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Research Article

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Antibacterial Activity of Four Different Libyan Origin Honey Against Salmonella Species

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ABSTRACT

Different studies have shown the antimicrobial activity of honey from different countries in the world. The objectives of this study were to conduct such studies and to compare the antimicrobial activity of four types of Libyan honey, AL-sader honey, spring honey, thyme honey and hanone honey against *Salmonella poona,* and *Salmonella typhimurium*. Screening the potential antibacterial activity of honey against *Salmonella poona,* and *Salmonella typhimurium* by *in vitro* methods was conducted in the experimental laboratories at the Microbiology Research Laboratory, University of Tripoli, Tripoli, Libya from January to May 2015. The microbiological tests such as disk diffusion method, cup cut agar method, minimum inhibitory concentration (MIC) and growth curve patterns were used for the determination of the microbial loads. There were non-significant differences between the four honey types against both bacteria species. For the activity of 100% pure Spring honey, the zones observed were against *S. poona* (2.2± 0.1mm) and *S. typhimurium* (2.1± 0.2mm), 100% pure Al-Sader honey *S. poona* (2.4± 1mm) and *S. typhimurium* (2.1± 0.15 mm), thyme honey *S. poona* (2.2 ± 0.08mm) and *S. typhimurium* (2.06± 0.08 mm), Hanon honey, *S. poona* (2.6± 0.1mm) and *S. typhimurium* (1.9± 0.15 mm). This study of Libyan honey shows that the highest activity was against *S. typhimurium* and *S. poons*. The Al-Sader honey, and Hanone honey had exceptionally high activity against *S. typhimurium* and *S. poons*.

Keywords- *Antibacterial, Honey, Salmonella.*

INTRODUCTION

Antibacterial agents are effectively decreasing the spreading of infectious diseases in the world. However, the effectiveness of the antibiotics is weakened as the resistance of bacteria develops and spreads. This type of bacterial resistance is a very serious threat to public health.[1] Consequently, substitute antimicrobial plans are immediately required, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey.[2] The highest components of honey are sugars (30.3% glucose ,18.4% fructose, 1.3% sucrose and 12% other carbohydrates). The other components include, 0.169% minerals, 169 mg/100 g of proteins, with a water content of about 17.2%.[3] Honey has been used for centuries as a food preservative and also as a gastrointestinal and topical remedy.[4]

The diversity of flora, gives the opportunity for producing a wide variety of honey from coniferous and citrus trees, thyme or other floral origin which gives to the final product, those special sensorial properties. But despite the variety and recognized quality, little research has been done towards the antibacterial properties of honey. Therefore in the current study we sought to study the antibacterial potency of natural honeys, against isolated pathogens and their respective reference bacterial strains.[5]

Previously, several studies have reported the antibacterial activity of honey and they found that natural unheated honey has some broad-spectrum antibacterial activity when tested against pathogenic bacteria.[6] Honey has been used for both nutritional and medical purposes. Medical treatments using honey and other bee products include fighting bacterial infections.[7] *Salmonella* is one of the major bacteria causing infections in humans, commonly causing an acute, self-limiting gastroenteritis which usually does not require treatment with antibiotics.[8] In this study the antibacterial activity of Spring honey, Al-Sader honey, Thyme honey and Hanone honey from Libya, against *Salmonella poona* and *Salmonella typhimurium* were determined.

MATERIALS AND METHODS

Microorganism

The bacterial strains used in this study were, *Salmonella poona,* NCTC 4840 and *Salmonella typhimurium* ATCC 14028, the standard bacterial strains were activated and cloned three successive times in nutrient agar and stored on nutrient agar slants at 4°C.

Preparation Honey samples

Four Libyan honeys from four floral sources were obtained, Spring honey from Tripoli, Al-Sader honey from Al Kamous, Thyme honey from Tarhona and Hanone honey from Al Beyda city.

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Immediately before conducting microbiological assays to determine if survival or growth of pathogens is influenced by honey, all test honeys and the control honey were adjusted in order to aid pipetting during preparation of diluted honey solutions. The solutions contained 25%, 50% 75% and 100% w/v. The honeys were prepared in sterile distilled water. These solutions were used to the saturate paper disks used in assays to determine zones of inhibition of growth.

