



RESEARCH ARTICLE

Different Concentrations of Honey's Antimicrobial Activity against *Staphylococcus aureus* by HPLC Determination

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ABSTRACT

Honey has been used in ancient times as a treatment. It is used for healing wounds and also can be used as antiseptic to kill bacteria and other microorganisms. The aim of this study was to examine the activity of honey as a bactericidal and bacteriostatic and to measure the most active compounds of honey with highly bacterial inhibition zone using high-performance liquid chromatography (HPLC) technique. In this experiment, the honey was used to check whether the honey can be used as an antimicrobial agent or not. First by well diffusion method is done by adding different concentrations of honey (25, 75, 100, 125, 150, and 200 ml). The results showed a little inhibition zone for the three types of honey but in different size for each type. The industrial honey showed the largest effect. Different concentrations were used (10%, 30%, and 50%). The antibiotic sensitivity was applied, the result showed only two resistant antibiotics (azithromycin and erythromycin). The concentration of catalase, amylase, invertase, and glucose oxidase in honey tested by HPLC and showed the concentration of each substance conc. of catalase = $(32877 \div 170,253) \times 20 = 3.862$ u/ml; conc. of amylase = $(136,985 \div 180,849) \times 20 = 15.149$ u/ml; conc. of invertase = $(58,466 \div 193,624) \times 20 = 6.039$ u/ml; and conc. of glucose peroxide = $(105,204 \div 163,245) \times 20 = 12.889$ u/ml. Moreover, the presence of gluconic acid "Organic acid" gives the honey its acidic characteristic which is about 3.2 pH.

Keywords: Honey, *Staphylococcus aureus*, antimicrobial activity, antibiotics, high-performance liquid chromatography test

INTRODUCTION

Honey could be a good health-care product in addition to its previously use as a part of the traditional medicine in the treatment of several wound's types.^[1] In the recent decade, the microbial resistance is becoming one of the serious concerns due to the extensive use of antimicrobial agents. As a part of the traditional antimicrobial preparations, honey has gotten more and more consideration as there is an increase in the investigations on its antimicrobial properties but is the honey have an effect on bacteria?^[2] Phytochemicals are already presents as they can thus affect the antimicrobial capacity.^[3] There are numerous *in vitro* and *in vivo* studies which were illustrated the antibacterial, antifungal, and even the antiviral activity of honey.^[4-6] However, the ability of honey to kill bacteria "Bactericidal effect" is due to the diversity of honey composition which includes about 181 constituents.^[7] One of the major compositions of honey is carbohydrates in addition to the vitamins, minerals, enzymes, amino acids, and water by 15–18%. Moreover, ripened honey consists of 80% sugars; mainly glucose and fructose as well as some sucrose and maltose along with 18% water. The high concentration of sugars combined with a low moisture content creates osmotic stress, which prevents spoilage of honey by microorganisms.

Only slight dilution of honey can result in yeast growth, but the sugar content of honey is sufficient to retain antibacterial activity of honey when diluted to approximately 30–40%. At higher dilution rates, the antibacterial activity could be due to the other compounds other than sugars.^[8,9] *Staphylococcus aureus* is Gram-positive pathogenic bacteria which causes a wide variety of diseases. Many methods were developed to treat *S. aureus*-related diseases but the treatment of this bacteria is still incomplete due to the appearance of different strains which are resistant to antibacterial agents

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Received: June 24, 2022

Accepted: August 12, 2022

Published: September 25, 2022

DOI: 10.24086/cuesj.v6n2y2022.pp112-118

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such as “Methicillin-Resistant *S. aureus*.”^[10] In addition, it can be found as a microbial flora on the mucosal membrane and skin.^[11] Some are aerobic and few are anaerobic and it can survive in high concentration of salt reaching about 10%.^[12] Honey is could be produced from various sources, and its antimicrobial activity varies significantly in response to its origin. The antibacterial activity of honey has been attributed by physical factors such as osmolarity, acidity, and chemical factors. Studies were reported that commercial honey had antimicrobial effects against *S. aureus*. The use of honey as an antimicrobial agent was started in 2000 BC as it showed some different results against different bacteria and depending on the honey that was used. The added value of honey has attracted the clinicians and scientists to concentrate on it and how to be used extensively.^[13] The second major advantage of the honey is that bacteria have not developed the same rate of resistance against the natural products as the one for the antibacterial agents.^[14,15] Furthermore, the bacterial resistance to many antibiotics can cause different chronic types of wounds which can sometimes progress to amputation or even death.^[13,16] *The aims of study were to examine the activity of honey as a bacterial inhibitor and to measure the most active compounds of honey with highly bacterial inhibition zone using high-performance liquid chromatography (HPLC).*

