cell wall was not disrupted upon treatment with ciprofloxacin though the cells appear hollow (Figure 4c). Similar effect but more aggressive was observed in the combination of MMH with ciprofloxacin where bacterial cells were completely fused and destructed (Figure 4d).

In order to investigate the main mechanism of honey inhibitory effect, the role of pH of different honey types and its influence on the
inhibition of different isolates had to be measured. Figure 5 is graphic representation of the pH values of the eleven different kinds of honey under research. It is noticeable from the figure that SSH, which is the most effective honey throughout our work had the highest pH (5.73) followed by the YSH and KSH. The range of pH values for YTH, MMH, SDH and CTH were between 4 and 5. On the other hand, CLH, HH, AMH, and NSH had the lowest pH (less than 3).

Since YSH and SSH had the most potent bactericidal effects on the isolates tested therefore, they were chosen to assess the mechanism of action that honey has on the bacterial strains.

The uptake of Crystal violet dye by the bacterial cells detects any alteration in the bacterial cell wall permeability, hence tried. The uptake of crystal violet by MRSA strain was 36.4% in the absence of honey treatment. This uptake was increased a little by the application of YSH where it reached 45.2% while SSH treatment increased crystal violet uptake into the cells to 58.4% (Figure 6). On the other hand, treatment of PA with the same two types of honey caused a value of 74% and 92.4% uptake of YSH and SSH, respectively, compared to only 43.4% uptake of the dye by the untreated strain.

The Effect of honey on leakage of 260nm absorbing materials from MRSA strain and PA strain is represented here in Figure 7. The measurement of release of UV-absorbing materials is an index of cell lysis after treatment with different types of honey (SSH and YSH). In the case of the MRSA strain tested against SSH, the OD only increased from 2.0 to 2.7 (Figure 7a) while there was no elevation in the OD by YSH treatment against the same isolate. However, a prominent effect was observed considering the other tested Gram negative isolate PA, where the treatment with YSH and SSH caused an OD reading of 1.2 and 1.7, respectively, compared to only 0.4 reading to the untreated control strain. These results supported the idea that honey damages cytoplasmic membrane and causes subsequent leakage of intracellular constituents.

Figure 8 a and b shows the induced cell wall disruption and release of intracellular proteins before and after honey treatment in both Gram positive and Gram negative strain tested. Figure 8 a, proves that proteins were released due to disruption in cell wall in the supernatants of MRSA by both YSH and SSH treatment though to a different extent. The figure clearly demonstrates that upon mixing the MRSA strain with honey, an increase in membrane protein release by 0.550 and 0.640 mg/ml than the control for SSH and YSH, respectively. On the other hand, a prominent amount of cell wall proteins released (3.167 mg/ml) was noticed upon SSH treatment compared to the untreated strain (1.175 mg/ml) while it was unnoticed upon treatment of the isolate with YSH.

Almost the same results were obtained in the case of the PA strain (fig 8b). The amount of the membrane proteins released after treatment with any of the honey types was higher compared to the Gram positive strain, reaching about 1 mg/ml in the case of SSH. Almost the same results were obtained in the case of the PA strain (fig 8b). The amount of the membrane proteins released after treatment with any of the honey types was higher compared to the Gram positive strain, reaching about 1 mg/ml in the case of SSH. Moreover, this difference was much higher in the case of cell wall proteins released by honey treatment compared to PA control.

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<tr>
<th>Types of Honey</th>
<th>Bacterial Strain [No. isolates]</th>
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<td>MSSA [4]</td>
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<td>MRSA [4]</td>
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<td>PA [4]</td>
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<tr>
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<td>SSH</td>
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<td>YTH</td>
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<td>SDH</td>
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<td>KSH</td>
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<tr>
<td>MMH</td>
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Table 1: Mean inhibition zone diameters (in mm) of different types of honey against MSSA, MRSA and PA

Figure 8 a and b shows the induced cell wall disruption and release of intracellular proteins before and after honey treatment in both Gram positive and Gram negative strain tested. Figure 8 a, proves that proteins were released due to disruption in cell wall in the supernatants of MRSA by both YSH and SSH treatment though to a different extent. The figure clearly demonstrates that upon mixing the MRSA strain with honey, an increase in membrane protein release by 0.550 and 0.640 mg/ml than the control for SSH and YSH, respectively. On the other hand, a prominent amount of cell wall proteins released (3.167 mg/ml) was noticed upon SSH treatment compared to the untreated strain (1.175 mg/ml) while it was unnoticed upon treatment of the isolate with YSH.
Figure 1. Mean diameter of inhibition zones of antibiotics alone (blue) and its combination with different types of honey (yellow) against (A) MSSA, (B) MRSA, (C) PA.

Figure 2: Bacterial isolates against honey alone, antibiotic alone and its combination.
Figure 3. Scanning electron micrographs of MRSA strains, (a) Untreated MRSA, (b) treated with Southern Sidr Honey; (c) treated with vancomycin and (d) their combination.

