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260

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RESEARCH ARTICLE

Antibacterial Activity of Honey against Methicillin-Resistant and Sensitive *Staphylococcus Aureus* Isolated from Patients with Diabetic Foot Ulcer

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Abstract:

Introduction:

Staphylococcus aureus is the most important causative agent of wound infections, including diabetic foot ulcers. Honey is a very useful nutrient with antimicrobial properties and other biological properties such as antitumor, anti-inflammatory, antioxidant and antiviral properties. The aim was to examine the antibacterial activity of honey against methicillin-resistant and sensitive *S. aureus* (MRSA and MSSA) isolated from patients with diabetic foot ulcers.

Methods:

This cross-sectional study was performed from January 2019 to December 2019. Twenty *S. aureus* isolates were collected from patients with diabetic foot ulcers. Different concentrations (100%, 70%, 50%, 25% vol/vol) of honey were studied. Dilutions of honey solutions were examined to determine the minimum inhibitory concentration (MIC) against *S. aureus*. MICs were determined by spectrophotometric assay at 620 nm.

Results:

All strains showed sensitivity to honey with MIC equal to 25% (vol/vol). The MIC (%) values of honey for all studied *S. aureus (*MRSA and MSSA) isolates ranged between 18-100% (v/v).

Conclusion:

Honey with confirmed, antibacterial activity has the potential to be an efficient treatment complementary for diabetic foot ulcers infected or at risk of infection with *S. aureus.*

Keyword: *Staphylococcus aureus*, Honey, Diabetic foot ulcers, Antibacterial activity, Antitumor, Anti-inflammatory, Antioxidant, Antiviral.

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1. INTRODUCTION

Diabetic foot infection is a major problem in the care of diabetic patients that results in amputation due to severe infection or peripheral ischemia in the leg [1]. These non-healing wounds state a remarkable risk of sepsis and can result in invasive inflammatory diseases such as infective endocarditis, which is related to high mortality and morbidity [2]. For suitable treatment of diabetic foot ulcers, we first should comprehend the microbiology of this type of infection. Wound infection is usually caused by a number of bacteria, including aerobic, optional anaerobes and anaerobes [3]. *Staphylococcus aureus* is the most important causative agent of wound infections, including diabetic foot ulcers [4 - 6]. This bacterium is one of the most common causes of blood and skin infections, ulcers, osteomyelitis, endocarditis, pneumonia, surgical infections and hospital infections [7]. Honey is the natural sweet substance obtained from the secretions of the living parts or excretions of plants that the honey bees (*Apis mellifera*) collect and store [8]. It contains 15% to 20% water and 80% to

