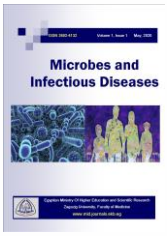
Microbes and Infectious Diseases 2025; 6(3): 3041-3050



Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Antibacterial activity of different types of honey on *Staphylococcus aureus* isolates

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ARTICLE INFO

Article history:
Received 22 October 2024
Received in revised form 12 November 2024
Accepted 24 November 2024

Keywords:
Manuka
Black seed
Fennel
S. aureus
Honey

ABSTRACT

Background: People have historically utilized honey as medicine due to its unique antimicrobial properties. Overused antibiotics have reduced therapeutic effectiveness for microorganisms with various kinds of resistance. *Staphylococcus aureus* (*S. aureus*) was used to test honey's antibacterial properties. It can withstand honey's high sugar and acidity levels and still be vulnerable to hydrogen peroxide's and non-peroxide honey's inhibitory effects. **Objectives:** This study examined how successfully Manuka UMF +20, fennel, and black seed honey destroy methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) and compared the three honeys' antimicrobial efficacy. **Methods**: Twenty *S. aureus* isolates were employed in this investigation. They were tested for antimicrobial susceptibility using the Kirby-Bauer disk diffusion technique. The sensitivity to methicillin was determined using a 30 µg cefoxitin disk. Manuka UMF +20, fennel, and black seed honeys were tested for their antimicrobial activity against *S. aureus* isolates by using agar well diffusion (AWD) and agar dilution (AD) methods. **Results**: According to this work, linezolid and gentamicin were the most sensitive MRSA/MSSA antibiotics. The AWD method showed that all honey types exhibited antibacterial activity against all clinical isolates at 75% (v/v). Concentrations of 18.75% to 37.5% (v/v) honey were required to totally inhibit all clinical isolates. Manuka UMF + 20 honey had the lowest minimum inhibitory concentration (MIC) of 18.75% (v/v). While fennel and black seed honeys had higher MIC values (MIC = 37.50% (v/v)). **Conclusion**: All honeys revealed the potential to suppress the growth of both MSSA and MRSA isolates. Compared to MRSA, Manuka MUF +20 and fennel honey were more efficient against MSSA.

Introduction

Honey has a long history of therapeutic applications, with its primary use being the treatment of infectious diseases, burns, ulcers, sore throats, digital dermatitis, and eye infections [1]. Many elements, including the honeybee's metabolism, floral source, surroundings, time of year, and weather, affect the honey's physical and chemical properties, which in turn affect the honey's medicinal effectiveness [2]. Production of hydrogen peroxide (H_2O_2), bee defensin-1, low pH, high osmolarity, and several phytochemicals, especially phenolic compounds, are key components responsible for honey's antibacterial efficacy, according to several studies [3].

The harmful overuse of antibiotics has led to several forms of bacterial resistance, which limits the effectiveness of these medications against resistant microbes [4]. Research on honey and other compounds with antibacterial properties is quite interesting due to their high probability of

DOI: 10.21608/MID.2024.330337.2303
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effectiveness against certain types of bacteria, especially drug-resistant strains [5].

One example of a Gram-positive bacterium with a widespread distribution is *Staphylococcus aureus* (*S. aureus*). This bacterium is currently a leading cause of healthcare-associated illnesses [6]. In addition, since this species is present on human skin and mucous membranes, it may enter a patient's circulation via surgical incisions, direct or indirect touch with contaminated objects, medical staff, or even another patient [7]. Treatment of these infections has become even more challenging due to the recent rise in methicillin-resistant *S. aureus* (MRSA) infections [8]. Researchers most frequently use *S. aureus* to test the antibacterial activity of honeys. This is due to the fact that *S. aureus* is susceptible to both the non-peroxide inhibitory action of honey and the antimicrobial activity of H_2O_2, yet it can withstand the high sugar and acidity levels of honey [9, 10]. So, the goal of this study is to compare the efficacy of three varieties of honey (Manuka UMF +20, fennel, and black seed) and evaluate their antibacterial activity against methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA).