METHODOLOGY

Collection and Preparation of Honey Test Solutions

There were three common honey samples collected. The honey samples were stored at 37°C for 24 h before the preparation process of different concentrations of each honey type. In addition, the honey types were kept in dark bottles to guarantee that they will be away from the sunlight.^[17]

Materials

1. Two types of agar were used
 - Nutrient agar
 - Mueller-Hinton agar
2. Three types of honey
 - Black honey
 - Industrial honey
 - Natural honey
3. Pure culture of *S. aureus*
4. Ten different antibiotics: (Cloxacillin, trimethoprim, azithromycin, ciprofloxacin, penicillin, rifampin, methicillin, erythromycin, clindamycin, and gentamicin)
5. Swabs and petri dishes
6. Needle and loop for culturing bacteria
7. Bunsen burner
8. Sterilizers (alcohol).

Isolation of Bacteria

Inactive pure culture of *S. aureus* was present, so the bacteria could be reactivated by making a new subculture on nutrient broth by transferring a sample from the inactive old culture to the new one using swabs to be subjected then for incubation at 37°C for 24 h.^[18]

Preparation of Nutrient Broth

A 28 g of nutrient broth powder was mixed with 1 L of distilled water and the required number of media is about 20 ml, to obtain the needed 20 ml, 0.56 g of nutrient broth powder was prepared following the ratio and proportion equation.

Addition of Honey on the Cultured *S. aureus*

Mueller-Hinton agar was prepared and then poured on the Petri dishes. First, about 18 Petri dishes were required with about 20 ml of media for each as the total media volume that will be needed is 360 ml.

In terms of the Mueller-Hinton agar composition, 38 g of the powder should be mixed with 1 L of distilled water. Accordingly, to have 18 Petri dishes with a total media volume of 360 ml; 13.68 g of Mueller-Hinton powder was required following the ratio and proportion equation. Finally, 13.68 g of powdered Mueller-Hinton was added to 360 ml of distilled water to a 500 ml flask followed by boiling on a Bunsen burner. For more sterilization, the flask was kept in the autoclave.^[19]

Agar Well Diffusion

A 20 ml of the media was poured into each Petri dish and remained for solidification to form the wells. All materials that were used had been sterilized in the autoclave for 15 min. Punching force was applied using a sterile tip with 5 mm width to develop on the plate to form wells with the same diameter. In addition, the excess agar was removed and the plates were subjected to incubation for 24 h at 37°C.

Culturing *S. aureus*

The bacteria were cultured using swabs by dipping them in the previously cultured test tubes. Furtherly, the bacteria were spread on the Petri dishes following the spread plate technique instructions to accomplish the culturing process.

Addition of Different Honey Concentration

Before adding various honey concentrations, the Petri dishes were labeled with the type and concentration of the honey to be added as follows: S1: Industrial honey, S2: Black honey, and S3: Natural honey.

Each type of honey was tested in terms of activity in different concentrations as these concentrations were, respectively; 25, 75, 100, 125, 150, and 200 in mcl. Furthermore, micropipette was used to add the different concentrations in the center of the wells and further incubation for 24 h.

Dilution Method

In the current method, the three types of honey were tested in different concentration using the nutrient broth and then cultured overnight in the tubes following a specific procedure.^[20]

Sterilization of Test Tubes

Heat proof glass test tubes were used to be subjected for high temperatures to guarantee a complete sterilization process using the autoclave at 121°C for about 15 min.