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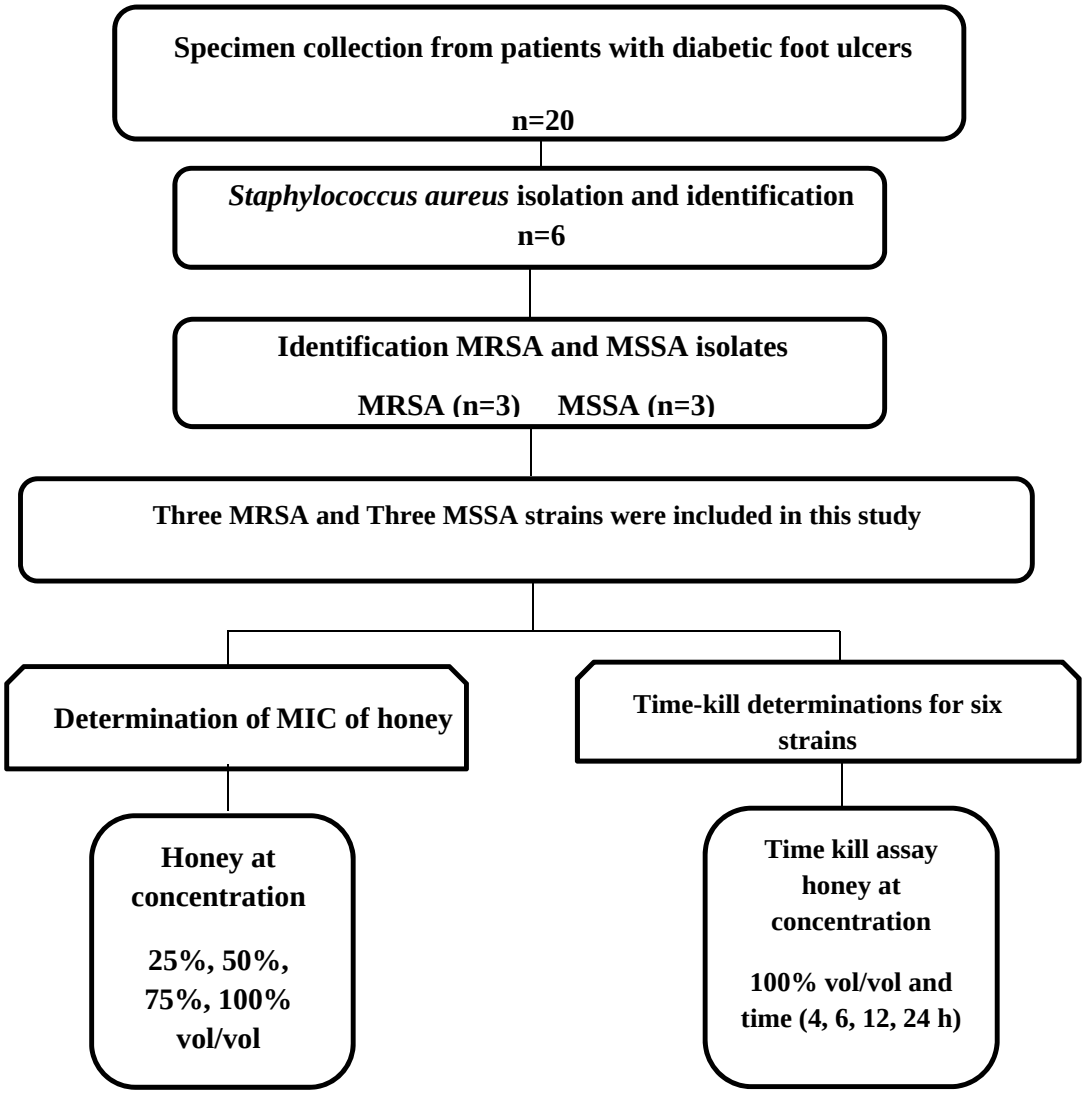

Fig. (1). Flowchart of the experimental steps.

85% sugar [9]. Several properties of honey-like enzymes are responsible for its bactericidal effect and wound healing properties. Glucose oxidase, which changes glucose into gluconolactone and then into hydrogen peroxide, is one of the enzymes found in honey. The release of H2O2 is slow and continuous for a constant antibacterial effect, successfully eliminating microorganisms but dilute enough not to damage host tissue. Honey having acidic pH of 3.2–4.5 is used to inhibit many pathogenic organisms and increase the wound healing process through epithelization. Honey is also one of the supersaturated solutions that inhibit bacterial growth due to this high osmolarity [10 - 15]. Naturally-derived compounds like honey have gained popularity as a substitute to antimicrobial compounds [16, 17]. Honey is a very useful nutrient with antimicrobial properties and other biological properties such as antitumor, anti-inflammatory activity, antioxidant and antiviral activity [18]. Honey wound gels and dressings have been approved by health authorities and are accessible to health professionals in several countries. It is useful to the topical treatment of infected chronic wounds [19]. Various studies showed that honey has broad-spectrum antibacterial activity against a wide range of gram-positive and gram-negative bacteria. The antibacterial effect of honey can be due to the inhibitory properties of factors such as peroxidases, flavonoids, and phenolic acids. Also, honey has an osmotic effect that disrupts the growth of microorganisms [20, 21]. According to studies, several ingredients have been identified that contribute to its antimicrobial activity, comprising high sugar content, low pH, low water activity, and the formation of hydrogen peroxide [22, 23]. Due to the fact that diabetic foot ulcer is one of the health problems in diabetic patients and considering the antibiotic properties of honey, the aim of the study was to investigate the antibacterial activity of honey against *S. aureus*-resistant and sensitive (MRSA, MSSA) isolated from diabetic foot ulcers.