Figure 4. Scanning electron micrographs of PA strains, (a) Untreated PA, (b) treated with Multi-Flower Mountain Honey, (c) treated with ciprofloxacin, and (d) their combination.
Figure 5. pH values of the different tested types of honey

Figure 6. Crystal violet uptake of honey (YSH and SSH) treated MRSA (dotted) and PA (Black).
Figure 7. Presence of 260nm absorbing material in the supernatants of MRSA (A) or PA (B) strain untreated with honey (control), treated with YSH or SSH.

Figure 8. Quantitative measurement of cell wall proteins (orange) and membrane proteins (blue) of MRSA strain (A) and PA strain (B) treated with honey (YSH and SSH) done by A205 Custom Method for Protein and Peptide Quantification method.


DISCUSSION

Despite recent advances in antimicrobial chemotherapy and wound management, infection continues to be an important problem in the treatment of wounds. A variety of topical agents as well as systemic agents has been used, but none has completely eliminated the problem of infection 27. Looking for a novel method to inhibit microbial growth has been an active area of research. An ideal agent would be one that is cost-effective and free of toxicity and allergy. Honey as a natural product is an agent that meets all these criteria. Honey is known to be a cheap, readily accessible and non-toxic remedy with significant antimicrobial effect on both Gram negative and Gram-positive bacteria especially *P. aeruginosa* and MRSA, which are considered the commonest pathogens affecting wounds. Moreover, honey does not affect human tissue unlike some other topical antimicrobial agents 14, 15.

In this study we tried some types of honeys available in the Saudi market and of different floral origin and compare them to a commercial Multi-Flower Mountain Honey MMH, with the aim to evaluate whether and to what extent they were able to reduce bacterial proliferation. *Pseudomonas aeruginosa* and *S. aureus* were used as test microorganisms to determine the antibacterial activity of the honeys. They were selected as representative of Gram-negative and Gram-positive wound pathogens.

The present work showed that there is a considerable variation in antimicrobial activity of each honey type on different bacterial strains (Table 1). These results were in accordance with findings of Stefan B. who stated that differences in antibacterial activity of unifloral honeys had been reported 25. Furthermore, Molan and Cooper reported that the difference in antimicrobial potency among the different honeys could be more than 100-fold, depending on its geographical, seasonal and botanical source 29.

Comparing natural honeys with commercial ones, Lusby et al. 8 mentioned that honeys other than the commercial available antibacterial honey could have equivalent antibacterial activity against bacterial pathogens. In addition, Deb Mandal and Mandal 1 stated that locally produced honey possess excellent antibacterial activity comparable to the commercial honeys. In our study, we tested ten medical grade honeys and one commercial grade and the results showed that commercial one; MMH, was among the best types of tested honeys. This commercial one has a potent killing effect on all bacterial strains (mean inhibition zone diameter is 13 ± 2.24 mm for Gram-positive and 11 ± 2.38 mm for Gram-negative) when applied alone, while CLH, CTH, and SDH; which are medical types, have 0 ± 0 mm mean diameter of inhibition for all strains. These variations between their results and ours, regarding the commercial honey, may be attributed to difference in harvesting, processing and storage conditions 29.

Gram-positive bacteria, including *S. aureus* affect burn wounds during the first week following a trauma. Colonization with *S. aureus* is often associated with delayed wound healing, an increase in the need for surgical interventions and prolonged stay in the burns center 30.

Abd-El Aal et al. 27 mentioned that the mean inhibition zone of honey on MRSA was significantly higher than that of VA, but less effective than AMC. They observed that on the addition of honey to the antibiotic discs, there was a synergistic effect with all antibiotics. Our study of the isolated MRSA revealed that SSS (15 ± 2.83 mm; which is the most effective medical type on MRSA, was less effective than VA or AMC; 16 mm or 25 mm, respectively. Synergistic effect of honey-antibiotic combinations are in agreement with VA; except for CTH, but honey-AMC combinations showed mainly antagonistic effects on MRSA although sometimes have additive effect with AMC alone.

*Pseudomonas aeruginosa* has long been recognized as a major burn pathogen. It has increased its presence not only in burns but also in other forms of trauma. Of all the Gram-negative aerobic rods, *Pseudomonas* species are the most repeatedly encountered and are chronic or acute 31. Resistance to the commonly used systemic antibiotics, especially betalactams and aminoglycosides, is a major problem. The effectiveness of multiple antibiotics had recently been observed to be decreasing 32. As the understanding of the mechanisms of resistance and the rapidity of development of *Pseudomonas* has increased, it has generally been accepted that serious *Pseudomonas* infections should be treated with two antibiotics that act with two different action mechanisms that are synergistic in their activity 33. To decrease side effects and toxicity of multidrug therapy, it is preferable to use antibiotic-natural product combination. Among the best natural product used in medical field is honey, as the resistance to it has never been reported, which makes it a very promising topical antimicrobial agent against the infection of antibiotic-resistant bacteria 1 as proved in our study.