Preparation of Nutrient Broth

Fifteen sterilized test tubes were needed for the whole three types of honey in addition to 150 ml of media as each test tube requires 10 ml of nutrient broth. To prepare 150 ml of nutrient broth media; following the previously discussed ration proportion equation, 4.2 g of the powder will be added to 150 ml of distilled water in a flask. In addition, the mixture was boiled using a Bunsen burner and then autoclaved for 15 min.

Dilution of Honey

The three types of honey were tested by taking 10%, 30%, and 50% concentrations respectively from each. Concentrations were expressed as percentage and weight based on the density of 1.42 g/ml as the 10% of honey equals 0.142 g/ml, 20% equals 0.284 g/ml, and 50% equals 0.701 g/ml of the honey.

Culturing of *S. aureus*

A swab from pure culture of *S. aureus* was obtained and then cultured in 10 ml of nutrient broth as the step was repeated for each test tube. Furthermore, different concentrations of honey were added to the cultured test tubes as the samples were incubated for 24 h and the results were checked.

Sterilization of Test Tubes

Heat proof glass test tubes were used to be subjected for high temperatures to guarantee a complete sterilization process using the autoclave at 121°C for about 30–60 min.

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Antibiotic Sensitivity Test

The effect of 10 types of antibiotics on *S. aureus* was tested after being cultured "*S. aureus*" on Mueller-Hinton agar. The reason behind using Mueller-Hinton agar is that it is inert; does

not have effect on the antibiotics. This test was done to show whether *S. aureus* is strong bacteria or not and compare the results the other tests that honey used as antimicrobial agent. There were two Petri dished used as five disks of antibiotics were put in each and then incubated for 24 h. In addition, the results were checked whether resistant or sensitive following the size of inhibition zone measurement. One factor at a time was used to improve the process parameters and the ideal circumstances for each type.

Inhibition Zone Measurement

The transparent area that surrounds the disks was measured using a ruler as the area refers to the sensitivity of bacteria to the antibiotics along with the measurement of the diameter of inhibition zone that has been subtracted from the disks.

HPLC Test Preparation

A 10 g of honey was shipped to Baghdad to be homogenized in 50 mL of 100 mM sodium sulfate buffer (pH 7.0), containing 1 mM ascorbic acid and 0.5% (W/V) polyvinylpyrrolidone, for 5 min at 4°C. The homogenate was filtered through three layers of cheesecloth, and the filtrate was then run at 5000× g for 15 min, and the supernatant was collected. Sample residue was then resuspended in 1.0 ml HPLC grade methanol by vortexing, and the mixture was then run through a disposable 2.5 µm filter. This was followed by storage at 4°C for further analysis, and finally, 20 µl of the sample was injected into the HPLC.^[21]

Equipment

Shimadzu 10 A V-LC with a binary delivery pump model LC-10 A was used for the separation, and a UV-Vis 10 A-SPD spectrophotometer was used to track the peak elution. The sequence of the eluted material of the standard mentioned in Table 1.

HPLC Determination of Enzymes in Honey

The extract was separated using an fast liquid chromatographic column with a 7 µm particle size, a NUCLOSIT 4000-7 PEI, anion exchange for protein and peptides (125 × 4.0 mm I.D) column, mobile phase, linear gradients from 0% B to 100% B in 10 min, and solvents A and B with different concentrations of tris-acetate at different pH levels. UV detection is set to 280 nm with a 1.5 ml/min flow rate.

RESULTS

Agar Well Diffusion Assay

24 h of incubation were illustrated that the bacteria were resistant to all samples "S 1, S2, and S3" at 25 µl concentration.

Table 1: The sequence of the eluted material of the standard

Seq.	Subjects	Retention time minute	Area
1	Catalase, CAT	2.02	170,253
2	Amylase	3.19	180,849
3	Invertase	4.10	193,624
4	Glucose oxidase	5.19	1632

The first sample (artificial honey) started its activity against bacteria at the concentrations 75 µl and 100 µl as the inhibition zone was 0.2 mm while at the same concentrations of the rest two samples, *S. aureus* was resistant. In addition, at 125 µl, the inhibition zone of *S. aureus* for the first sample was about 0.3 mm in the same time of being resistant in the rest two samples. At 150 µl, samples 2 and 3 (black and natural honey) started their activity against bacteria as the inhibition zone for the second sample was 0.2 mm while for the third sample was 0.1 mm as per the Figures 1-4.^[22]

