INTERNATIONAL BACCALAURATE BIOLOGY EXTENDED ESSAY

Investigating the Antibacterial Effect of Flower Honey and Pine Honey on the Growth of *Staphylococcus aureus*

Session May 2022

Word Count: 3571 (when references and tables are substracted)

“To what extent does flower honey types and pine honey types have an antibacterial effect on wound infection agent *Staphylococcus aureus,* considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion?“

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Research Question

Research Question is “To what extent does flower honey types and pine honey types have an antibacterial effect on wound infection agent *Staphylococcus aureus,* considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion? “

Different methods and different concentrations of honey were used to answer this question.

- In this study, two methods, agar well diffusion and disc diffusion, were used to determine the antibacterial effect.
- Concentrations were prepared to determine the intensity of antibacterial effect of flower and pine honey.

Introduction

One day my friend spilled tea on his hand and it burned. His mother immediately rubbed honey on the burned area. When I asked her why she applied honey, she said that honey heals the burn quickly and prevents it from getting infected. This caught my attention. I started researching. I learned that honey is used in the treatment of burns and wounds, that it heals quickly and prevents infection. In my research, it is reported in the literature that the antibacterial action mechanisms of honey are speculative. Namely, honey can inhibit bacterial growth through a number of different mechanisms. High sugar concentration, low pH, hydrogen peroxide formation, proteinaceous compounds, phytochemicals, or other unidentified components found in honey can all confer antimicrobial activity. It is reported that the dominant antibacterial mechanism of honey is caused by hydrogen peroxide, which is formed as a result of the oxidation of glucose in honey by the glucose oxidase enzyme produced in the hypopharyngeal glands of bees and defined as an inhibitor. In honeys with high hydrogen peroxide content, hydroxyl radicals cause oxidative damage to DNA and bacterial growth is restricted (13). Hydrogen peroxide is released gradually and after 12 hours its concentration is 4 to 5 μg per gram of honey, after 24 hours it rises to 25 μg/gram, which is sufficient to disinfect the wound. (19) The antibacterial effect of honey varies according to its origin, vegetation, climate and geographical conditions. Therefore, it is thought that flower and pine honeys will have different antibacterial effects (7).

In the literature, it has been reported that *Staphylococcus aureus* frequently cause infection in wounds anda burns (13,21). Agar well diffusion and disc diffusion methods are frequently used to determine the antibacterial effect of honey on this bacteria.

Hypothesis

H_1: The hypothesis put forward in this study is that different concentrations and types of honey (pine honey and flower honey) have a different antibacterial effect on *Staphylococcus aureus* by considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion.

H_0: The hypothesis put forward in this study is that different concentrations and types of honey (pine honey and flower honey) don't have a different antibacterial effect on *Staphylococcus aureus* by considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion.

Background Information

Today, beekeeping is one of the most common agricultural activities in the world. Our country is one of the largest honey producers in the world, and it has a structure suitable for beekeeping and honey production in terms of geography and climate, and rich in vegetation. Honey is produced in almost every region of our contry. (4).

Honey, which is one of the most consumed bee products, has become a part of traditional medicine since ancient times, not only as a nutrient and sweetener, but also to provide wound healing and tissue regeneration, to alleviate gastrointestinal disorders, gingivitis and various other pathologies. The composition, taste and color of honey vary according to the type of flower sources, geographical region, climate, bee species and processes of honey (12). It is a natural functional food that is nutritious, protective against many diseases, and helpful in the treatment of some diseases due to more than 200 different components in its content (16). Basically, about 82% of its composition is carbohydrates, 17% is water, 0.7% is mineral matter, 0.3% is protein, vitamins, organic acids, free amino acids and various types of

flavonoids, phenolic acids, carotenoids, a-tocopherol. Phytochemicals, volatile compounds, form from minerals. More than 5000 phenolic compounds have been reported in honey, which is the source of many bioactive compounds, and most of these compounds are in the form of flavonoids, the main ones being apigenin, pinocembrin and quercetin. There are also phenolic acids such as caffeic, ferrulic, coumaric and benzoic acids (7,25).

In recent years, an alternative medicine called apitherapy has developed based on the use of honey and bee products against many diseases. Various in vitro and in vivo studies show the antioxidant, antibacterial, anti-inflammatory, antimicrobial, antiviral, antifungal, anticancer, and antidiabetic effects of honey (11). Honey for therapeutic purposes; It is used in the treatment of ulcers, skin infections caused by wounds and burns, and bedsores. When the literature is examined, it is seen that honey has inhibitory effects on bacteria, viruses and fungi (7,15,22). Due to the increasing incidence of antibiotic-resistant bacteria worldwide, the use of naturally occurring antimicrobial compounds in foods is also of great interest. Honey is a food with promising antibacterial effects in the fight against antimicrobial resistance (18). For example, Manuka honey, obtained from a tree called Leptospermum scoparium, produced in New Zealand and Australia, is the honey best known worldwide and sold as a therapeutic agent. Tualang honey, like Manuka honey, is one of the globally documented honeys, which has made a name for itself with its antibacterial activity. Structural differences of honey can affect its medicinal value. (6,10). The antibacterial effect of each honey is not the same. Therefore, it is important to analyze the honey to be used in apitherapy (6,10,15).

