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Article *in* Journal of Advances in Medicine and Medical Research · March 2018

DOI: 10.9734/JAMMR/2018/39349

1 author:

Basma Doro
Pharmacy school, University of Tripoli
27 PUBLICATIONS **79** CITATIONS

Journal of Advances in Medicine and Medical Research

25(7): 1-10, 2018; Article no.JAMMR.39349
ISSN: 2456-8899
(Past name: British Journal of Medicine and Medical Research, Past ISSN: 2231-0614, NLM ID: 101570965)

The Antibacterial Activity of Libyan Honey against Gram Negative Bacilli: Potential Treatment Agent for Infectious Diseases?

Nouha Elmarbet[1], Mahmoud Ben Shaban[1], Basma Doro[2*], Bashir A. Lwaleed[3], Sarah Fouch[4] and Mohamed Elemam[5]

[1]Department of Food Science and Technology, Faculty of Agriculture, University of Tripoli, Tripoli, Libya.
[2]Department of Microbiology and Immunology, Faculty of Pharmacy, University of Tripoli, Tripoli, Libya.
[3]Faculty of Health Sciences, University of Southampton, Southampton, UK.
[4]School of Biomedical Science, Pharmacy and Medical Biotechnology, University of Portsmouth, Portsmouth, UK.
[5]Department of Microbiology, Faculty of Dentistry, University of Tripoli, Tripoli, Libya.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ME and MBS designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors BD and NE managed the literature searches; analyses of the study performed the spectroscopy analysis. Authors BAL and SF did the analyses of the study with help of statisticians. Authors BD, NE and ME supervised the laboratory work. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2018/39349
Editor(s):
(1) Kalpy Julien Coulibaly, Département Environnement et Santé (Intérim), Institut Pasteur de Côte d'Ivoire, Université Félix Houphouet-Boigny, Abidjan, Côte d'Ivoire.
Reviewers:
(1) Belal J. Muhialdin, Universiti Putra Malaysia, Malaysia.
(2) Giuseppe Pipicelli, Italy.
(3) R. Prabha, Karnataka Veterinary, Animal and Fisheries Sciences University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23450

Original Research Article

Received 18th November 2017
Accepted 12th February 2018
Published 7th March 2018

ABSTRACT

Aims: Honey has is a rich source of many compounds that exhibit anti-microbial, anti-inflammatory and pro-angiogenic properties. The emergence of antibiotic resistance in a wide variety of bacterial pathogens has generated renewed interest in natural antimicrobials. The aim of the present study

**Corresponding author: E-mail: basmadoro@yahoo.co.uk;*

was to assess the antibacterial activity of Libyan honey *in vitro*.
Methodology: The antibacterial activities of selected Libyan origin honey including Libyan Spring, AL-Sader, Thyme and Al-Hanone (at 25%, 50%, 75% and 100 % (w/v)) were tested against *Yersinia enterocolitica*, *Escherichia coli* and *Proteus mirabilis* using agar well-diffusion assay. The measurement of exponential bacterial growth curves was used to determine the effects on the microbial growth pattern spectrophotometrically at 600 nm. In addition, plate count methods were used to enumerate the effects of honey on the viable bacterial count.
Results and Discussion: Honey progressively inhibited bacterial growth at higher concentrations. This effect was variable depending on the honey type. For example, Al Sader honey showed the highest inhibition zones 21.3 mm ±0.8 against *P. mirabilis* followed by Thyme (21 mm ±0.5) and spring (20 mm ±0.5). Al-Hanone honey only exhibited effects against *E. coli* (14 mm ±0.5 and 8 mm ±0.4 at 100% w/v and 75% w/v, respectively). The Al Sader, spring and Thyme honey significantly reduced the *Yersinia enterocolitica* bacteria growth curve ($p<0.05$). All tested honey significant reduced *E. coli* growth from 5hrs compared to the control samples ($p<0.05$).
Conclusion: All honey tested showed inhibition of bacterial growth. Concentrated honeys were more effective against *Yersinia enterocolitica*, *Escherichia coli* and *Proteus mirabilis*. The efficacy of different types of honey was dependent on the honey concentration and origin.

Keywords: Antibacterial activity; gram negative; Libyan honey; Escherichia coli; Proteus mirabilis; Yersinia enterocolitica.