Dilution Method

The first honey sample (industrial honey) on a certain concentration was tested and then mixed it with nutrient broth and *S. aureus* as well as the same concentration from

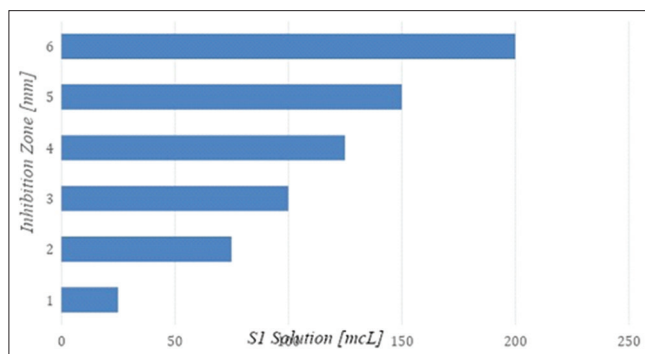


Figure 1: Effect of different concentrations of industrial honey “S 1” on *Staphylococcus aureus*

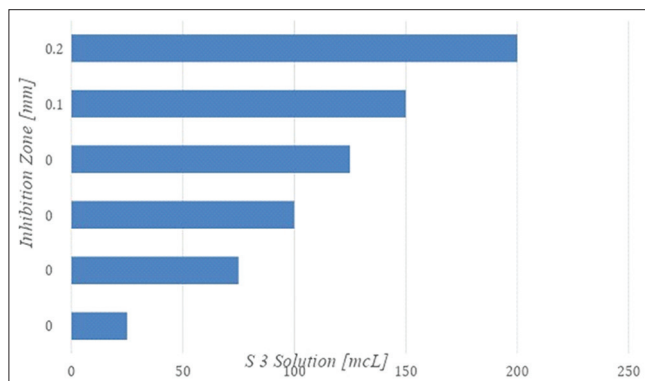


Figure 2: Effect of different concentrations of natural honey on *Staphylococcus aureus*



Figure 3: Antibiotic sensitivity test

the other two samples as they were subjected for incubation for 24 h. The results showed that the bacteria were resistant based on the observed growth in the three test tubes. When the concentration of all honey samples was increased, the bacterial growth was detected but in lower rates. The lowest concentration of the honey in clear test tube was considered as the minimum inhibitory concentration “MIC,” Table 2.

Aureus Resistance against Antibiotics

The effect of 10 types of antibiotics on *S. aureus* was tested after being cultured *S. aureus* on Mueller-Hinton agar. The reason behind using Mueller-Hinton agar is that it is inert; does not have effect on the antibiotics. The resistance and sensitivity of *S. aureus* were tested to some of the antibiotics including; rifampin, clindamycin, gentamicin, methicillin, ciprofloxacin, penicillin, cloxacillin, trimethoprim, azithromycin, and erythromycin as the results are illustrated in Table 3.

HPLC Test of Honey

The concentration of catalase, amylase, invertase, and glucose oxidase in honey tested by HPLC and by following the equation below shows the concentration of each substance:

$$\text{Conc. of sample} = (\text{Area of sample} \div \text{Area of standard}) \times \text{cons. of standard} \times \text{dilution factor} :-$$

$$\text{Conc. of catalase} = (32877 \div 170253) \times 20 = 3.862 \text{ u/ml}$$

$$\text{Conc. of amylase} = (136985 \div 180849) \times 20 = 15.149 \text{ u/ml}$$

$$\text{Conc. of invertase} = (58466 \div 193624) \times 20 = 6.039 \text{ u/ml}$$

$$\text{Conc. of glucose peroxide} = (105204 \div 163245) \times 20 = 12.889 \text{ u/ml}$$

DISCUSSION

The findings showed that the three different honey samples have antibacterial action against the Gram-positive bacterium *S. aureus*. The possible reason could be that honey consists mainly of carbohydrates (approximately 82%), water and other minor components including; proteins, minerals, and phytochemicals.^[23] The water activity of honey ranges from 0.5 to 0.6 which could be low enough to prevent bacterial growth. Furthermore, the high sugar content of honey can induce the osmotic pressure on the bacterial cells the thing that will initiate the process of water flow to the outside of the cell, leading to the dehydration and shrinkage of the cell thus the inability to survive.^[24]