Scientific studies indicate that the antimicrobial activity of honey is due to hydrogen peroxide, osmolarity, acidity, aromatic acids, polyphenols, phenolic compounds, flavonoids and maillard reaction products. In addition, lysozyme and volatile compounds are thought to play a role in bacterial inhibition. Thanks to these properties, honey prepares an inhibitory environment for the development of pathogens that cause disease in humans (2,9). Antibacterial activity due to hydrogen peroxide is adversely affected by high heat treatment, storage in unsuitable conditions and exposure to light. Hydrogen peroxide is defined as the main component responsible for the antibacterial activity of honey. Hydrogen peroxide is a potent antimicrobial agent produced mainly during glucose oxidation, catalyzed by the action of glucose oxidase. Glucose oxidase secreted from the pharyngeal glands of bees is known as bee enzyme and is transferred to honey by bees during nectar harvest. The concentration of hydrogen peroxide in honey is determined by the rate at which it is produced by glucose oxidase and destroyed by catalases. Honey has a structure that varies according to its botanical origin. Therefore, the amount of hydrogen peroxide in honey varies from honey to

honey. The higher the hydrogen peroxide concentration, the higher the antibacterial effect of honey (5,9).

However, honey have a very complex structure that includes many components of the structures. Therefore, its antibacterial activity is not only dependent on the presence of antibacterial activity in dark-colored honeys was found to be higher than in light-colored honey, and it was stated that this difference was due to the excess of phenolic compounds. Pine honey is a darker honey than flower honey. It has been reported that there is an antibacterial effect of varying degrees depending on the origin (24).

Many studies have shown sensitivity to honey of antibiotic-resistant and multi-drug-resistant strains. *S. aureus* is important pathogen that spread easily and cause infection in wounds and burns if hygiene is not observed widely in the environment.

Methodology

Variables

Table 1- Identifying Variables

Variable Type	Variable	Method of Management and/or Measurement
Independent	Concentrations (50%, 25%, 12.5%, 6.25%, 3.13%, 1.56%, 0.78%) Types of honey (pine or flower)	Observation and calculating in numbers by lenght equipment.
Dependent	Inhibition zone radius	Types of procedure • Agar well diffusion • Disc diffusion
Controlled	Temperature of incubation Bacterial culture incubation time Depth of incision holes (agar well diffusion) The size of the discs used in the disc diffusion method	37°C (to reflect temperature of human body) 24 hours 8 mm cavities 6 mm diameter
Uncontrolled	Climate, atmospheric pressure, relative humidity	

Materials and Equipments Used in the Experiment

Table 2- List of Materials and Equipment Used in the Experiment

Name of the Material	Explanation
Absolute alcohol (% 96)	The spoon used in honey weighing was sterilized by flaming with ethanol.
Sterile distilled water	Distilled water was sterilized in an autoclave at 121°C for 15 minutes and used for concenration of honey samples, preparation of media and artificial honey.
Pepton Water (OXOID CM 0009)	Dehydrated culture media. It is a liquid medium used for the concenration of microorganisms in standard microbiological analyzes performed in vitro (outside of living cells)
Brain Heart Infusion Broth (OXOID CM1135)	It is a ready-made liquid medium used for the reproduction of microorganisms that are difficult to grow in standard microbiological analyzes performed in vitro (outside of living cells).
Nutrient Agar (MERCK 105450)	It is a multi-purpose, ready-to-use solid medium used in standard microbiological analyzes performed in vitro (outside of living cells)
Fructose (MERCK 57-48-7)	It is a monosaccharide, also known as fruit sugar, and was used in artificial honey mixture
Maltose (MERCK 6363-53-7)	It is a disaccharide consisting of two glucose molecules and was used in artificial honey mixture
Glucose (MERCK 14431-43-7)	It is a monosaccharide consisting of two glucose molecules and was used in artificial honey mixture
Sucrose (MERCK 57-50-1)	Also known as tea sugar (sucrose), it is a disaccharide consisting of glucose and fructose molecules and was used in artificial honey mixture
Volumetric flask (±0.1)	100.0 ml volumetric flask was used in the preparation of honey concenrations
Sensitive scale	Honey samples, chemicals and medium were weighed in a sensitive scale that allows precise and small amounts of measurement (Photo 7)
Lab spoon	It was used for weighing honey samples and medium
Erlemeyer flask	It was used for the preparation of 25%, 12.5%, 6%,