1. INTRODUCTION

Increased public health awareness of the pathogenic effects of bacteria, and the increased use, and inappropriate prescribing of antibiotics promoting resistance, has necessitated the investigation of alternative antibacterial compounds for use in the healthcare environment. Treatment of infections associated with medical devices and the formation of biofilms has been further complicated by the identification and increased prevalence of multi-resistant organisms, significantly limiting antimicrobial therapy choices [1-3]. Exposure to resistant organisms coupled with inappropriate prescribing may also increase the risk of patient colonization. This is not only a risk factor for future infections but enhances the transmission of antibiotic resistance determinants, through mechanisms such as conjugation and mutation, between bacterial species resulting in sensitive organisms becoming resistant to commonly used antibiotics [4-7]. While it is necessary to develop novel antibiotics for the treatment of various infectious diseases, chemical compounds can have a variety of side effects and may interact if the patient receives poly-pharmacy. Alternative effective antimicrobial substances from natural sources, such as honey, may play an indispensable role in supporting the treatment of bacterial infections, potentially reducing patient side effects and increasing compatibility with other currently prescribed medications.

The importance of honey as a therapeutic agent was reported as early as 4000 years ago by the Egyptians and Sumerian Physicians [9]. Honey was used as an antibiotic to reduce the risk of human infections and for wound healing, as part of what was then considered traditional medicine [10,11].

Manufactured by bees, honey is a natural sweet substance produced from nectar, from the secretions of living plants or excretions of plant sucking insects [8]. The main plants associated with honey production in Libya include *Acacia spp, Pinus spp, Cupressus spp, Thymus vulgaris, Lantana camara, Hisbiscusrosa-sinensis, Eucalyptus cawaldulensis,* and *Medicago sativa* [15].

The composition of honey is mainly a variety of sugars and water. Other constituents include amino acids, antibiotic rich inhibitors, proteins, phenol antioxidants, vitamins, minerals and micronutrients [13]. Some of the vitamins found in honey include ascorbic acid, pantothenic acid, niacin and riboflavin, the minerals include calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc. [14]. The sugars in honey provide a naturally sweet flavor and generate more energy in comparison to artificial sweeteners [13]. Several components of honey are thought to contribute to its antimicrobial activity. Complex factors such as phenolic compounds, hydrogen peroxide, high osmolality and acidity provide mechanisms of action against a number of human pathogens,

making honey a suitable alternative treatment option [12].

The aim of this study was to evaluate the antibacterial activity of selected Libyan origin honey; Spring honey, Thyme honey, Al-Sader honey and Al-Hanone honey, against Gram negative bacteria; *Yersinia enterocolitica*, *Escherichia coli* and *Proteus mirabilis*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains and Media

The bacterial strains used in this study were *Yersinia enterocolitica* ATCC 9610*, Escherichia coli* ATCC 25922 and *Proteus mirabilis* ATCC 14153. Bacterial strains were streaked onto nutrient agar, incubated for 24 hrs at 37°C then stored at 4°C. The media used in this study were nutrient broth (NB), nutrient agar (NA) and Mueller Hilton agar (Oxoid). All media was prepared according to the manufacturer's instructions.

2.2 Preparation of Bacterial Cultures

Liquid cultures of *Yersinia enterocolitica, Escherichia coli,* and *Proteus mirabilis* were prepared by inoculating a single colony of bacteria into 20 ml NB (Oxoid), in a sterile Pyrex conical flask, the inoculated broth was then incubated at 37 ºC overnight (18 hr).

2.3 Honey Sample Collection

Four representative samples of honey were collected from different geographic locations in Libya. Spring honey was collected from Tripoli, Libya. The city of Tripoli is located in the northwestern part of Libya at 32°62' 54" N and 13°22′ 75″ E. AL-Hanone honey was obtained from Al Beyda city, located in the northeast of Libya at 32° 45′ 59″ N and 21° 44′ 30″ E. Thyme honey was collected from the southeast of Tripoli, Tarhona 32° 26′ 02″ N and 13° 38′ 04″ E, and Al-Sader honey was obtained from Al Khoms, in the northeast, 32° 38′ 59″ N and 14° 15′ 52″ E. All the samples were stored at room temperature in the dark until tested.

2.4 Preparation of the Honey Samples

The honey samples were diluted in sterile distilled water to achieve different concentrations constituting, 25%, 50%, 75% and 100% (w/v). These concentrations were expressed as the percentage of honey weight per total reaction volume and used to determine the antimicrobial activity using agar well-duffision, optical density and plate count methods.