Determination of activity by disk method

Antimicrobial susceptibility was tested using the paper disc agar diffusion method.[9] Paper discs (6 mm in diameter, Whatman No.1) were sterilized by autoclave (120°C/ 15 min) and soaked in a honey solution with different concentrations (25%, 50% 75% and 100% w/v). The solutions at varying concentrations were placed separately in the plate, under aseptic conditions. Antibiotics such as Tetracycline, Amoxicillin, and Cephalexin (5 µg/ disk) were used as a standard. The controls were prepared without honey. Triple plates were used for each concentration. The agar plates were maintained at room temperature for 2 h, allowing the diffusion of the solution. All plates were then incubated at 37°C for 24 h. The zones of inhibition were subsequently measured in millimeters.[10]

Cup cut agar method

The cup-cut agar method was used throughout this study to find out if the honey has the ability to inhibit bacteria growth.[11] This method was performed using freshly prepared Mueller Hinton agar with overnight culture of bacteria inoculums, which were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. On each plate, wells (5 mm) were made by a sterile cork borer. Each well was filled with 100µL of the honey solution and the plates were then re-incubated for 24 h at 37°C. The diameter of the zones of inhibition was measured.[11] The inhibition zones were then measured in millimeters. Inhibition zones indicated a lack of microbial growth due to inhibitory concentrations. The antibiotics: Tetracycline, Amoxicillin, Cephalexin (5 µg/ disk) were used as standards to compare the activity of honey in inhibiting the growth of bacteria. Each experiment was carried out three times.

Minimum Inhibitory Concentration (MIC)

All the materials and equipment that were used were sterilized using an autoclave. Inoculates were prepared by growing each strain of tested microorganism in nutrient broth adjusted to a turbidity equal to that of a No. 0.5 McFarland standard, by using a blank nutrient broth (to get the bacteria number of about $1x10^8$ CFU /ml). The honeys were prepared by taking the calculated weight of honey and adding 1ml sterile nutrient broth, to give a final concentration equal to 80% w/v. Then, serial dilutions of solution were prepared in 1ml nutrient broth. Positive control tubes were also inoculated in the same manner, all tubes were incubated at 37°C. Control tubes were tested after 24 h to determine whether the solution-containing tubes were ready to be read. This was accomplished by adding 0.02 ml of Aalamar blue (at the concentration of 0.0125% w/v resazurin salt in PBS solution) to the positive control tube and incubating it for 10 min at 37°C. When the color in the control tube changed from blue to pink after 10 min of incubation, then Alamar blue was added to the honey-containing tubes and these were incubated for 10 min at 37°C. The absorbance was measured at 600nm using a UV spectrophotometer.

The results were expressed as percentage reduction in bacteria viability compared to controls and concentration that gave 50% inhibition (IC_{50}), this was calculated by Probit analysis.[12] The mean value was calculated from three separate experiments.

Determining the growth curve of bacterial cell exposed to honey

The growth curve of eight bacteria was drawn per standard methods.[13] Liquid culture of bacteria was prepared by inoculating a single colony into 20 ml nutrient broth medium in a sterilized conical flask and then it was incubated for 24h at 37˚C. The culture was diluted (1:50) into 0.4 ml for 20 ml culture. 1 ml of honey solution (75% w/v) was added and then incubated at 37˚C in a water bath, with shaking. The time–zero absorbance was read at 600 nm on a UV spectrophotometer (Biochrom, UK). Subsequent absorbance readings were recorded in 1-hour intervals for 7 h, after 24/ 25 h. The results were transferred to Excel, and the mean absorbance values were calculated.

Statistical analysis of data

The data was tested for normality using a QC Analyses/K-S Normality Test. Normally distributed data was analyzed by student's t-test using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, Inc., Berkeley, CA, USA). A *P* value of < 0.05 was considered significant.