	3%, 1.5%, 0.75% concentrations of honey samples, except for 50% concentrations, for the preparation of media and for mixing all the materials prepared in artificial honey production
Bunsen burner	These are the stoves that work with gas and can have a controlled flame. It was used in the analysis of honey samples in the microbiology laboratory (Photo 8)
Digital pH meter	The pH values of the media were measured (Photo 9)
Magnetic stirrer	It was used to mix the media weighed into the flasks so that they could be dissolved in distilled water and to prepare the artificial honey mixture (Photo 10)
Water bath	It was used to dissolve the media (Photo 11)
Autoclave	It was used in the sterilization of media and sterile distilled water by providing it for 15-20 minutes at 121 °C in an environment saturated with pressurized water vapor (Photo 12)
Vortex mixer	It was used to mix tube contents (Photo 13)
Nanodrop ND 100 Spectrophotometer	It was used to determine the inoculation levels of test microorganisms (Photo 14)
Petri dish	It was used in agar well diffusion and disc diffusion tests (Photo 15)
Drigalski spatula	It was used to spread test bacteria on media (Photo 16)
Sterile glass pipette (1-10ml)	It was used for microbiological inoculation (Photo 17)
Sterile glass tube	It was used for the production of microorganisms (Photo 18)
Automatic pipette	It was used to prepare low honey concenrations (Photo 19)
Eppendorf tube	Used to prepare honey dilutions (Photo 20)
Sterile blank discs	These are small discs with a diameter of 6 mm, obtained from special absorbent papers for use in laboratories and used to absorb desired substances. It was used in the disc diffusion test in this study
Inoculation loop	It was used for transferring samples or microorganisms to the medium, (Photo 21)
Digital caliper	It was used to measure the zones formed in this study (Photo 3)
Bacterial Cultures Used	Staphylococcus aureus ATCC 25923

Method Development

1- Preparation of Bacterial Culture

The antibacterial properties of the collected honey have been tested against *S. aureus* that cause infections in wounds. *Staphylococcus aureus* ATCC 25923 was used as reference strains. It was inoculated into Brain Heart Infusion Broth (Oxoid, UK) and incubated at 37°C for 24 hours. The bacterial culture was then adjusted to have an optical density of 0.5 at 450 nm in Peptone Water (Oxoid, UK).

2- Methods used in Antibacterial Activity

The antibacterial activity of honey was evaluated using 2 different methods. In this context, Disc Diffusion and Agar-Well Diffusion methods were preferred. In each method, it was investigated up to which concenration the honey showed antibacterial activity. For this purpose, each honey sample was diluted to 7 serial concentrations. Then, each honey concentration was tested on microorganism with these 2 different methods (Table 3).

3- Collection of Pine Honey and Flower Honey Samples

This study was carried out with unpasteurized, unprocessed, 15 flower and 15 pine honey samples from local beekeepers. Each sample was collected in sterile glass containers and kept in the dark at room temperature (23-25°C) until testing (Photo 4,5,6).

Procedure

Preparation of Honey Samples

1- Honey concenrations for use in all methods were prepared aseptically at rates ranging from 50% to 0.75%. 50% honey concenration prepared in sterile balloon flask was diluted with sterile distilled water and 7 series were made; 50%, 25%, 12.5%, 6%, 3%, 1.5%, 0.75%.

- For this purpose, 50 g honey sample added to 100 volumetric flask was made up to 100 ml with distilled water and mixed until dissolved. Then, 50% honey sample was diluted at a ratio of 1:1 in a sterile flask and 25% concentration was obtained. Other concentrations were also obtained by concentration the previous dilution. (Photo 23, 24)

2- In addition, sugar solution was used as a control group in order to reveal whether the antibacterial activity is due to the components found in the structure of honey or due to the high sugar concentration.

-In this context, it was prepared by dissolving 40.5% fructose, 7.5% maltose, 33.5% glucose and 1.5% sucrose in 100 ml sterile distilled water in the final volume (26). Serial concentrations from 50% to 0.75% were applied in artificial honey, in the same way as in other honeys.

Preparation of bacterial strains: The antibacterial properties of honey were tested against a common wound pathogen. *Staphylococcus aureus* ATCC 25923 was used as reference strains.

-Each of the strains was inoculated into Brain Heart Infusion Broth (Oxoid, UK), and incubated overnight at 37 °C.

-The concentration of bacterial strains was quantified using Nanodrop spectrophotometer (ND-1000 spectrophotometer) at 450 nm. In this context, the blank was made twice with Brain Heart Infusion Broth. Then 2-3 microliters of bacterial cultures were taken using an automatic pipette and placed in the chamber of the device. After the measurement of samples was made, the concentration of each strains was adjusted to 0.5 optical density at 450 nm in sterile peptone water.

Disc Diffusion Method

- Test pathogen (*S. aureus*) adjusted to have an optical density of 0.5 at 450 nm in Peptone Water was inoculated onto the surface of petri dishes containing Nutrient agar (Oxoid, UK) using sterile swabs.

- Petri dishes were then dried at room temperature for 15 minutes. Each honey concentration was poured into sterile empty petri dishes.

-Sterile absorbent blank discs with a diameter of 6 mm were placed in these honey concentrations for 10 minutes and the discs were allowed to absorb the honey.

-All discs were then placed in petri dishes inoculated with bacterial cultures and gently pressed to ensure full contact with the medium.