Material and method

This cross-sectional investigation included 20 isolates of *S. aureus* obtained from clinical specimens inside the Clinical Laboratories of Kasr AL-Ainy University Hospitals from September 2023 to June 2024. The research was performed with adherence to the Helsinki Declaration, and the protocol obtained clearance from the Research Ethical Committee of Cairo University's Faculty of Medicine (approval number: N460-2023). Due to the research being conducted on bacterial isolates, the ethical committee specifically exempted the need for informed consent. The isolates were subcultured on blood agar, nutrient agar, and mannitol salt agar media (Oxoid, UK) and then incubated aerobically at 37°C for 24 to 48 hours. The obtained bacteria were identified by colony characters, Gram staining, and standard biochemical testing [11].

Antibiotic susceptibility testing

The antimicrobial susceptibility of the Staphylococcal isolates was assessed using commercially available antibiotic discs (Himedia, India) on Mueller-Hinton agar (MHA) (Oxoid, UK), following the guidelines set by CLSI [12]. The studied antibiotic discs included Penicillin (P) at a dosage of 10 units, gentamicin (GEN) at 10µg, tetracycline (TE) at 30µg, levofloxacin (LE) at 5µg, cotrimoxazole (COT) at 25µg, erythromycin (E) at 15µg, clindamycin (CD) at 2µg, and linezolid (LZ) at 30µg. A D test was used to identify the presence of inducible clindamycin resistance. The CLSI breakpoints were utilized to examine the sensitivities [12].

Assessment of methicillin susceptibility

The susceptibility to methicillin was assessed using the Kirby-Bauer disk diffusion technique with a cefoxitin disk (Fox) containing 30 µg (Oxoid, Altrincham, UK) and interpreted according to CLSI breakpoint references. The inhibition zone diameter was measured in millimeters; a zone diameter equal to or more than the susceptible breakpoint is considered indicative of susceptibility in isolates; otherwise, the isolate is classified as resistant [12]. Hence, the twenty isolates of *S. aureus* were categorized into 10 isolates of MRSA and 10 isolates of MSSA.

Honey samples

The investigation was carried out using three distinct honey brands: fennel, black seed (obtained from apiaries planted in Egypt), and Manuka honey UMF +20 (Steens Honey, New Zealand). All the honey was stored in opaque containers, shielded from direct sunlight or any heat source.

Antimicrobial activity of honey

We tested the antibacterial activity of honey using the methods outlined by **Osés *et al.*** [13] which included agar well diffusion (AWD) and agar dilution (AD) in sequence, to determine the minimum inhibitory concentration (MIC). Fresh-daily serial honey dilutions (75%, 37.50%, 18.75%, 9.38%, and 4.69% (v/v)) that were aseptically made in sterile distilled water were used to develop the MIC assay. A honey dilution of 75% (v/v) was used for the AWD procedure. To create 75% honey solutions, 0.75 milliliters of honey will be mixed with 0.25 milliliters of sterile distilled water [13].

Agar well diffusion

A broth culture of *S. aureus*, adjusted to a 0.5 McFarland Turbidity standard, was inoculated onto MHA (Oxoid, UK) by swabbing. Next, 8 mm diameter wells were made in the agar surface using the back of a sterile blue tip. Each well was then filled with 150 µl of 75% (v/v) honey. Following 24-hour incubation at 37° C, the zones of inhibition were measured. The measurements of zone

diameters, including the diameter of the well, were documented.

Determination of minimum inhibitory concentration using AD method

The Agar dilution method was used in this experiment to determine MIC. The MIC was defined as the lowest concentration of honey at which no visible signs of *S. aureus* growth were detected.

Each honey was subjected to consecutive serial half-dilutions using four tubes filled with 10 milliliters of sterile nutrient broth (Oxoid, UK), achieving final concentrations ranging from 37.50% to 2.35% (v/v). A volume of 10 ml of each honey dilution, ranging from 75% to 4.69% [v/v], was introduced into 10 ml of sterile liquid double-strength nutrient agar at a temperature of 50 °C. A vortex mixer was used to homogenize the mixture before placing it onto plates and allowing it to harden. Each plate was then spotted with 5 µl of each isolate broth culture adjusted to 0.5 McFarland turbidity standards. A control plate with no added antimicrobial was made and inoculated to guarantee sufficient growth of the isolates. Plates were incubated at 37 ◦C for a duration of 24 hours.