2. MATERIALS AND METHODS

2.1. Bacterial Isolation and Identification

This cross-sectional study of wound swab versus tissue

sampling in infected diabetic foot ulcers was performed from January 2019 to December 2019 (Fig. **1**). This study was approved by the Ethical Committee (license number: 5572/9/35/17/p) of the Hamadan University of Medical Sciences. Twenty specimen from patients with diabetic foot ulcers were collected. *S. aureus* isolates were identified using the biochemical and polymerase chain reaction (PCR) tests [7].

2.2. Identification of Methicillin-Resistant *S. aureus*

All S. *aureus* isolates for which the MIC of cefoxitin was ≤8 µg/ml were classified as MRSA. Methicillin resistance was identified by the presence of the *mec A* gene by PCR, as explained previously [7]. *S. aureus* isolates were tested for the presence of the 310 base pair (bp) PCR product of *mec A* gene, using the following primers: forward (5′- GTAGAAATGACT GAACGTCCGATAA-3′) and reverse (5′- CCAATTC CACATTGTTTCGGTCTAA -3′). *S. aureus* ATCC 25923 was included as a positive control.

2.3. Preparation of Honey Solutions

Honey (100% purity) (Iran, Nahavand city), with yellowish-brown color, was used in the study. The honey solution was prepared immediately prior to testing by diluting honey to the required concentrations (25%, 50%, 75%, 100% vol/vol).

2.4. The Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) of honey was determined by agar dilution methods [16]. Honey was weighed and dissolved in sterile deionized water to prepare a stock solution of 25%, 50%, 75%, and 100% (vol/vol) honey immediately before use.

Up to 0.4 ml of the cell suspension was inoculated into 6 ml volume of honey concentration in a test tube while inoculation of 6 ml volume of nutrient broth with 0.4 ml of the cell suspension served as control. The optical density (OD) was determined in a spectrophotometer (UV/VIS, USA) at 620 nm prior to incubation (T) and recorded after which the cultures were incubated for 24 hours in the dark at 37 ºC with constant shaking to prevent adherence and clumping. After 24 hours of incubation, the optical densities were again determined (T_{24}) and recorded. The OD for each replicate at T was subtracted from the OD for each replicate using the formula:

Percentage inhibition= 1- (OD test/OD control) ×100

Where the resulting measurement recorded a negative inhibition value (growth promotion), this was reported as stimulation using the formula:

Percentage inhibition= (OD test/OD control) ×100

The minimum and maximum values were 0% and 100%, respectively [16].

2.5. Statistical Analysis

Statistical analysis was performed using SPSS 23.0 (SPSS, Chicago, IL, USA). To compare categorical variables, chi-square or Fisher's exact tests were performed. All of the analyses were performed with a confidence level of 95%. P values < 0.05 were considered statistically significant.

3. RESULTS

A total of six strains of *S. aureus* isolated from patients with diabetic foot ulcers were studied over a period of 3 months. Based on the result of polymerase chain reaction (PCR) and disc diffusion agar, three strains with resistance to cefoxitin and *mec A*+ and three strains sensitive to cefoxitin and *mec A*- were selected for further study (Fig. **2**).