-Distances of 15 mm were placed on the edges of the petri dishes and between the discs to avoid overlapping of the inhibition zones.

-A sterile blank disc was used as the control group. Petri dishes with discs were incubated at 37°C for 24 hours.

-After the incubation period, the diameters of the inhibitions were measured using an electronic caliper (23) (Photo 3)

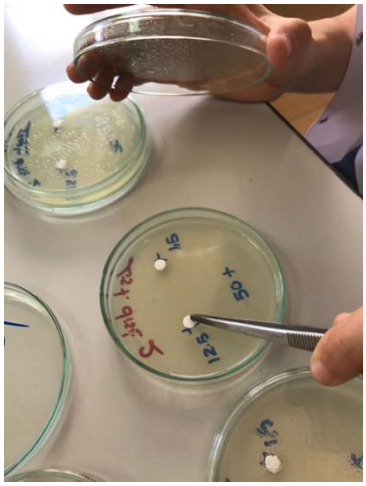

Photo 1- Disc Diffusion Method

Agar-Well Diffusion Method

- The test pathogen (*Staphylococcus aureus*) adjusted to have an optical density of 0.5 at 450 nm in Peptone Water was mixed with freshly prepared 20 ml Nutrient agar and immediately poured into empty sterile petri dishes.

-8 mm diameter cavities were drilled on their surfaces. Distances of 15 mm were placed on the edges of the petri dishes and between the wells to avoid overlapping of the inhibition zones.

-180 microliters of each dilution of diluted honey was inoculated into each well.

-Petri dishes were then incubated at 37°C for 24 hours.

-After incubation, zones of inhibition were measured using an digital caliper (23) (Photo 2, Photo 3)

Photo 2- Agar Well Diffusion Method

Photo 3- Digital caliper measuring

Safety Issues and Ethical Considerations

Glass materials, devices and test bacteria used in the study were obtained from Ankara University Veterinary Faculty laboratories and no patient or a person was harmed during the study. Analyzes were performed by wearing a lab coat, goggles, and gloves, in compliance with biosafety rules. In addition, since the work was carried out during the pandemic period, it was worked in accordance with the mask and distance rules. Due to the fact that the pathogen microorganism was studied, the study was carried out under the supervision of an expert.

Results and Analysis

Table 3- Antibacterial effect of flower honey samples on *Staphylococcus aureus* and the diameters of the inhibition zones formed

Different Brand and Same type of Honey Sample (Numbers)	Independent Variable Concentrations of Honey	METHODS (Pine Honey Samples) (inhibition zones in cm)		METHODS (Flower Honey Samples) (inhibition zones in cm)	
		Disc Diffusion(cm) (± 0.01)	Agar-Well Diffusion (cm) (± 0.01)	Disc Diffusion(cm) (± 0.01)	Agar-Well Diffusion (cm) (± 0.01)
1	50 %	2.87	4.98	1.82	3.81
	25 %	2.52	4.21	1.01	3.26
	12.5 %	1.84	3.63	-	2.63
	6.25 %	1.02	2.81	-	1.72
	3.13 %	-	2.13	-	-
	1.56 %	-	1.05	-	-
	0.78 %	-	-	-	-
	50 %	2.85	5.32	2.33	3.65
	25 %	2.14	4.58	1.66	3.12
	12.5 %	1.23	3.76	1.03	2.65
	6.25 %	-	2.43	-	2.13

2	3.13 %	-	1.84	-	1.12
	1.56 %	-	1.03	-	-
	0.78 %	-	-	-	-
3	50 %	2.22	4.87	2.42	3.21
	25 %	1.28	3.74	1.35	2.49
	12.5 %	-	3.16	-	1.62
	6.25 %	-	2.59	-	1.12
	3.13 %	-	1.82	-	-
	1.56 %	-	1.02	-	-
	0.78 %	-	-	-	-
4	50 %	2.11	3.72	2.63	3.57
	25 %	1.33	2.63	2.16	3.24
	12.5 %	1.04	1.85	1.59	2.64
	6.25 %	-	1.19	-	2.13
	3.13 %	-	-	-	1.87
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-
5	50 %	2.82	3.42	2.62	3.45
	25 %	1.86	2.65	2.16	3.12
	12.5 %	1.05	1.87	1.89	2.48
	6.25 %	-	1.12	1.04	1.79
	3.13 %	-	-	-	1.0
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-
	50 %	2.97	4.69	1.82	3.64
	25 %	2.27	3.98	0.97	3.28
	12.5 %	1.38	3.14	-	2.65