2.5 Agar Well-diffusion Assay

The antimicrobial activity of honey was tested *in vitro* using the agar well-diffusion assay. This method was performed using freshly prepared Mueller Hinton agar inoculated with an overnight culture of bacteria suspended in sterile saline and adjusted to a 0.5 McFarland standard. Prior to inoculation, 6 mm diameter wells were punched into the Mueller Hinton agar plates [16]. After inoculation each well was filled with 100 µl of the honey solution (25%, 50%, 75% and 100% w/v) and then incubated for 24 h at 37 °C. The inhibition zones were measured in millimeters. Antibiotics, including Tetracycline, Amoxicillin, and Cephalexin (5 µg/ disk) were used as a standard (Thermo Scientific ™Oxoid ™Antimicrobial Susceptibility Disks). Negative controls were prepared without honey. All experiments were carried out in triplicate to ensure the reproducibility.

2.6 Optical Density 600 Measurements and Test Bacterial Viable Count

Twenty ml of an overnight bacterial culture was prepared and diluted to 1:50, to give an OD_{600} (600 nm) ≈ 0.05. Dilutions of honey were added and the inoculated cultures were incubated at 37 °C in an orbital shaker. Growth was measured, in triplicate, at an optical density of 600_{nm} using a UV spectrophotometer (Biochrom UK) at 1, 2, 3, 4, 5, 24 and 25 hrs. The Plate count method was used to enumerate the microbial count. One ml of each sample was serially diluted (10^{-1} to 10^{-8}) with 9.0 ml of 0.1% peptone water. Then, 0.1 ml of the diluent was inoculated onto Plate Count Agar (Oxoid). All the plates were incubated at 30°C for 24-48 hrs. The Colony counts were converted to CFU per ml according to the criteria specified by ISO, 2003 [17].

2.7 Statistical Analysis of Data

The data were tested for normality using a QC Analyses/K-S Normality Test. Normally distributed data were analyzed using the Student's T-Test performed by 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.

3. RESULTS AND DISCUSSION

Many studies have evaluated the antibacterial, antifungal and antiviral properties of honey against a range of microorganisms [18]. This study used the agar well-diffusion assay to compare the zone inhibition diameter values (average ± standard error) of four selected Libyan origin honey types against *Yersinia enterocolitica*, *Escherichia coli* and *Proteus mirabilis.* Results obtained from this experiment are shown in Table 1. All four honey types had an effect against *Yersinia enterocolitica, Escherichia coli* and *Proteus mirabilis.* The largest inhibition zone diameters for the activity of the 100% (undiluted) honey against *P. mirabilis* were; Al-Sader, Thyme and spring honey (21.3 mm±0.8, 21±0.5 mm and 20mm±0.5 respectively). The 75% concentration of the Al-Sader honey produced a zone of inhibition of 12.0 mm±6.1, slightly lower in comparison to the 100% concentration. The other samples of honey were at an insufficient concentration to inhibit the bacterial growth. The largest zones of inhibition were found to be 18.3 mm ±2.0 and 17.3 mm ±0.3 against *Y. enterocolitica* and *E. coli*, respectively. Results obtained from the 25% and 50% concentrations of honey, suggest that the bacteria used in this study were able to resistant any antimicrobial properties, as no inhibition zones were recorded (0.00 ± 0.00mm), with the exception of Al-Sader at 50% w/v. These results clearly suggest that the higher concentration of all four honey types could possibly be used as antibacterial agents.

The results of this study were also compared with the inhibition zone diameters of commonly used antibiotics (Table 2). All the tested bacteria were affected by Cephalexin with zones of inhibition measuring 28.3 mm ±3.06, 18.6 mm ±0.03 and 16 mm ±0.05, respectively. Analysis of tetracycline against *Y. enterocolitica* revealed a large zone of inhibition (31.1 mm ±0.01) in comparison to *P. mirabilis* that appeared to be resistant (0±0.00). Amoxicillin showed zones of inhibition measuring 23 ±0.05 mm, 19 ±0.05 mm.