Controlling quality of data

The quality of data was guaranteed throughout the experiment by adhering to a set standard operating procedure (SOP). The manufacturer's instructions were followed for preparing the culture media, and the sterility was verified by overnight incubation of a representative sample of the batch at 35-37°C and observation of bacterial growth. The batches of media that showed signs of growth were disposed away. The medium quality and antibiotic efficacy of the positive controls were evaluated using a reference *S. aureus* strain (ATCC 25923).

Statistical methods

Data was coded and entered using the statistical package for the Social Sciences (SPSS) version 28 (IBM Corp., Armonk, NY, USA). Quantitative data were summarized with the mean and standard deviation, as well as categorical data with frequencies and relative frequencies. For two groups, an unpaired t-test was used, and for more than two groups, an analysis of variance (ANOVA) with multiple comparisons post hoc test was used [14]. The chi-square ($\chi 2$) test was used to analyze categorical data. The exact test was used instead when the expected frequency is less than 5 [15]. *P*-values less than 0.05 were considered statistically significant.

Results

Antimicrobial susceptibility profile of MSSA and MRSA isolates

The tested isolates showed varying levels of susceptibility to the antimicrobials investigated, as shown in **table (1)**. The MRSA and MSSA isolates had the highest susceptibility profiles for linezolid and gentamicin among all the antibiotics that were examined. Out of the MRSA isolates, around 90% and 100% of the isolates tested showed sensitivity to linezolid and gentamicin, respectively. Similarly, among the MSSA isolates, around 90% and 80% of the isolates tested were susceptible to both antimicrobial agents, respectively. Contrarily, MRSA isolates showed the lowest susceptibility to penicillin and erythromycin. All antibiotic-resistant MRSA isolates exhibited resistance to penicillin, but only 20% showed sensitivity to erythromycin. All examined MSSA isolates exhibited penicillin resistance, but only 10% of the MSSA isolates tested showed sensitivity to tetracycline. Among the 10 MRSA isolates, 5 presented with resistance to clindamycin. Among these isolates, 4 showed constitutive resistance, while only one revealed inducible clindamycin resistance. From a total of 10 MSSA isolates, 4 showed resistance to clindamycin. Among these isolates, 3 displayed constitutive resistance, while only one showed inducible resistance. Comparisons of susceptibilities to various antimicrobial drugs between MSSA and MRSA did not show any statistically significant differences.

Antimicrobial effect of the honey on MSSA and MRSA

At a concentration of 75% (v/v), all honey types examined by the AWD technique exhibited antibacterial activity against both MSSA and MRSA. The most potent antibacterial activity was shown by Manuka MUF +20, followed by black seed and fennel honey. The inhibition zone diameters ranged from 15mm to 24mm, 11mm to 18mm, and 11mm to 15mm, respectively. The mean inhibition zone diameters were 19.80mm, 13.23mm, and 12.42mm, respectively, (**Figures 1, 2)**. The same observation was made using the AD technique, where the Manuka MUF +20 showed the lowest MIC value compared to black seed and fennel (18.75% (v/v) for Manuka MUF +20 and 37.50% (v/v) for both black seed and fennel).

Comparing honey types in MSSA and MRSA isolates

With respect to MSSA isolates, Manuka MUF +20 honey exhibited a statistically significant antibacterial effect compared to fennel and black seed honeys using the AWD method. The inhibition zone diameter ranges were 17mm-24mm, 11mm-15mm, and 12mm-14mm, with mean inhibition zone diameters of 20.7mm, 12.8mm, and 12.71mm, respectively. The P-value was less than 0.001, as shown in **figure (3)**. Although fennel honey exhibited more antibacterial activity than black seed honey, the difference between the two was not statistically significant. Utilizing the AD approach, Manuka MUF +20 honey exhibited a statistically significant reduction in bacterial growth compared to both fennel and black seed honeys. The minimum inhibitory concentration (MIC) for Manuka MUF +20 was 18.75% (v/v), whereas for both fennel and black seed honeys it was 37.50% (v/v). The *P* value was less than 0.001, as shown in **table (2)**.