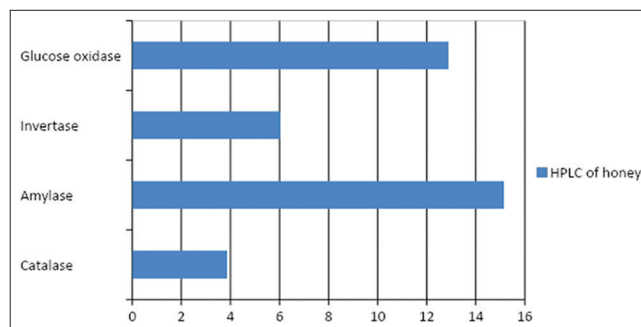


Figure 4: Concentration of enzymes found in industrial honey

Table 2: MICs of honey against *Staphylococcus aureus* growth

Solution %	MIC	Result
S1		
10	No MIC	Negative
20	No MIC	Negative
50	MIC	Positive
S2		
10	No MIC	Negative
20	No MIC	Negative
50	MIC	Positive
S3		
10	No MIC	Negative
20	No MIC	Negative
50	MIC	Positive

MIC: Minimum inhibitory concentration

Table 3: Antibacterial activity of antibiotics against *Staphylococcus aureus*

Antibiotic (mcg)	Sensitivity	Inhibition zone
Cloxacillin (10 mcg)	Sensitive	8.1 cm
Trimethoprim (10 mcg)	Sensitive	6.2 cm
Azithromycin (15 mcg)	Resistant	No inhibition
Ciprofloxacin (10 mcg)	Sensitive	10.4 cm
Penicillin G (10 mcg)	Sensitive	5.3 cm
Rifampin (5 mcg)	Sensitive	8.1 cm
Methicillin (10 mcg)	Sensitive	3.5 cm
Gentamicin (10 mcg)	Sensitive	7.8 cm
Clindamycin (10 mcg)	Sensitive	6.5cm
Erythromycin (10 mcg)	Resistant	No inhibition

Moreover, the presence of gluconic acid “Organic acid” gives the honey its acidic characteristic which is about 3.2 pH the thing that is not optimal for further bacterial growth which is provided by neutral PH.^[25] The degree of antibacterial action changed agreeing according to the type of bacteria and honey. The observed variations in the behavior of distinct honeys types at vary concentrations as well as the nature of honey production’s impact such an effective pattern on their inhibitory activities that were directly proportional to the increased honey concentrations respectively.^[24] It has been detected that these phytochemicals are the ones responsible for medical and biological activities of honey within the treatment of infections, burns, wounds, and ulcers.

Using CYBOW 11 test strips, the pH was determined for all varieties of honey and their concentrations, and it was found to be 5, indicating that the honey is acidic. In addition, honey’s significant osmotic impact (approximately 80% wt/vol of concentrated sugars) and microorganism susceptibility to hydrogen peroxide act as inhibitors of bacterial development.^[26]

In this research, agar well diffusion and the dilution method were both employed to assess the antibacterial activity of honey. Acidity, non-hydrogen peroxide activity, high osmotic effect, and

the presence of phytochemical components, which are helpful in regulating bacterial dispersal, are the main actions of honey. Because honey has a significant amount of sugar, bacteria cannot grow in it because the pH is low enough to prevent many types of bacteria from growing in it. Honey’s primary antibacterial component, hydrogen peroxide, is produced when the enzyme glucose oxidase is triggered by honey dilutions. Furthermore, other components found in honey and give it the ability to kill or inhibit the growth of bacteria.^[27,28]

All of the investigated honeys’ MIC and inhibitory zone values against *S. aureus* demonstrated bactericidal and bacteriostatic activity. Our findings are comparable to those of other studies.^[29] The bacteria were resistant to the honey sample if any zone had a diameter of <3 mm. The bacterium appeared to be sensitive to the honey sample, though, as shown by a zone diameter higher than 7 mm.^[30] These honey samples might be used topically to heal wounds because the bacteria are known to infect wounds and are sensitive to the honey samples. It is known that honey may be used to cure an infected wound.^[31]