Table 1. The mean diameter, in millimeters, of the bacterial inhibition zones of the honey tested against *Yersinia enterocolitica, Escherichia coli* and *Proteus mirabilis*

Concentration W/V%	Mean zone of inhibition (mm± SE)					
	Spring honey			AL-Sader honey		
	Y. enterocolitica	*E. coli*	*P. mirabilis*	*Y. enterocolitica*	*E. coli*	*P. mirabilis*
0	0±0	0±0	0±0	0±0	0±0	0±0
25	0±0	0±0	0±0	3.0±0.3	0±0	0±0
50	0±0	0±0	0±0	4.0±0.4	6.6±0.3	0±0
75	13.6±0.25	11.3±0.3	0±0	12.6±0.5	14±0.5	12±0.16
100	18.2±0.2	14.6±0.3	20±0.5	18.2±0.2	17.3±0.3	21.3±0.8
Concentration W/V%	**Mean zone of inhibition (mm± SE)**					
	Thyme honey			**AL Hanone honey**		
	Y. enterocolitica	***E. coli***	***P. mirabilis***	***Y. enterocolitica***	***E. coli***	***P. mirabilis***
0	0±0	0±0	0±0	0±0	0±0	0±0
25	0±0	0±0	0±0	0±0	0±0	0±0
50	0±0	0±0	0±0	0±0	0±0	0±0
75	14±0.5	12.3±0.8	0±0	0±0	8.0±0.4	0±0
100	18.3±0.2	15.6±0.3	21±0.5	0±0	14.0±0.5	0±0

Table 2. Inhibitory Zones of antibiotics against *Yersinia enterocolitica, Escherichia coli* and *Proteus mirabilis*

Type of antibiotics	Mean zone of inhibition (MM± SE)		
	Y. enterocolitica	***E. coli***	***P. mirabilis***
Tetracycline	31.1±0.1	19.6±0.3	0±0
Amoxicillin	0±0	19±0.5	23±0.5
Cephalexin	28.3±3.6	18.6±0.3	16±0.5

The variation of antibacterial activity of the different types of honey was attributed to a range of previously mentioned factors [19] such as osmotic pressures, pH [20], the activity of glucose oxidase, hydrogen peroxide [21], non peroxide substances and presence of propolis, which contain flavinoides and volatile antibacterial substances [22].

Samples of 100% honey were highly viscous and difficult to manipulate [23], therefore the 75% w/v concentration was selected to investigate the effects of the different honey types on the bacterial growth at different time points. The time points selected were every hour for the first 5 hrs, 24 and 25 hrs. The first five hour measurements aimed to demonstrate the antibacterial effects of the honeys during the different phases of the bacterial growth curve. The 24 and 25 hour readings would demonstrate any lasting antimicrobial effects. Previous studies measuring the effects of bacterial exposure to Penicillin G and Erythromycin have reported maximal suppression between 2 and 5 hrs, respectively. These effects were observed over a range of different bacterial inoculum [24].

The inhibitory effects of all four honey types, at 75% w/v concentration, against *Yersinia enterocolitica, Escherichia coli,* and *Proteus mirabilis* are shown in Figures 1, 2 and 3. Bacterial cultures were grown overnight in nutrient broth and then diluted 1:50 into fresh media. Spring, Al-Sader, Thyme and AL-Hanone honeys were added to investigate the antimicrobial effects of the honeys on bacterial cells in liquid culture. The samples were incubated at 37 °C with continuous shaking (~200 rpm) in an orbital shaker. The optical density of the suspension was measured at 600 nm and monitored every 1hr for the first 5hrs, then again at 24 and 25 hrs.

Fig. 1 illustrates that the Al-Sader, Spring and Thyme honey were more effective at inhibiting the growth of *Y. enterocolitica,* with little difference in growth inhibition when using the Al Hanone honey compared to the control. Treatment with these honeys gave a level of inhibition, indicating that the different honey types had elicited their antibacterial effects on the bacterial cells. Almost the same effect was observed for the Al-Sader, Thyme and Spring honey against *E.coli*. The Al Hanone honey's inhibition against this organism demonstrated greater activity compared to *Yersinia enterocolitica,* however this was less effective overall, Fig. 2.

Al Sader honey showed the highest antimicrobial activity against *P. mirabilis* compared to the Thyme, Spring and AL-Hanone honey, Fig. 3. It was clear from the significant interaction of the honey, that different honey types have different antimicrobial properties.

The activity of the tested honey was also analyzed using the total viable bacteria count at 0, 1, 2, 3, 4, 5 and 24 hrs. Bacterial cultures were prepared as described in the bacterial growth analysis section and the different honey was added to investigate their antimicrobial priorities.