RESULTS

Interest lies in using the natural antimicrobials in honey- antibacterial, antifungal and antiviral; to eliminate foodborne pathogens or control their growth.[14] In this study, we compared the zone inhibition diameter values of four honey types determined by well diffusion assay against *Salmonella poona* and *Salmonella typhimurium*. The inhibitory effect of the tested honeys at various concentrations is shown in Table 1. The disk method results and cup cut diffusion explained that the four honey types have an effect against *S. poona* and *S. typhimurium*. There are non-significant differences between the four honey types against both bacteria species. As for the activity of 100% pure spring honey, the zones observed were against *S. poona* (2.2± 0.1mm) and *S. typhimurium* (2.1± 0.2mm), the 100% pure Al-Sader honey had *S. poona* (2.4± 1mm) and *S. typhimurium* (2.1± 0.15 mm), Thyme honey, *S. poona* (2.2 ± 0.08mm) and *S. typhimurium* (2.06± 0.08 mm), and Hanon honey: *S. poona* (2.6± 0.1mm) and *S. typhimurium* (1.9± 0.15 mm). The 25% of honey concentration was not effective on either bacteria species nor for the four honey samples. In the concentration of honey at 50 % there was only an effect on the *S. typhimurium* and no effect on *S. poona*.

The effect of honey on the growth curve of bacteria shows that the four types of honey reduced the OD value of bacteria culture, which is considered a significant reduction compared to control samples without honey. In *Salmonella typhimurium,* the Spring honey had the highest

Table 1: Mean diameter (mm) of inhibition zones by honey samples against *Salmonella poona* and *Salmonella typhimurium* reference strains

Honey type	Concentration W/V%	Mean Zone of inhibition (mm± SE)			
		Salmonella poons		*Salmonella typhimurium*	
		Cup cut agar method	Disk method	Cup cut agar method	Disk method
Spring	25%	0±0	0 ±0	0 ±0	0±0
	50%	0±0	0 ±0	1.7 ± 0.1	1.06 ± 0.01
	75%	0±0	0±0	1.86 ± 0.06	1.5±0.01
	100%	2.2 ± 0.1	1.53±0.01	2.1 ± 0.2	1.5±0.02
AL-Sader	25%	0 ±0	0 ±0	0 ±0	0 ±0
	50%	0 ±0	0 ±0	1.53 ± 0.08	0.5 ±0.02
	75%	1.7±0.01	0 ±0	2 ±0.05	0.8±0.02
	100%	2.4 ± 0.1	0.5±0.05	2.1 ±0.15	1.2 ± 0.05
Thyme	25%	0 ±0	0 ±0	0 ±0	0 ±0
	50%	0 ±0	0 ±0	1.63 ±0.03	1.13 ± 0.08
	75%	0 ±0	0 ±0	1.9 ±0.15	1 ± 0.03
	100%	2.2 ±0.08	1.06±0.01	2.06 ±0.08	1.5 ± 0.03
Hanone	25%	0 ±0	0 ±0	0 ±0	0 ±0
	50%	0 ±0	0 ±0	1.56 ± 0.18	0.56 ± 0.09
	75%	0 ±0	0 ±0	1.8 ±0.1	0.9 ±0.01
	100%	2.6 ±0.1	1.13 ±0.08	1.9 ± 0.15	1 ±0.08
Tetracycline		1.6 ± 0.05		2 ± 0.05	
Amoxicillin		1.7 ±0.06		1.9 ±0.05	
Cephalexin		2.1 ± 0.03		2 ± 0.05	

effect on the reduction of the bacteria growth curve, then Al Sader, Hanon and the Thyme honey (Figure 1) and the percentage of growth inhibition (Figure 2). However, the Al Sader honey had the highest effect on the reduction of *S. poons* bacteria growth curve, then Hanon, Al Sader, and the Spring honey (Figure 1), as well as the % of growth inhibition (Figure 2). In *S. poons*, the Hanone honey had less IC_{50} (49.2±2 w/v%) than Al Sader (60.9±2 w/v%), Thyme (69 ±1 w/v%), Spring honey (72 ±1 w/v%). In *S. typhimurium,* the Hanone honey had less IC_{50} (16±3 w/v%) than Al Sader and Spring honey (13.8±4 w/v%), Thyme (44.1 ±2 w/v%) (Table 2). The result showed that the Al Sader honey had significantly *(P***<0.0.5)* reduced the *S. poons* growth at 4 h. compared to the control (non-treated bacteria with honey in both *S. typhimurium* and *S. poons*.