6	6.25 %	-	2.78	-	2.10
	3.13 %	-	2.15	-	1.03
	1.56 %	-	1.13	-	-
	0.78 %	-	-	-	-
7	50 %	2.86	4.28	1.83	3.59
	25 %	2.43	3.76	1.02	2.61
	12.5 %	1.84	2.93	-	1.78
	6.25 %	1.03	2.17	-	1.01
	3.13 %	-	1.69	-	-
	1.56 %	-	1.05	-	-
	0.78 %	-	-	-	-
8	50 %	2.95	5.28	1.76	3.19
	25 %	2.21	4.37	1.09	3.54
	12.5 %	1.63	3.41	-	2.37
	6.25 %	1.02	2.81	-	2.12
	3.13 %	-	1.36	-	1.41
	1.56 %	-	1.18	-	-
	0.78 %	-	-	-	-
9	50 %	2.38	3.57	2.53	3.23
	25 %	1.76	3.23	1.72	2.64
	12.5 %	1.19	2.65	1.11	1.78
	6.25 %	-	1.86	-	0.96
	3.13 %	-	1.12	-	-
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-
	50 %	1.98	3.52	1.57	3.17
	25 %	1.05	2.26	1.02	2.38

10	12.5 %	-	1.63	0.87	1.67
	6.25 %	-	1.08	-	1.01
	3.13 %	-	-	-	-
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-
11	50 %	2.74	4.63	1.98	3.62
	25 %	2.01	4.11	1.01	3.23
	12.5 %	1.13	3.72	-	2.15
	6.25 %	-	3.21	-	1.28
	3.13 %	-	2.54	-	-
	1.56 %	-	1.12	-	-
	0.78 %	-	-	-	-
12	50 %	2.59	4.17	1.21	3.01
	25 %	2.02	3.82	-	2.14
	12.5 %	1.51	3.29	-	1.63
	6.25 %	-	2.86	-	-
	3.13 %	-	2.18	-	-
	1.56 %	-	1.56	-	-
	0.78 %	-	-	-	-
13	50 %	2.21	3.71	2.39	3.72
	25 %	1.24	3.04	1.32	3.26
	12.5 %	-	2.73	1.03	2.79
	6.25 %	-	2.03	-	2.15
	3.13 %	-	1.18	-	1.09
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-
	50 %	2.85	4.59	2.68	3.63

14	25 %	2.24	3.98	1.53	3.21
	12.5 %	1.42	3.41	1.03	2.62
	6.25 %	-	2.31	-	1.79
	3.13 %	-	1.63	-	1.02
	1.56 %	-	1.02	-	-
	0.78 %	-	-	-	-
15	50 %	2.98	3.56	2.10	3.48
	25 %	1.94	3.23	1.75	3.62
	12.5 %	1.01	2.16	1.02	2.17
	6.25 %	-	1.72	-	1.03
	3.13 %	-	1.06	-	-
	1.56 %	-	-	-	-
	0.78 %	-	-	-	-

Diameter in millimeters of the zone of inhibition, (–) = negative

Table 4 - Antibacterial effect of sugar solution on *S. aureus* and the diameters of the inhibition zones formed

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Concentrations	*Staphylococcus aureus*	
	Agar Disc Diffusion (inhibition zone in cm)	Agar Well Diffusion (inhibition zone in cm)
% 50	1.32	2.63
% 25	-	2.02
% 12.5	-	1.21
% 6.25	-	-
% 3.13	-	-
% 1.56	-	-
% 0.78	-	-

Diameter in millimeters of the zone of inhibition, (–) = negative

The results obtained in the study were evaluated by making statistics (14). In this context, the antibacterial effect difference between flower honey and pine honey, the comparison of the methods in terms of sensitivity and the change of inhibition zones according to concentration are given in Table 5.