Concerning MRSA isolates, Manuka MUF +20 honey exhibited a statistically significant antibacterial effect compared to fennel and black seed honeys using the AWD method. The inhibition zone diameter ranges were 15mm-22mm, 11mm-13mm, and 11mm-18mm, with mean inhibition zone diameters of 18.90mm, 12.00mm, and 13.83mm, respectively. The P-value was less than 0.001, as shown in **figure (4)**. While black seed honey really exhibited more antibacterial activity compared to fennel honey, there was no statistically significant difference between the two. Utilizing the AD technique, Manuka MUF +20 honey exhibited a statistically significant reduction in bacterial growth compared to both fennel and black seed honeys. The minimum inhibitory concentration (MIC) for Manuka was 18.75% (v/v), whereas for both fennel and black seed honeys it was 37.50% (v/v). The *P*-value for this reduction was less than 0.001, as shown in **table (3)**.

Comparing between MSSA and MRSA for the antibacterial activity of different honeys

The results of the present study indicate that MSSA exhibited greater susceptibility to Manuka MUF +20 and fennel honeys compared to MRSA. The inhibition zone diameters for MSSA ranged from 17mm to 24mm (mean diameter = 20.7mm), while for MRSA it ranged from 15mm to 22mm (mean diameter = 18.9mm). However, there was no statistically significant difference observed between MSSA and MRSA. Furthermore, MRSA exhibited greater susceptibility to black seed honey compared to MSSA, as shown by inhibition zone widths ranging from 11mm to 18mm (mean diameter = 13.8mm). However, there was no statistically significant difference between MSSA and MRSA (**Table 4)**.

Relation between susceptibility of antimicrobial agents and antibacterial activity of honey in MSSA and MRSA

There was no significant difference in the susceptibility of the antimicrobial agents used and the antibacterial activity of the three types of honey in either MSSA or MRSA, except for the presence of a statistically significant difference in the susceptibility of the only linezolid-resistant MRSA isolate to black seed honey compared to the sensitive ones, with a *P*-value of 0.045 **(Figure 5)**.

Table 1. Antimicrobial susceptibility profile of MSSA and MRSA isolates.

Isolate		**Antimicrobial agents (no. (%))**							
		P	**GEN**	**TE**	**LE**	**COT**	**E**	**CD**	**LZ**
MSSA (n =10)	**S**	0 (0)	8 (80)	1 (10)	3 (30)	7 (70)	3 (30)	5 (50)	9 (90)
	I	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)	0 (0)	1 (10)	0 (0)
	R	10 (100)	2 (20)	9 (90)	7 (70)	1 (10)	7 (70)	4 (40)	1 (10)
MRSA (n =10)	**S**	0 (0)	10 (100)	5 (50)	3 (30)	7 (70)	2 (20)	5 (50)	9 (90)
	I	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	2 (20)	0 (0)	0 (0)
	R	10 (100)	0 (0)	5 (50)	6 (60)	3 (30)	6 (60)	5 (50)	1 (10)
Total (n =20)	**S**	0 (0)	18 (90)	6 (30)	6 (30)	14 (70)	5 (25)	10 (50)	18 (90)
	I	0 (0)	0 (0)	0 (0)	1 (10)	2 (20)	2 (20)	1 (10)	0 (0)
	R	20 (100)	2 (10)	14 (70)	13 (65)	4 (20)	13 (65)	9 (45)	2(10)

P =Penicillin, GEN =gentamicin, TE =tetracycline, LE =levofloxacin, COT =cotrimoxazole, E =erythromycin, CD =clindamycin, LZ =linezolid, MSSA= methicillin sensitive *Staphylococcus aureus*, MRSA= methicillin resistant *Staphylococcus aureus*

Table 2. Comparing between MIC of honey types in MSSA isolates.

	MSSA Strains				
	Mean	**SD**	**Minimum**	**Maximum**	***P* value**
Manuka 20+ MIC	26.25	9.68	18.75	37.50	< 0.001
Fennel MIC	37.50	0.00	37.50	37.50	
Black seed MIC	37.50	0.00	37.50	37.50	

SD= Standard Deviation

Table 3. Comparing between MIC of honey types in MRSA isolates.