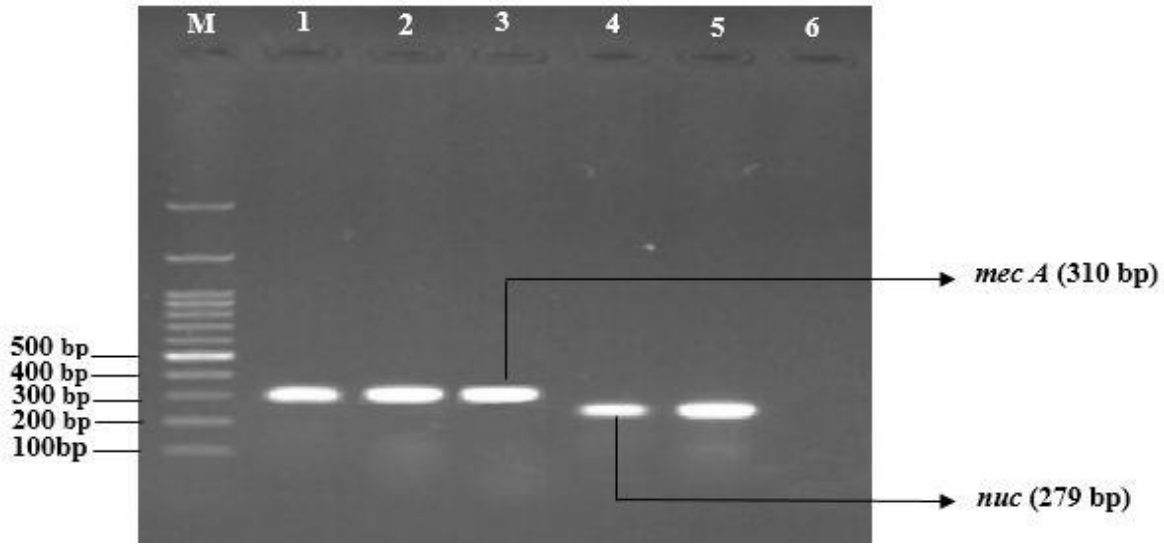

Fig. (2). Agarose gel electrophoresis of *mec A* and *nuc* genes in *S. aureus* isolates. **Lane 1-3:***mec A* gene in clinical isolates of *S. aureus;***Lane 3-4:***nuc* gene in clinical isolates of *S. aureus*; **Lane 6:** Negative control**; M:** DNA marker (100 bp).

Table 1. The time-kill assay for the antibacterial effect of 100% honey on *S. aureus* isolates.

Strains	4h Log_{10} CFU/ml×10^5	6h Log_{10} CFU/ml×10^5	12h Log_{10} CFU/ml×10^5	24h Log_{10} CFU/ml×10^5
0	5	5	5	5
MRSA-S1	1.2	2.1	3.5	4.2
MRSA-S2	1	2.2	3.9	4
MRSA-S3	1.3	2.4	3.2	4.5
MSSA-S1	1.9	2.8	3.9	4.1
MSSA-S2	2	2.5	3.6	4.8
MSSA-S3	1.8	2.9	3.9	4.9
Strains	4h Log_{10} CFU/ml×10^5	6h Log_{10} CFU/ml×10^5	12h Log_{10} CFU/ml×10^5	24h Log_{10} CFU/ml×10^5
0	5	5	5	5
MRSA-S1	1.2	2.1	3.5	4.2
MRSA-S2	1	2.2	3.9	4
MRSA-S3	1.3	2.4	3.2	4.5
MSSA-S1	1.9	2.8	3.9	4.1
MSSA-S2	2	2.5	3.6	4.8
MSSA-S3	1.8	2.9	3.9	4.9

Table 2. Antibacterial effect of MIC% (vol/vol) of honey on *S. aureus* isolated from patients with diabetic foot ulcers.

Concentration of honey (vol/vol)	Spectrophotometry MIC% (vol/vol)					
	MRSA-S1	**MRSA-S2**	**MRSA-S3**	**MSSA-S1**	**MSSA-S2**	**MSSA-S3**
25%	23	18	22	45	35	40
50%	30	28	34	55	50	52
70%	55	60	52	68	70	75
100%	85	78	75	95	100	100

The time-kill assay for the bactericidal effect was carried out on six isolates of *S. aureus*. The results showed that with increasing time, more bacteria were killed (Table **1**).

The effects of honey on all the isolates of *S. aureus* isolated from patients with diabetic foot ulcers were studied by the determination of MIC, indicating the highest dilution of honey in the culture medium, which inhibited the growth of *S. aureus* isolates. Antibacterial activity of honey was observed in four concentrations i.e, 25% (vol/vol), 50% (vol/vol), 70% (vol/vol), and 100% (vol/vol); the properties are shown in Table **2**. All the strains showed sensitivity to honey with MIC equal to 25% (vol/vol). The MIC (%) values of honey for all the studied *S. aureus (*MRSA and MSSA) isolates ranged between 18-100% (v/v). The results showed that with increasing the concentration of honey, the MIC (%) value increased (Table **2**).