When we applied different eleven types of honey to Gram-negative bacteria, we found that SSH, the most effective type had mean diameter zone (18 ± 3.44 mm) less than CIP (34 mm), while higher than SXT and CRO that exhibited 8 and 17 mm inhibition zone, respectively. Testing different combinations of honey types with CIP showed variable synergistic effect varying from highly potent like AMH to no effect like SSH. While CRO displayed antagonistic effect with all tested honey types. The above observations indicated that some types of honey like YSH, SSH, and MMH had a more inhibitory effect on isolated Gram-negative bacilli than SXT, but are less effective than CIP or CRO. With the addition of honey to antibiotics, there were some synergistic and slightly antagonistic effects. These findings are in contrast to Abd-El Aal et al. 7 who further reported that the mean inhibition zone (in mm) of honey when applied on isolated Gram-negative bacteria was significantly higher than that of CRO and non-significantly higher than that of CIP and there was a significant increase when honey was added to antibiotic discs of CIP and CRO. With the addition of honey to antibiotics, there were some synergistic and slightly antagonistic effects.

The effect of honey on Gram-negative bacteria was explained by Taormina et al. who attributed it to the presence of hydrogen peroxide and powerful antioxidants in bee honey, to a naturally low pH, which is unsuitable for bacterial growth and to the presence of phenolic acids, lysozyme and flavonoids 34.

Hanyeh et al. 35 reported that Honey is characteristically acidic with pH between 3.2 and 4.5, which is low enough to be inhibitory to several bacterial pathogens and they further stated that the minimum pH values for growth of *P. aeruginosa* (4.4), *S. aureus* (4.5) and thus the acidity is a significant antibacterial factor. On the other hand, Stefan B. 36 reported that antibacterial activity correlated significantly with free and total acidity but did not correlate with honey pH. He concluded that although honey acids; which have a bee origin, exert the main antibacterial action, honey pH could additionally act as an antibacterial factor. Our results match the last conclusions where, the most effective honey types on Gram-positive and negative strains; YSH, SSH, and MMH, have pH ranged from 4.33 to 5.73. Although pH value of KSH was 5.11, it had limited activity on sensitive strains of Gram-positive cocci. This contradicts the relationship between antibacterial activity and honey pH (Figure 5).

Den Mandal and Mandal 1 reported that antibacterial property of honey is derived from the osmotic effect of its high sugar content and low moisture content. According to their study, bacterial cell should shrink due to water loss. The changes in bacterial surface morphology and cell damage were studied by SEM 30. SEM images demonstrated that Gram-positive and Negative bacterial cells got swollen significantly with the two different types of honey we tested,
indicating that killing effect of honey is not due to osmolality (Figure 3b & 4b).

SEM examination revealed that rough surface morphology and swelling of cell was apparent in the cells treated with honey, when compared to the untreated ones. Loss of membrane integrity and damaged cell surface further supports the evidence that the mode of bactericidal action of honey against either Staphylococcus aureus or Pseudomonas aeruginosa is through membrane disruption and further blocking the cell growth. Since there has been a limited study on the mechanism of action of honey against wound pathogen like MRSA and PA, the present study validates the mode of action of honey on the cell wall of both isolates under test.

For understanding the mechanism of action of honey, the ability of honey to damage the cell wall of both MRSA and PA strains were evaluated by crystal violet assay, release of UV absorbing materials and membrane protein quantification upon treatment with YSH and SSH.

The effect of honey on cell wall permeability was evidenced by the uptake of the crystal violet dye. Generally, crystal violet penetrates the cell wall poorly, but it easily enters when the membrane is defective. A significant enhancement in the uptake of crystal violet was observed in both MRSA and PA, though to a different extent, treated with either YSH or SSH when compared to control cells. This shows that honey alters membrane permeability and makes the cells hyperpermeable to solutes, which are generally less permeable.

Release of UV-absorbing materials is an index of cell lysis and nonselective pore formation. Leakage of intracellular components suggests that the crucial effect of honey on both strains could be the formation of pores in the plasma membrane. Moreover, release of intracellular proteins is another hallmark for the cell wall damage and loss of membrane integrity.

CONCLUSION

It is definitely worthy to consider honey as a promising future antibiotic to be tested and studied. Rediscovering honey as a natural remedy for wound pathogens proved its effectiveness on antibiotic resistant strains of bacteria including MRSA and PA. In this research, we tried to focus more on whether honey when combined with the antibiotic has synergistic, antagonistic, additive effect on the isolates under study and we made some assumptions and conclusions of the possible mechanism of action that the honey could have exerted on them. Certain tests were conducted for that purpose as well, and although they did not specify the exact mechanism of it, they helped in ruling out some of the mechanisms that are most commonly attributed to honey, leaving this field open for other possible options to be further studied.

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Cite this article as:

Source of support: Nil, Conflict of interest: None Declared

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