It was shown that 50% (v/v) of the three samples’ concentration of honey that prevented the growth of which 90% of *Staphylococcus aureus* accordingly. The findings of earlier studies reflect our ideals. These variations in honey’s antibacterial action may result from its diverse bee sources or species.^[32] In addition, samples of honey included alkaloids, tannins, and flavonoids, all of which are known to have antimicrobial properties.^[19]

Data from both the agar diffusion and broth dilution methods indicated that bacteria were more resistant to the second and third samples more than industrial honey, despite the fact that this result is supernatural, according to previous research, the natural honey has greater antibacterial activity than modified honey. This may be due to fraud by the companies exporting these two samples and selling them as a natural honey. However, in our studied if we take into account that the two samples are natural, the second sample was having greater antibacterial activity than the third, the reason is due to the different nectar source of the bees or the change in acidity and water rate in both samples.

The industrial honey is tampered with honey. It started off as pure honey, but later other ingredients – glucose, dextrose, molasses, sugar syrup, flour, corn syrup, starch, or any other product – were added to boost its volume or alter its qualities. Inverted sucrose from beet or cane sugar is artificial honey. To mimic genuine honey, it has been altered in look, flavor, and odor.^[33] There are various researches studied the chemical components of industrial honey in general, regardless of the company that manufactured it to distinguish between natural honey and artificial honey. A total of 800 samples of industrial honey were examined for this reason. Since the designs of honey with 0 and 7 and 0 and 0 and C-4 sugar content (percent) were so similar, it was possible to determine that honey with 7 and C-4 sugar content (percent) was also free of C-4 sugars. The 13C protein levels of both honey groups and the 13C honey values show a very good association. A helpful characteristic for contaminated honey with a C-4 sugar content (percent) 7 is that the C-4 sugar content (percent) 7 group had lower (p 0.05) values (16.30) for the 18O value compared to other honey.

A HPLC test was carried out to determine the components of industrial honey and their concentrations to determine why industrial honey had a greater impact on honey. This test's results revealed that industrial honey is primarily composed of carbohydrates, catalase, and invertase, and that its low water content also plays a role in the concentration-dependent killing of bacteria. HPLC test showed that the most common type of enzymes present in the honey is organic acidic and is explained the acidity of honey. It also showed a high amount of glucose oxidase which plays an important role in killing bacteria by oxidation of glucose to H_2O_2 .^[7]

Many researches have employed the earlier techniques while using the agar diffusion method to measure the activity of honey. When researchers researched honey or the natural antibacterial components of honey, they discovered a number of issues with the agar diffusion method. A few modifications in the experimental settings, such as agar volume, inoculation concentration, and incubation conditions, may cause significantly different findings. These include insensitivity, wherein modest levels of antimicrobial activity are not always evident.^[34]

Finally, the data acquired by the dilution techniques and time-kill assay did not confirm the results of the agar diffusion obtained in the current investigation, which indicated limited activity. It was obvious that there was no direct correlation between MIC and zone size. This shows that results from the agar diffusion technique may not always be an accurate reflection of overall antibacterial activity.

This research also studied the effect of some antibiotics on bacteria to see if *S. aureus* is resistant or sensitive to it. We have noticed that *S. aureus* was sensitive to some antibiotics, including rifampin, clindamycin, gentamicin, methicillin, ciprofloxacin, penicillin, cloxacillin, and trimethoprim, and it was resistant to azithromycin and erythromycin.

CONCLUSION

All tubes that were cultured with addition of honey showed a level of turbidity except 10% diluted tubes which was a clear tube (no turbidity) that refers to MIC. In the well diffusion method, the three types of honey had different effects at the same concentration of honey. Low concentration had not any effect and high concentration of honey showed a different effect. Although the industrial honey had more effect (larger inhibition zone), we cannot consider it as an antimicrobial agent because the inhibition zone was not large enough compared to the concentration of honey and other antibiotics that were used.

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