4. DISCUSSION

Due to the increase in antibiotic resistance and the decrease in the development of newer antimicrobial agents, the search for new antibiotics with a natural source can provide a new method for the treatment of drug-resistant infections. These natural antibiotics can be called plant compounds and natural ingredients such as honey [24]. In the antibacterial studies of honey, the lowest concentration of honey that inhibited the growth of bacteria was determined in the range of 20-90%. The antibacterial effect of honey has been found to be concentration-dependent and the bactericidal effect was observed at a concentration of 20% or more for *S.aureus* isolates tested. There are several laboratory studies that have shown the efficiency of medical use of honey on MRSA and *P. aeruginosa* [25, 26]. Shenoy *et al.*, evaluated the antimicrobial activity of honey agent *P. aeruginosa.* Their results showed that all the isolates of *P. aeruginosa* tested were killed in 12-24 h [27]. In another study, Moussa *et al.*, assessed the antimicrobial activity of honey against *S. aureus*. Their findings showed that the MIC (%) ranged from 12%-95% for *S. aureus*. The findings were similar to the current study [24]. Nzeako *et al.*, and Iurlina *et al.*, in their research works showed that honey can inhibit the growth of *S. aureus* [28, 29]. In addition, its antimicrobial properties have been approved in various studies. Honey can clear infections in a number of ways; *in vivo*, through anti-inflammatory activity, by promoting the immune system, through the osmotic effect and antioxidant activities as well as through the stimulation of cell growth, enhancing the rate of healing [30, 31]. Cooper *et al.*, evaluated the antibacterial activity of honey against *S. aureus*. Their results showed the sensitivity of the isolates to honey: the MIC values observed were all between 2 and 3% for honey [32]. In the current study, the honey inhibited *S. aureus* completely at much greater dilution. This is due to the fact its mode of action is not exclusively based on its osmolality. Cooper *et al.*, reported that the strains of *P. aeruginosa* were

observed to be inhibited at a concentration of 4%–9% v/v (32). In the present study, *S. aureus* has been shown to be inhibited by relatively low concentrations of honey. Henriques *et al.*, in a recent study showed that the growth of *S. aureus* species isolated from wounds was inhibited by honey [33]. In recent years, *in vitro* studies have been designed to investigate the mode of action of honey, and have confirmed that cell division in MSSA and MRSA is interrupted by exposure to honey [34, 35]. Cooper *et al.*, evaluated the antibacterial activity of honey against Gram-positive cocci of clinically isolates of wounds. Their finding showed that for all of the strains tested, the MIC values against honey were below 10% (v/v), which confirmed the results of this current study [36]. The findings of this study show that honey is an effective wound antiseptic, with broad-spectrum antimicrobial activity. George *et al.*, investigated antibacterial effects of honey against clinical isolates of MRSA; the mean MIC values of 4% v/v was obtained for *S aureus* ATCC 25923. All the test strains of MRSA were observed to be inhibited at a concentration of 4% v/v irrespective of antimicrobial phenotype [22]. Unlike the use of antibiotics in treating wounds, in vitro assessment of sensitivity to honey would not be required before the beginning of treatment. Furthermore, honey does not have an adverse affect on human tissues, unlike other topical antimicrobial agents. It not only has the potential to limit the growth of wound pathogens, but there is evidence that honey has the potential to promote healing. No other antimicrobial agent possesses these characteristics.

CONCLUSION

Honey with confirmed antibacterial activity has the potential to be an efficient treatment complementary for diabetic foot ulcers infected or at risk of infection with *S. aureus.*

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures were approved by Ethical Committee (license number: 5572/9/35/17/p) of Hamadan University of Medical Sciences, Hamadan, Iran.

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013.

CONSENT FOR PUBLICATION

Informed consent was received from all participants.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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