	MRSA Strains				
	Mean	**SD**	**Minimum**	**Maximum**	***P* value**
Manuka 20+ MIC	24.38	9.06	18.75	37.50	< 0.001
Fennel MIC	37.50	0.00	37.50	37.50	
Black seed MIC	37.50	0.00	37.50	37.50	

SD= Standard Deviation

Table 4. Comparing MSSA and MRSA for the antibacterial activity of different honeys.

	MRSA Strains				MSSA Strains				***P***
	Mean	**SD**	**Minimum**	**Maximum**	**Mean**	**SD**	**Minimum**	**Maximum**	**value**
Manuka 20+ honey samples (inhibition zones in mm)	18.90	2.23	15.00	22.00	20.70	2.26	17.00	24.00	0.090
Fennel honey samples (inhibition zones in mm)	12.00	0.50	11.00	13.00	12.80	1.32	11.00	15.00	0.100
Black seed honey samples (inhibition zones in mm)	13.83	2.48	11.00	18.00	12.71	0.95	12.00	14.00	0.292

SD= Standard Deviation

Figure 1. Antimicrobial effect of honey on MSSA.

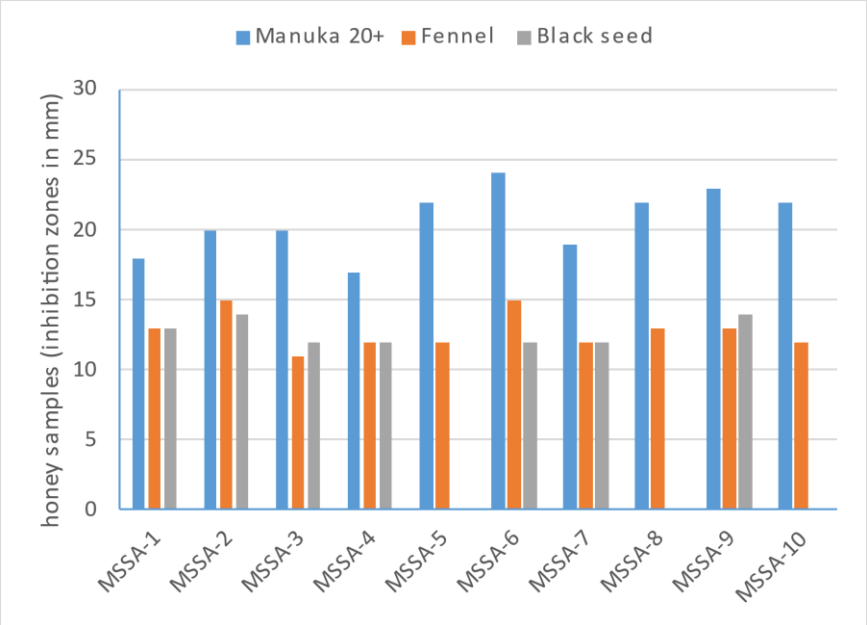


Figure 2. Antimicrobial effect of honey on MRSA.