Table 5. Comparison results of honey types and methods

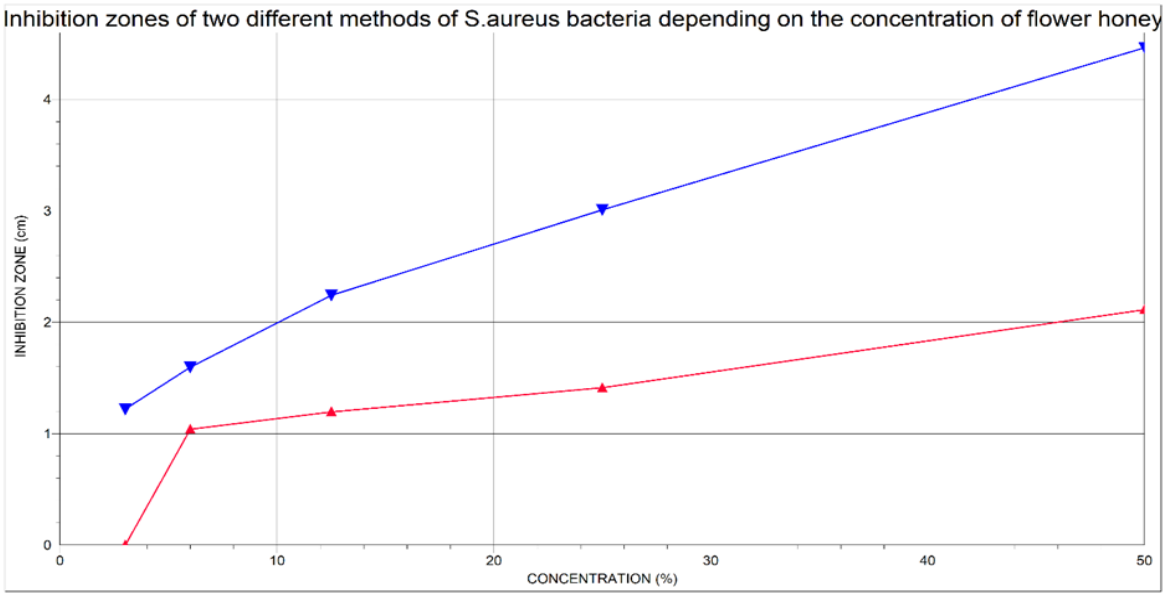
Concentration	Flower Honey							Pine Honey						
	Method	Inbition zones (cm)					p1	Inbition zones (cm)					p1	P2
		N	Min	Max	Mean	St.Dev		N	Min	Max	Mean	St.Dev		
% 50	Disc Diffusion	15	1,21	2,68	2,113	0,443	**0,001**	15	1,98	2,98	2,625	0,348	**0,001**	**0,001**
	Agar Well Diffusion	15	3,01	3,81	3,465	0,242		15	3,42	5,32	4,287	0,671	-	**0,001**
% 25	Disc diffusion	14	0,97	2,16	1,412	0,425	**0,001**	15	1,05	2,52	1,887	0,462	**0,001**	**0,007**
	Agar Well Diffusion	15	2,14	3,62	3,009	0,442		15	2,26	4,58	3,573	0,696	-	**0,023**
% 12.5	Disc Diffusion	8	0,87	1,89	1,196	0,351	**0,012**	12	1,01	1,84	1,356	0,298	**0,002**	**0,135**
	Agar Well Diffusion	15	1,62	2,79	2,242	0,438		15	1,63	3,76	2,889	0,715	-	**0,004**
% 6.0	Disc Diffusion	1	1,04	1,04	1,040	.	-	3	1,02	1,03	1,023	0,006	-	-
	Agar Well Diffusion	14	0,96	2,15	1,596	0,499	-	15	1,08	3,21	2,198	0,685	-	**0,009**
% 3.0	Disc Diffusion	-	-	-	-	-	-	-	-	-	-	-	-	-
	Agar Well Diffusion	7	1	1,87	1,220	0,319	-	12	1,06	2,54	1,725	0,475	-	**0,017**

- **N** Number of samples with zones of inhibition
- **p1** Difference between methods
- **P2** The difference between flower and pine honey

Statistical data analysis

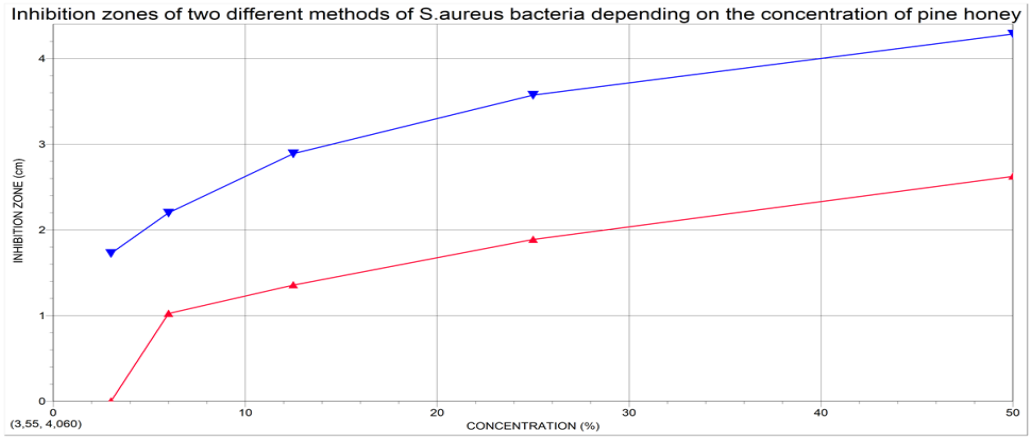
In the study, the antibacterial effect of flower honey types and pine honey types was tested by T test in dependent groups, difference between methods T test in independent groups and analysis of variance between dilutions (14)

Graphic 1- Inhibition zones of *S.aureus* formed in two different methods depending on the flower honey concentration.