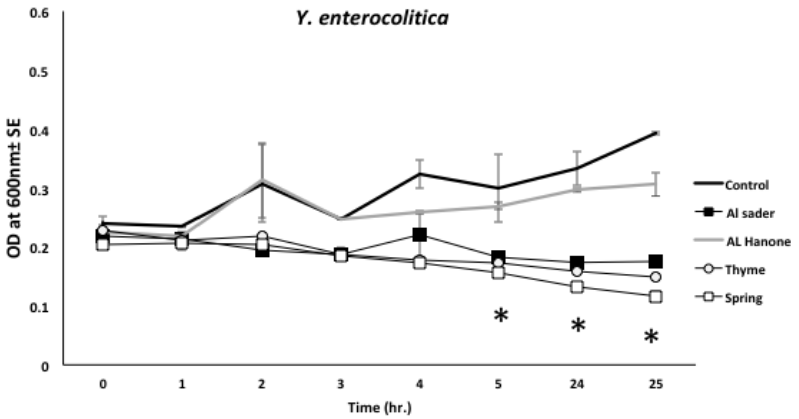

Fig. 1. Optical density of growth of *Yersinia enterocolitica* in nutrient broth with the incorporation of 75% w/v of each honey sample (*p<0.05 Al sader, spring and thyme honey comparing to control)**

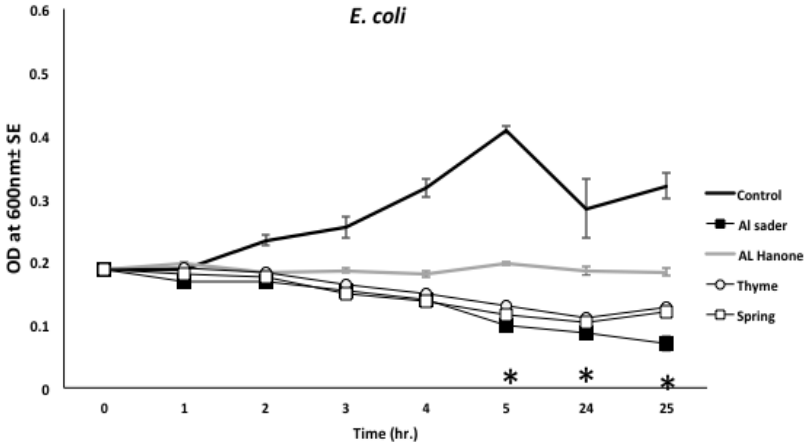

Fig. 2. Optical density growth of *E. coli* in nutrient broth with the incorporation of 75% w/v concentration of honey samples (*p<0.05 All honey tested type comparing to control).**

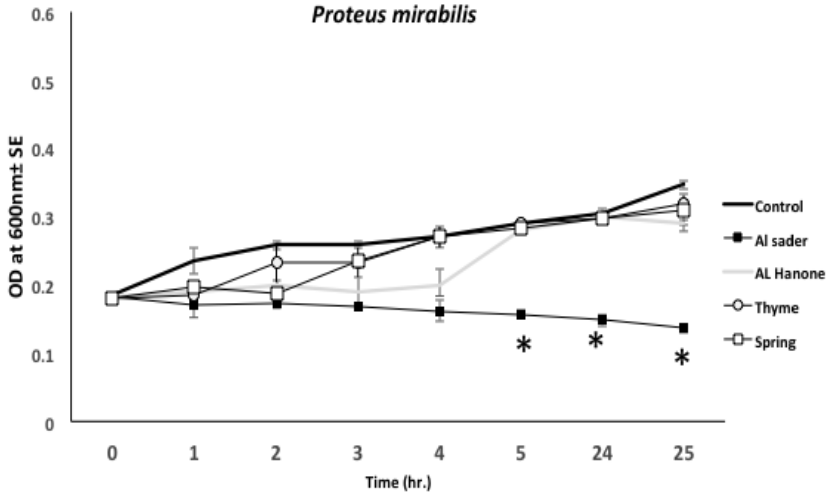

Fig. 3. Optical density growth of *Proteus mirabilis* in nutrient broth with the incorporation of 75% w/v concentrations of honey samples (*p<0.05 Al sader honey comparing to control).**

In order to determine the viable colony counts, decimal dilutions from 10^{-1} to 10^{-8} were used. The mean data (Log_{10} CFU/ml) against time was recorded for each bacterial strain (Table 3). In order to assure the sterility of the tested honey, 0.1 ml of each honey sample was streaked onto Muller Hinton agar and incubated at 37 °C for 24 hrs. Table 3 shows the number of viable cells in both the control sample and the different honey treatments. The results showed that the different honey, all at 75% w/v concentration, had greatly reduced the viable count of *Yersinia enterocolitica*, *E. coli* and *Proteus mirabilis*, at different times, when compared to the control.