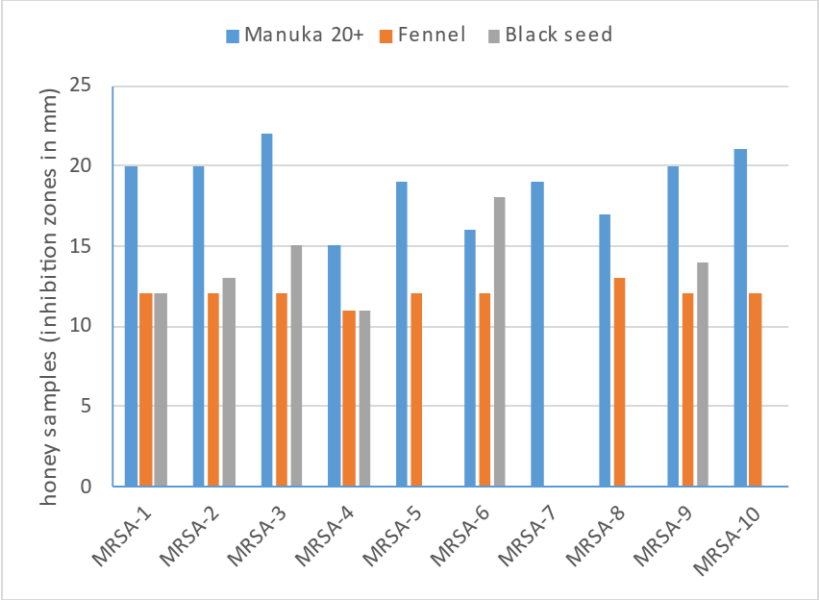


Figure 3. Comparing honey types in MSSA.

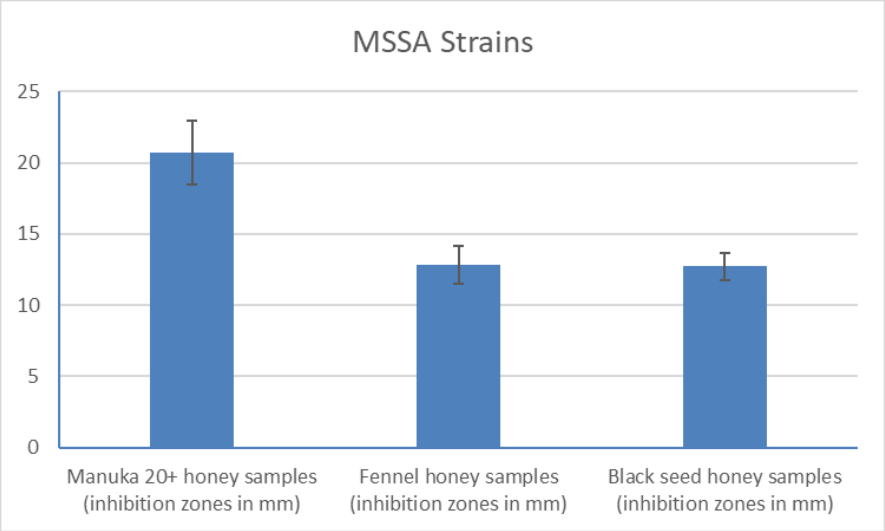


Figure 4. Comparing honey types in MRSA

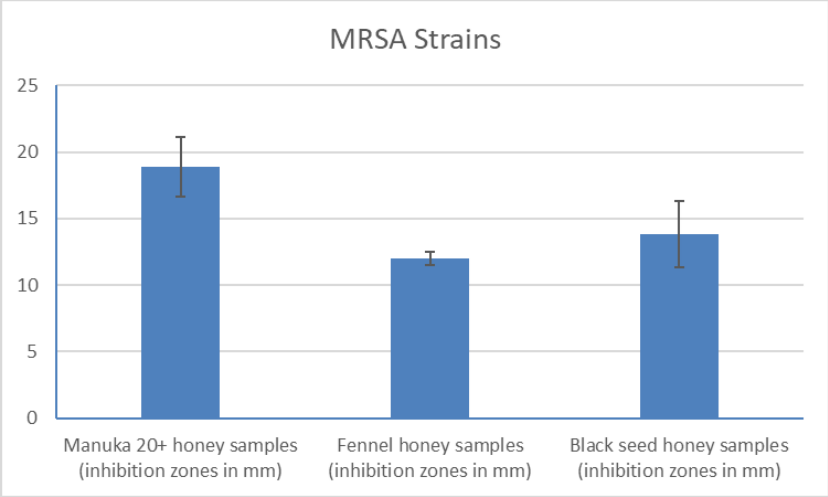
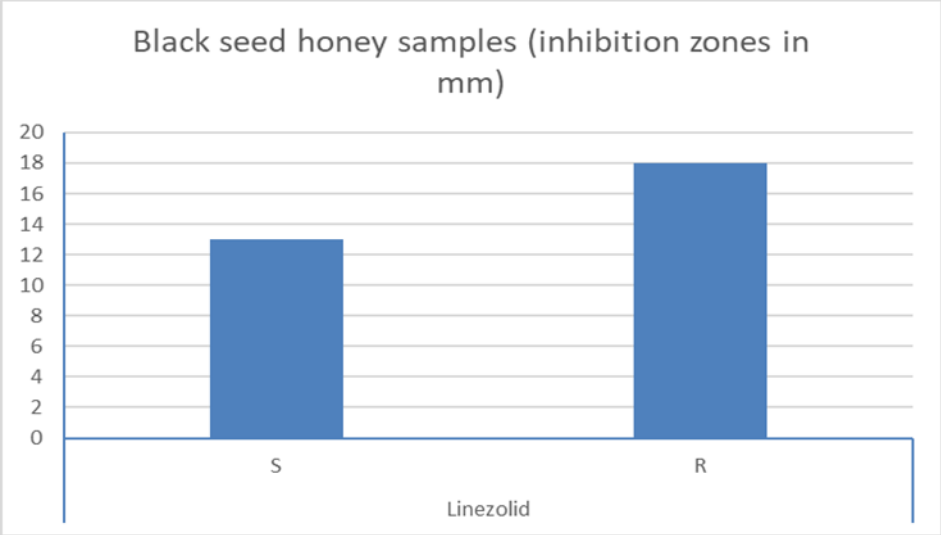