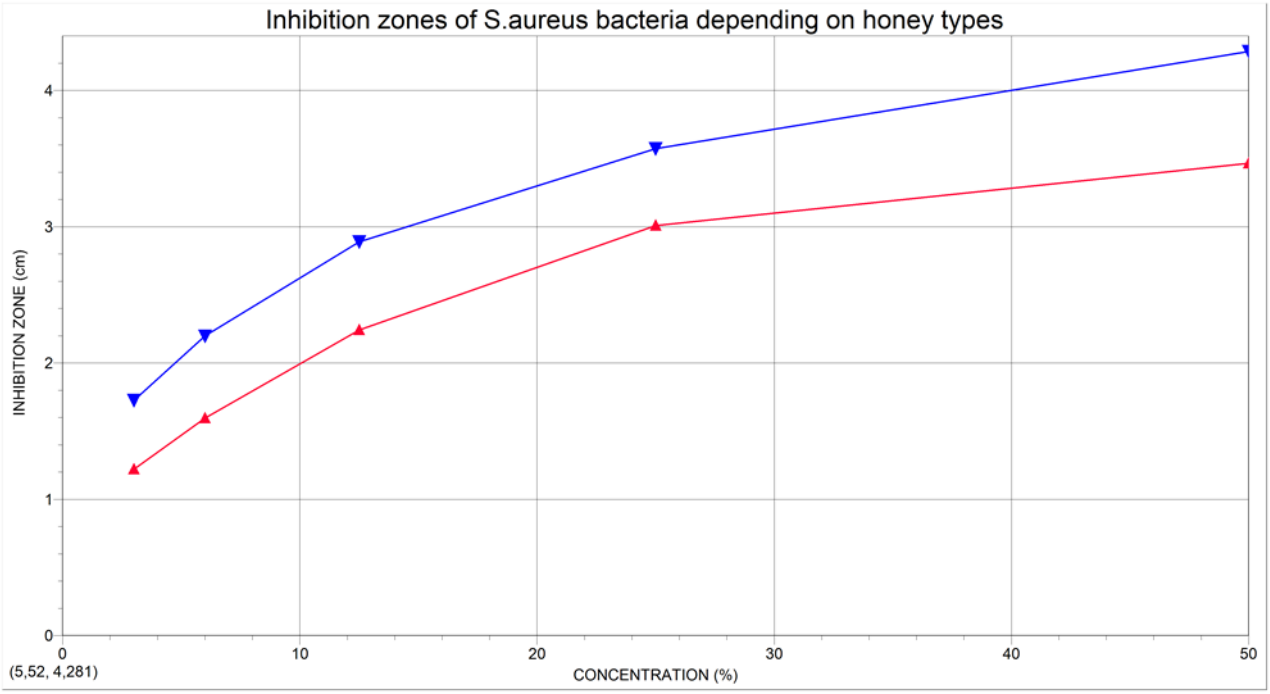
- DISC DIFFUSION METHOD
- AGAR WELL DIFFUSION

Graphic 2- Inhibition zones of *S.aureus* formed in two different methods depending on the pine honey concentration.



- DISC DIFFUSION METHOD
- AGAR WELL DIFFUSION

Graphic 3- Values of inhibition zones of *S. aureus* depending on honey species



- FLOWER HONEY
- PINE HONEY

Analysis and Discussion

My aim in this experiment was to examine flower honey types and pine honey types which have an antibacterial effect on wound infection agent *Staphylococcus aureus*, considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion.

When the inhibition zones on *S. aureus* were examined in the comparison of honeys according to their origins, an inhibition zone with a diameter of 2.11 cm at 50% flower honey concentration and 2.62 cm at 50% pine honey concentration in disc diffusion method. In the agar well diffusion method, an inhibition zone with a diameter of 3.46 cm with flower honey and 4.28 cm with pine honey was created on *S. aureus* at the same concentration. When considering the 25% concentration, pine honey showed more inhibitory effect for *S. aureus* in both methods and showed a significant difference with flower honey ($p<0.01$).

When a general evaluation is made, it is seen that the antibacterial effect of pine honey is higher than flower honey and the difference is significant ($p<0.01$). When the changes in the antibacterial effects of honey according to concentration are examined, it is seen that the degree of inhibition decreases proportionally with concentration ($p<0.001$). The highest inhibition was found in both pine and flower honey at 50% concentration in both microorganisms. In flower honey, there was no inhibition in both microorganisms, in the last two concentration (1.5%, 0.75%) and in both methods, while no inhibition was observed in pine honey at only 0.75% concentration. Considering the diameter of the inhibition zones and the concentration in which the final inhibition is seen, it is seen that pine honey has a stronger antibacterial effect compared to flower honey (Graph.3) As seen in the Table 5, since P-value being smaller than 0.05, there is statistically significant mean difference amongst groups.

As a result, H_1: "The hypothesis put forward in this study is that different concentrations and types of honey (pine honey and flower honey) have a different antibacterial effect on *Staphylococcus aureus* by considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion. " is accepted and H_0: "The hypothesis put forward in this study is that different concentrations and types of honey (pine honey and flower honey) don't have a different antibacterial effect on *Staphylococcus aureus* by considering the inhibition zone formed using two different methods- agar well diffusion and disc diffusion." is rejected.

Osmotic pressure is an important factor that inhibits the growth of microorganisms. The high osmotic pressure of honey may also provide an antibacterial effect (3,5). In order to distinguish the antibacterial activity of honey from osmolarity, artificial honey (sugar solution) was made and tested on *S. aureus* with the same methods. Examining the results, *S. aureus* was inhibited at 50% and 25% concentration of artificial honey in the agar well diffusion method, while inhibition occurred at only 50% concentration in the disc diffusion method. When compared with the flower and pine honeys examined, it is understood that there is no inhibition zone in low concentrations of artificial honey and the antibacterial effect of honey samples is due to components other than osmotic pressure. In a study, it was stated that *S. aureus*, an osmotolerant bacterium, can grow at different osmotic pressures (3,24).