Our results demonstrated that approximately 7 log_{10} reductions in the viable count of *Y. enterocolitica* were observed when treating with Spring honey and 8-log_{10} reductions with Al-Sader, Thyme and AL-Hanone honey. Moreover, there was a significant effect on the viability of the *E. coli* cells, with a reduction of 7 -8 log_{10} for all tested samples. Similarly, the results showed that honey had reduced the viability of *P. mirabilis* by approximately 7 -8 log_{10} (Table 3). Therefore, it can be concluded that all the types of honey demonstrated antibacterial activity against the tested organisms.

Table 3. Total viable count of *Yersinia enterocolitica*, *E. coli* and *Proteus mirabilis* inhibited by the different honey tested (75%w/v concentration) at 0, 1, 2, 3, 4, 5 and 24 hrs

Yersinia enterocolitica							
Mean Log_{10} (CFU/ml ± SE)							
	Time (hour)						
Honey type	**0**	**1**	**2**	**3**	**4**	**5**	**24**
Control	7.60±0.8	7.77±0.8	8.00±0.8	8.59±31.8	8.82±0.1	8.90±1.5	8.97±1.1
Spring	7.00±0.8	0±0	0.84±0.09	0±0	1.42±0.3	1.63±1.8	1.33±0.28
AL-Sader	7.39±2.0	0±0	0.60±0.32	0±0	1.13±0.07	1.04±0.6	0.97±0.09
Thyme	7.00±0.8	0.30±0.8	0±0	0.5±0.4	0.17±0.04	1.61±0.7	0.47± 0.08
AL Hanone	7.00±0.8	0±0	2.5±2.0	0±0	0.39±0.02	1.11±0.7	0.87±0.02
Escherichia coli							
Control	9.42±0.76	9.51±0.1	9.57±0.5	9.58±0.69	9.53±0.9	9.63±0.4	9.70±0.8
Spring	9.40±0.95	2.40±0.5	2.57±0.23	2.42±1.7	1.53±0.5	1.88±0.5	1.43±0.5
AL-Sader	8.77±0.42	2.46±0.2	2.31±0.8	2.31±1.8	1.76±0.8	2.46±0.8	1.97±0.5
Thyme	9.36±1.7	2.26±1.7	2.40±0.1	1.86±0.5	2.43±0.2	2.57±0.6	2.11±0.3
AL Hanone	8.94±0.41	2.27±1.4	2.32±0.13	2.46±0.6	2.46±0.9	2.55±1.8	1.60±0.4
Proteus mirabilis							
Control	9.22±0.13	9.23±0.38	9.26±1.1	9.47±0.1	9.48±0.6	9.50±0.6	9.51±0.49
Spring	9.25±0.52	2.21±0.7	2.19±0.8	1.51±0.6	2.32±0.7	2.26±0.18	2.14±0.82
AL-Sader	8.91±0.73	0.95±0.1	2.07±0.1	1.41±0.89	2.31±0.92	2.28±0.71	1.39±0.8
Thyme	9.23±0.31	0.95±0.8	1.79±0.56	1.91±0.44	2.34±4.4	2.20±0.2	1.56±0.26
AL Hanone	9.07±0.78	2.14±0.28	0.54±0.4	2.41±0.58	2.37±0.28	2.28±3.2	2.11±0.81

Emerging evidence from clinical studies has shown that honey has enormous potential, a complex composition and numerous interesting clinical properties. Honey has been used as a therapeutic agent against different types of pathogenic bacteria since ancient times [25]. Evidence has identified a number of antimicrobial properties, making it a compelling alternative/addition to chemical antibiotics [11]. Therefore, these antibacterial properties are increasingly valued, notably so with the increased prevalence of antibiotic resistance and the failure of antibiotic treatments to eliminate certain bacterial infections. In order to quantify the antibacterial activity of honey, this study was carried out to investigate the bacterial inhibition of Libyan Spring, Al-Sader, Thyme and Al-Hanone honey in different concentrations against *Yersinia enterocolitica, Escherichia coli* and *Proteus mirabilis*.