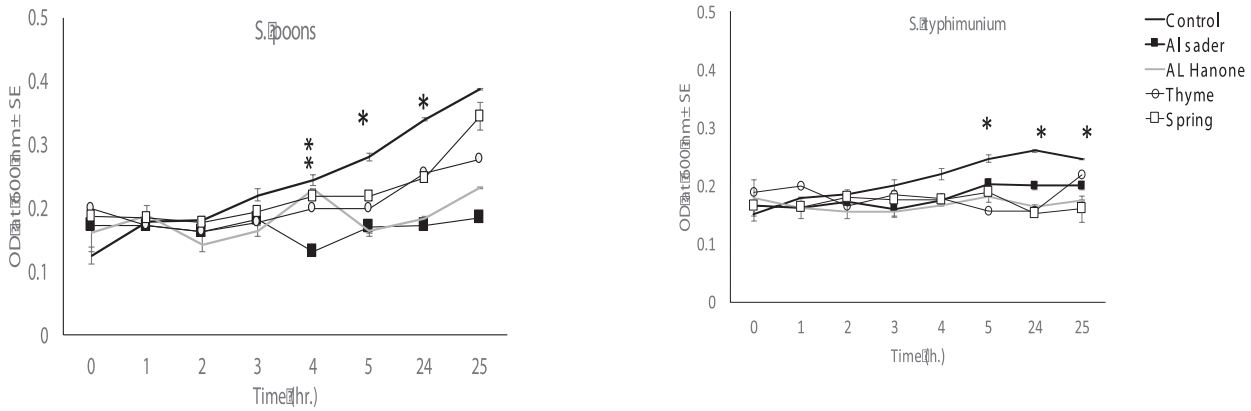

Figure 1: The effect of four type of honey on the bacteria growth curve (*P**<0.05 comparing all honey type to control) (*P***<0.05 comparing control to AL- Hanone only).

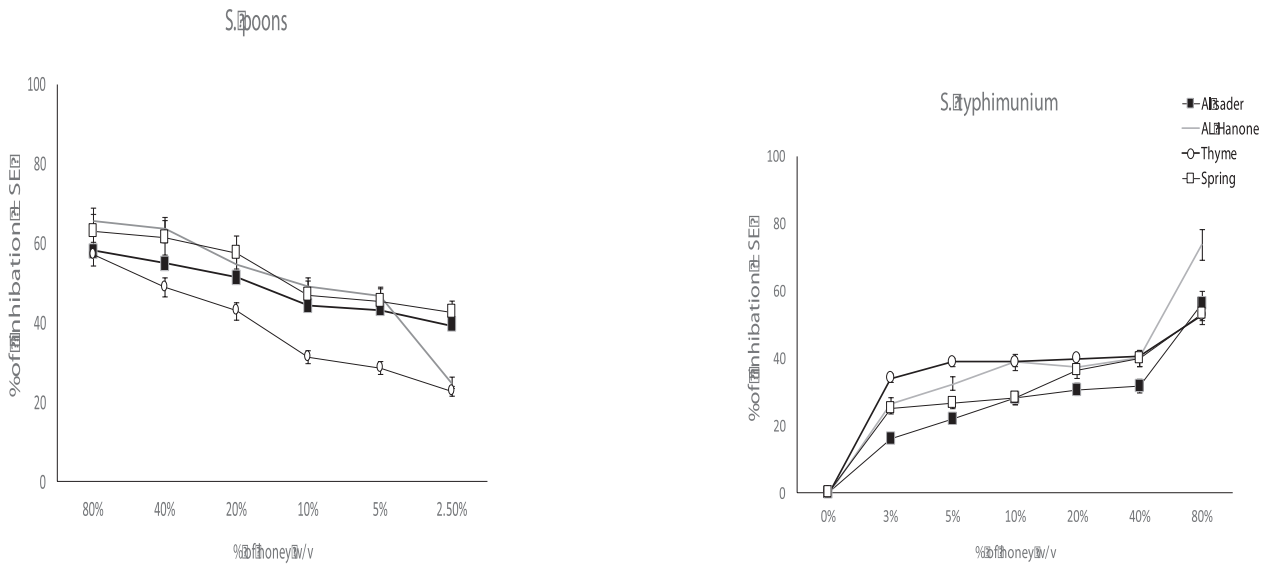

Figure 2: The percentage of inhibition of four type of honey against *Salmonella poona* and *Salmonella typhimurium* reference strains.