Evaluation

When the methods are compared, the value graph of the Agar well diffusion method has been taken as a more reliable, consistent and sensitive method since the value graph of the disc diffusion method shows a more linear graph and the inhibition zone values are evident (Graph.1, Graph.2). For example, honey sample with 50% concentration in flower honey created an inhibition zone with a diameter of 3.465 cm on *S. aureus* in agar well diffusion, while a zone with a diameter of 2.113 cm in disc diffusion method was found to be significant between the methods ($p<0.001$). While a similar situation was observed at 25% dilution, the difference was found to be significant ($p<0.01$). In addition, the zone of inhibition in the Agar well diffusion method also occurred at low concentration. It has been reported that the agar well diffusion method mimics the spread in the tissue similar to wound treatment (20). The low sensitivity of the disc diffusion method has been attributed to the inability of the large molecules in honey to be fully absorbed by the disc (26). Although it does not give very good results in determining the antibacterial effect in honey samples, the disc diffusion method is currently the most used method in antibiotic tests.

Since the experiment I did was done during the Corona Virus Pandemic, the number of trials was limited due to the lack of equipment used during the experiment. Increasing numbers of trials could be made to elimite the errors.

Another problem encountered in this study is that it is somewhat difficult to set the agar hardness very well and to drill the wells properly in the agar well diffusion method. Many attempts have been made for this. In addition, since honey is a viscous food when weighing, its temperature being between 20-25 °C provides easy and error-free weighing. It is necessary to pay attention to this.

Conclusion

Nowadays, due to the side effects of antibiotic use and antibiotic resistance developed by pathogens that cause infections, the interest in alternative substances to antibiotics is increasing day by day. Among these substances, one of the most noted foods with antibacterial activity is honey. However, its mechanism is still not fully understood and its

effectiveness on pathogens has not been standardized (18). Honey is a natural product and is produced from a variety of plants, so the nutritional and medicinal profile of each honey varies. The properties of honey associated with its antibacterial activity are significantly affected by geographical location and botanical origin (1). Studies have revealed that honeys obtained from different geographical regions show significant and variable antibacterial activity against bacteria. In this context, in this study, the in vitro antibacterial effect of flower honey and pine honey against *S. aureus*, which is a wound pathogen, was investigated and the efficacy of the selected tests to determine the antibacterial activity were compared. As predicted in the hypothesis, pine honey and flower honey were found to have a different antibacterial effect on S. aureus in the study. It was determined that the test microorganism, S. aureus, was sensitive to the tested honey in direct proportion to the concentration. And it seen that pine honey has a better antibacterial effect on S. aureus rather than flower honey in the study.

Since this experiment was carried out with honey samples taken from honey growers in the same province, the antibacterial effect of honey from different regions may also differ. Due to the composition of honey, it may be difficult to apply on the wound compared to other medicinal drugs. Because there may be different substances in it, it can cause allergic reactions when used for therapeutic purposes. For this reason, more studies are needed for the use of honey for therapeutic purposes.

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Appendix

Appendix 1

Photo 4- Flower honey samples

Photo 5- Pine honey samples

Photo 6- Flower and pine honey samples

Photo 7- Sensitive Scale

Photo 8- Bunsen Burner

Photo 9- pH meter

Photo 10- Magnetic stirrer

Photo 11- Water bath

Photo 12- Autoclave

Photo 13- Vortex mixer

Photo 14- Nanodrop ND100 spektrophotometer

Photo 15- Petri dish

Photo 16- Drigalski spatula

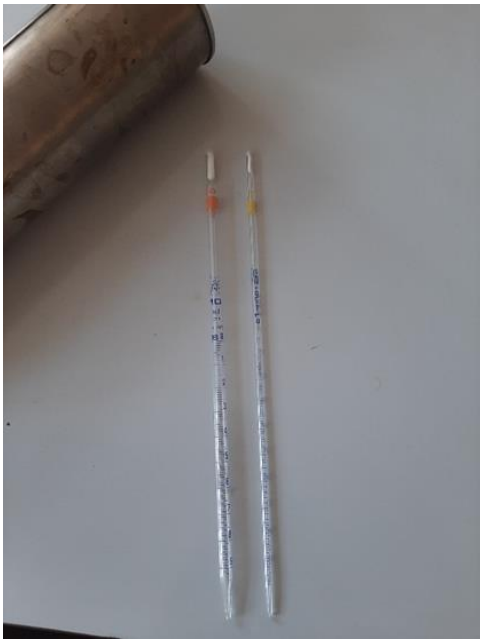

Photo 17- glass pipette

Photo 18- Glass tube

Photo 19- Automatic pipette

Photo 20- Eppendorf tube

Photo 21 - Inoculation loop

Photo 22- Digital caliper

Photo 23- Pine honey concentrations

Photo 24- Flower honey concentrations

Appendix 2

The student has performed their experiment in Ankara University, Department of Food Hygiene and Technology themselves with supervision from our staff.

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