Figure 5. Relation between resistance and antibacterial activity black seed honey in MSSA and MRSA (S= Sensitive, R= Resistant).



Discussion

The Gram-positive bacterium *S. aureus* may cause numerous skin infections, including impetigo, furuncles, boils, styes, pustules, burns, and wounds. The main cause of infections, especially in healthcare facilities, is the development of antibiotic resistance in *S. aureus* strains. Newly developed strains of *S. aureus* have developed resistance to methicillin and other antibiotics that were formerly effective against *S. aureus* [16].

The most sensitive antibiotics against both MRSA and MSSA, according to the present study, were linezolid and gentamicin. These results agreed with those of earlier research [17, 18]. In contrast, isolates of MRSA showed the least sensitivity to erythromycin and penicillin, whereas isolates of MSSA showed the least sensitivity to tetracycline and penicillin. Contrary to what we found, researchers have found that MRSA isolates and MSSA isolates have higher erythromycin and tetracycline sensitivity, respectively [17, 18]. The isolates of MRSA have a higher incidence of clindamycin resistance. However, the specific pattern of clindamycin resistance was not statistically significantly associated with methicillin resistance. Other investigations [18, 19] also supported this result. Geographical distribution, public health, infection control programs, and community understanding primarily cause variations in *S. aureus* susceptibility to various antibiotics.

Depending on the type of honey, the region of production, and its floral origin, its antimicrobial properties can vary [20]. So, the goal of this study was to find out how well three different honeys, Manuka UMF +20, fennel, and black seed honeys, affected the growth of clinical isolates of MSSA and MRSA.

Using the AWD method, the present study demonstrated that at a concentration of 75% (v/v), Manuka MUF +20 showed the most potent antibacterial activity, followed by black seed and fennel honey. According to earlier studies [20, 21], Manuka UMF + 20 honey had the strongest antibacterial activity. A different study [22] revealed that fennel honey exhibited more antibacterial activity than Manuka +20 honey.

The current study found that honey concentrations ranging from 18.75 to 37.5% (v/v) were necessary to completely suppress the growth of MSSA and MRSA. This finding was consistent with **Mama *et al.*'s** results [16]. According to an Ethiopian study [23], the honey concentration required to completely prevent *S. aureus* growth was 6.5% (v/v), which is lower than our matching result.

We observed that Manuka UMF +20 exhibited the best antibacterial efficacy, with the lowest MIC value of 18.75% (v/v) against both MSSA and MRSA isolates. This finding agreed with that of **Sherlock *et al.*** [20], who found that Manuka honey could only inhibit MRSA growth at concentrations greater than 12.5% (v/v). On the other hand, another study indicated that Manuka

honey significantly reduced MSSA and MRSA when administered at a 10% (v/v) concentration [21]. In a similar vein, Willix ***et al.*** [24] found that 1.8% (v/v) of Manuka honey was necessary to totally prevent the growth of *S. aureus*.