Table 2: The value of $IC_{50,}$ that four type of honey against *Salmonella poona* and *Salmonella typhimurium* reference strains.

Honey Type	IC_{50}(w/v%)± SE	
	Salmonella poons	*Salmonella typhimurium*
AL Hanone	49.2±2	16.0±3
Thyme	69.2±1	44.1±2
Al Sader	60.9±2	13.8±4
Spring	72.3±1	13.4±2

DISCUSSION

There are many studies on honey microbial activities like antibacterial, antifungal and antiviral.[14] In this study, we compared the zone inhibition diameter values of four honey types determined by well diffusion assay against *Salmonella poona* and *Salmonella typhimurium*. The inhibitory effect of the tested honeys at various concentrations using disk method and cup cut diffusion explained that the four honey types have an effect against *S. poona* and *S. typhimurium*. There are non-significant differences between the four honey types against both bacteria species. It was clear that all honey types at 100% showed inhibition of bacterial growth. Also it was obvious that the inhibition of *S. typhimurium* and *S. poons* was dependent on the honey origin. The result was supported by a number of previous studies that have demonstrated that various honeys, have antibacterial activity. Alqurashi A. M. Meaama (2013) in their study of different types of Saudi honey found that the inhibition of *E. coli* was less than other bacteria.[15] All the tested bacteria were sensitive to Al Sader and Mountain honeys at 40 to 80% concentrations. The antibacterial activity of Al Sader honey was higher than those obtained by Mountain honey.[15]

Also, the research about the antibacterial activity of different honey origins found that the highest antibacterial activity was against Corynebacterium. This was observed in spring honey pride, then Sair honey (0.233±0.021) and Abu Roses honey (0.294±0.002) Al Sader honey (0.333±0.012), Shamar Mount honey (0.379±0.023), Spring Lena honey (0.381±0.002) and Agaa Mount honey (0.397±0.003) and where the moderate activity was displayed by Hegaz spring honey (0.425±0.002), Spring Hospitality honey (0.425±0.002) and Rok honey (0.439±0.001). The lower activity was demonstrated by Tabah honey (0.682±0.011) and Valley Offense honey (0.701±0.001). While the lowest activity was observed in the case of Harbingers honey (0.859±0.003). Tetracycline (50ug) inhibited Corynebacterium (0.326±0.002).[16]

The effect of honey on the growth curve of bacteria, which is considered a significant reduction compared to control samples without honey. In *Salmonella typhimurium,* the Spring honey had the highest effect on the reduction of *S. typhimurium* growth curve, then Al Sader, Hanon and the Thyme honey. Al Sader honey had the highest effect on the reduction of *S. poons* bacteria growth curve, then Hanon. In previous studies, they used Saudi honey samples, Al Sader and Mountain honey, which were tested for their antimicrobial activity against *E. coli, K. pneumoniae, P. aeruginosa* and *A. baumannii*. The study showed varying degrees of *in vitro* growth inhibition activity of Al Sader and Mountain honeys against the tested organisms. They concluded that it could be due to the osmotic effect, the effect of pH, and the sensitivity of these organisms to hydrogen peroxide, which are unsuitable for bacterial growth, and represented as an inhibition factor in honey.[17] All the different concentrations of both honey samples (10 to 80%) showed growth inhibitory activity against bacteria.[18]

The variation of the activity of different honeys was attributed to the previously mentioned factors which

influence the antibacterial activity[19] as osmotic properties of honey and honey pH[20], or activity of glucose oxidase, hydrogen peroxide[21], non peroxide substances and presence of propolis, which contains flavinoides and volatile antibacterial substances.[16]

CONCLUSION

It can be concluded that all studied honey types showed inhibition of bacterial growth. Moreover, it was clear that the inhibition of the studied strains was dependent on the type of honey origin. Honey has a good antibacterial effect, sterility, and no or minimal side effects in comparison to many other antibacterial drugs, which makes it an ideal antibacterial agent. This study revealed that Libyan honeys have the highest activity against *S. typhimurium* and *S. poons*. Therefore, increasing the honey concentration increased the inhibition of growth of the tested bacteria. However, it is necessary that further investigations be undertaken to discover the mechanisms involved in the antibacterial activity of honey, and the possible ways of its clinical use.

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