We observed varying degrees of antibacterial activity against the tested organisms. Most of the bacteria were affected by the Spring, Al-Sader, Thyme and AL-Hanone honey specifically at 75% and 100% concentrations. Undiluted honey showed strong antibacterial activities, with the largest zone of inhibition measuring 21.3±0.8 mm. The present findings are supported by Basualdo et al [26], particularly in relation to *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results from this study showed slight differences in the inhibitory properties between the different honey types. However, in accord to the difference between the honey concentrations, undiluted (100% concentrations) were the most effective. Several authors have reported that different honey types vary substantially in the potency of their antibacterial activities, probably attributed to the natural variations in floral sources of nectar and geographical origins or chemical composition [27-29]. Likewise, the results generated from this study may be due to the collection of the representative samples of honey from different geographical locations in Libya. The susceptibility of *Yersinia enterocolitica, Escherichia coli and, Proteus mirabilis* was also compared with three types of commonly used antibiotics. It has been shown that bacterial growth of *Yersinia enterocolitica* was affected by tetracycline, amoxicillin and cephalexin. *Proteus mirabilis* and *Escherichia coli* were not inhibited by amoxicillin and tetracycline.

The present study was also designed to investigate the effects of the different honey types on the bacterial growth curve. The bacterial species demonstrated differences in the presence of honey (Fig. 1, 2 and 3). These results indicate that bacterial activity is influenced by Libyan Spring, Al-Sader, Thyme and AL-Hanone honey. Undoubtedly, the monitoring of bacterial growth and physical features, under which species of microorganisms can survive in nature provides a better understanding of the

conditions that determine these organism's survival and reproductive success. This study clearly suggests that different honey types have varying effects on the bacterial growth curve. Nevertheless, some of the bacterial species were affected as soon as one hour after the addition of the honey, this could possibly be due to the active ingredient in honey itself, inhibiting bacterial growth.

The antibacterial activity of the different honey resulted in a reduction of the total viable counts for all of the bacterial strains by approximately 7-8 $\log_{10}$ cfu/ ml, with almost a 90% growth inhibition over the full experimental time period (initial concentrations of *Yersinia enterocolitica*, *E .coli* and *Proteus mirabilis*, were 7.77± 0.8, 9.51±53.1 and 9.23± 38.4 $\log_{10}$ cfu/ ml. After exposure to spring, Al-Sader, Thyme and AL-Hanone honey, these values were reduced by about 7-8 $\log_{10}$ cfu/ml between 1 to 24 hrs). Thus, it is possible to note that the potency of the anti-microbial effects of honey has remained considerably stable. In contrast, many other studies have reported that the antibacterial properties of honey change overtime, the efficiency of honey in some cases noticeably increase, while others, report decreases [30]. In addition, different honeys inherit the particular plant properties associated with their production, such as color, aroma, flavor, density, and physical and chemical properties. A number of studies have demonstrated that weather conditions, as well as processing, influence composition and chemical properties [31]. As a result, the nutritional values and profiles of honey vary accordingly and this can influence the value of a specific honey for health promoting purposes [32].

Our study demonstrates the antimicrobial stability of honey over time is highly promising, as these agents could be used in clinical applications, as potential medicinal agents. Therefore, the clinical benefits of medical honey including its antibacterial protection, anti-biofilm formation and wound healing and cleaning properties [33] could revolutionise the treatment of infectious diseases.

4. CONCLUSION

All of the studied Libyan origin honey types showed bacterial growth inhibition. Moreover, it was clear that the inhibition of the studied strains was dependent on the honey origin. Honey has good antibacterial effects, sterility, and no or minimal side effects in comparison to many other antibacterial drugs, which makes it an ideal therapeutic agent. This study revealed that Libyan honey had potent activity against *Yersinia enterocolitica, Escherichia coli,* and *Proteus mirabilis*. Results also suggest that increasing the honey concentration increased the bacterial growth inhibition. It is necessary to undertake further investigations to identify the mechanisms involved in the antibacterial activity of honey, and the possible implications of its use in the clinical setting.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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DOI: 10.1002/ptr.4957. PubMed PMID: 23494861.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23450