Manuka honey, a monofloral honey, originates from the nectar of the Manuka tree's blossoms. Apis mellifera honeybees produce this specific kind from the Leptospermum scoparium, which grows on Manuka trees in New Zealand [25]. In addition to phenolic and flavonoid components, carbohydrates, minerals, proteins, and fatty acids make up Manuka honey [26]. The high antibacterial activity of this honey is not associated with H_2O_2, but rather because Manuka honey has extraordinarily high amounts of the antibacterial component methylglyoxal, known as the unique Manuka factor (UMF) [27].

Regarding fennel and black seed honey, we found that both types exhibited a higher MIC value than Manuka UMF +20 honey (MIC = 37.50% (v/v)), which means that both types have lower antimicrobial potency. According to Sherlock ***et al.*** [20], it was found that black seed honey significantly reduced the growth of MSSA and MRSA with honey at 20% (v/v) concentration. Remarkably, **Hossain *et al.*'s** study [28] found that black seed honey demonstrated its inhibitory potential at 1.56% (v/v) concentration for all tested bacteria, including *S. aureus*. Regarding fennel honey, **Zhang *et al.*** [22] found that it had a lower minimum bactericidal concentration (MBC) for *S. aureus* (25.0–40.0% (v/v)) than other honeys, such as Manuka honey (>50.0% (v/v)). **Hamouda *et al.*** [29] reported that fennel honey had MIC values (7.91±3.5 & 8.71±3.3% (v/v)) for all tested MRSA strains. Similar findings were made in an Egyptian study, which tested several Egyptian honey brands, including fennel honey, and discovered that all of them had strong antimicrobial activity with MIC values ranging from 6 to 15% (v/v), where the MIC value with the use of fennel was 8% (v/v) for MRSA [30], which is significantly lower than our results.

Some believe that a combination of factors works together to determine the antibacterial activity of honey. Important factors such as sugar content, high viscosity, mild acidity, and H_2O_2 release influence the antibacterial activity of honey. Additionally, it could vary based on the floral and/or geographic source [31]. Thermal circumstances reduced the efficacy of H_2O_2-derived honey, according to **Irish *et al.*** [27], but had no impact on non- H_2O_2-derived honey. This highlights the varying antibacterial characteristics of different types of honey, influenced by factors such as storage duration, nectar composition and source on which the reared bees were fed [32].

It was found in the current investigation that MSSA isolates were more sensitive than MRSA isolates to fennel honey and Manuka MUF +20. Nonetheless, compared to MSSA, MRSA isolates showed a greater sensitivity to black seed honey. Since no prior study had noted this finding, the precise reason for the variation in bacterial susceptibility to distinct honeys remains unclear.

Honey made from nectars and pollens from various parts of the world may have varied antibacterial characteristics, which may explain why the results of different investigations have shown conflicting conclusions. Other possible explanations include variances in bee species, testing methods, and test organisms. Research has demonstrated that honey possesses antibacterial characteristics, yet different honey samples exhibit varying levels of antibacterial activity [18]. So, it is not possible to judge the sensitivity of MSSA and MRSA isolates based on the results of just a few studies, since the honey used in those studies may have had very different antibacterial properties.

Notably, in the current study it was observed that the only linezolid-resistant MRSA exhibited higher sensitivity to black seed honey than the MRSA isolates sensitive to it. This observation was not reported by any other studies, and it can give hope for utilization of black seed honey as an alternative therapy or in combination with other antimicrobials for the treatment of linezolid-resistant *S. aureus*.

In conclusion, the results of this investigation demonstrated that the three varieties of honey were capable of suppressing the growth of MRSA and MSSA isolates. The type and concentration of the honey that was used determined its antibacterial activity. When compared to black seed and fennel honeys, manuka MUF +20 had the strongest antibacterial action.

This study has several limitations that should be taken into account. Firstly, patients' clinical history and risk factors were not included. Secondly, the number of clinical isolates tested against the honey samples was limited. Lastly, testing the antibacterial activity against gram-

negative bacteria as well as the synergistic effect of used honeys with other antimicrobial agents are recommended in the upcoming studies.

Conflict of interest

There are not any financial or personal relationships with other people or organizations that could inappropriately influence the authors' actions.

Financial disclosures

This work was completely self-funded by the authors.

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