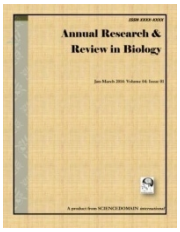

Annual Research & Review in Biology
4(22): 3335-3344, 2014

SCIENCEDOMAIN *international*
www.sciencedomain.org

Antibacterial Activity of Milk Vetch Flower Honey against Four Bacteria of Human Oral Flora: *Streptococcus mutans, Lactobacillus casei, Lactobasillus rhamnosus* and *Lactobasillus plantarum*

Faezeh Kgozeimeh[1], Zahra Golestannejad[2], Marzieh Tofighi[3], Azadeh Ayen[3], Mohsen Doost Mohammadi[4], Shahin Gavanji[5*] and Azizollah Bakhtari[6]

[1]Oral Medicine Department, Dental School and Torabinejad Research Center, Isfahan, Iran.
[2]Dental Implant Research Center, Department of Oral Medicine, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran.
[3]Dental Student's Research Center, school of dentistry, Isfahan University of Medical Sciences, Isfahan, Iran.
[4]Department of Biotechnology, Faculty of Advanced Sciences and Technologies, University of Isfahan, Isfahan, Iran.
[5]Young Researchers and Elite Club, Khorasgan Branch, Islamic Azad University, Khorasgan, Isfahan, Iran.
[6]Department of Animal Science, Isfahan University of Technology, Iran Animal Science, Iran.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Original Research Article

Received 10th March 2014
Accepted 29th April 2014
Published 13th June 2014

ABSTRACT

Aims: Milk vetch flower honey has valuable therapeutic effects, however, its antibacterial effect is not well understood. In present study, milk vetch flower honey was assessed for antibacterial activity against four bacterial species: *Streptococcus mutans, Lactobacillus casei, Lactobasillus rhamnosus and Lactobasillus plantarum,* which are the main causes of oral cavity infection.

**Corresponding author: Email: shahin.gavanji@yahoo.com;*

Methodology: Honey solutions were prepared by diluting with sterile water to the final test concentrations (9.3, 18.75, 37.5, 75, 150, 300, 600 and 1200ppm) immediately before testing. Antimicrobial activity was determined by serial dilution and the disk diffusion method.
Results: Although a honey concentration of1200 ppm strongly inhibited growth of all four bacterial species, concentrations below 37.5 ppm were more efficient as antibacterials. We determined the minimum inhibitory concentrations (MIC) for honey against *S. mutans, L. casei, L. rhamnosus and L. plantarum* were 75, 75, 100 and 100 ppm, respectively. *S. mutans* was the most resistance species with a zone of inhibition of 6.81 millimetres (mm) while *L. casei* showed significant sensitivity with a zone of inhibition of approximately 11.3 mm.
Conclusion: To conclude, the reasonable antibacterial effect of milk vetch flower honey against mentioned bacteria species indicated that this type of honey could be used as a natural antibiotic, however, it need more studies for finding its effective agents.

Keywords: Antibacterial; infection; traditional medicine.

1. INTRODUCTION

One of the major concerns to the global health community is the spread of antibiotic resistance. Effective disease treatment depends on new and more effective pharmaceuticals, of which traditional medicine is a useful potential source. Traditional medicine has been used since ancient times and honey is one of oldest components of such medicine [1]. Many ancient communities, such as the ancient Egyptians, Chinese and Sumerians used honey for medicinal purposes [2]. Many studies have been performed to identify the component(s) that confer honey's antibacterial activity. There are four main factors that contribute to the antibacterial effects of honey, which are: 1) its strong osmotic effect, 2) Its naturally low pH [3], 3) production of hydrogen peroxide, and [4] 4) photochemical factors. Hydrogen peroxide is the major determinant of honey's antimicrobial activity and, as different types of honey have varying concentrations of this compound, their antimicrobial activities differ from one another [5]. To illustrate, Manuka honey has high antibacterial activity do to an unidentified component [6]. It is important to note that, although honeys with higher concentrations of hydrogen peroxide have increased antibacterial activity, they are also more sensitive to heat and light. This is because the main enzyme required to generate hydrogen peroxide is inactivated by these factors (2<3<9). Environmental conditions also effect on honey's properties, including light, temperature, storage conditions and processing These factors especially affect its antibacterial activity. Interestingly, according to the study of Allen et al., age does not have any effect on honey's antibacterial activity [7]. Recently, it has been reported that honey has an inhibitory effect on approximately 60 bacterial species, including Gram negative, Gram positive, aerobic and anaerobic [8]. *Streptococcus mutans* and the *Lactobacillus* species cariogenic bacteria, which are involved in the formation of oral cavities. This demonstrates the importance of studying the antibacterial effect of honeys against these bacteria. S. mutans is a facultative anaerobic, Gram positive bacteria predominantly found in the human oral cavity and plays a major role in tooth decay [9]. *Lactobacillus species are* another Gram positive bacteria and are mainly present in the vagina and gastrointestinal tract where they make up a small portion of the gut flora. These bacteria in the mouth are associated with tooth decay [10]. As mentioned above, these two bacteria are the main cause of dental caries and finding novel effective therapeutics for preventing their negative effects is of great importance. The aim of

this study was to determine whether milk vetch flower honey has inhibitory or toxic effects on these two bacterial species.

2. MATERIALS AND METHODS

2.1 Honey Sample

Honey was obtained from local apiarists throughout Shahrekord Iran and samples were aseptically prepared and protected from sunlight. Honey solutions were prepared with sterile water and samples were assayed immediately after dilution.

2.1.1 Measuring water content

The water content of the honey samples was measured using a refract meter set to 20ºC [11] and the moisture content was determined by comparison with a reference table (standard NO. 92 Institute of Standard and Industrial Research of Iran).

2.1.2 Colour measurement

Five grams of honey sample was diluted with 50mL water and centrifuged for 15 min at 1500RPM. The supernatants were collected and their absorbance measured at 385nm using spectrophotometer [12].

2.1.3 Determination of free acidity

10 grams of each honey sample was diluted in 80mL of distilled water and the pH was measured at 20ºC. The pH of each solution was adjusted to 8.3 using 1N NaOH. Water was used as control. The free acidity content was determined using the equation:

Free acidity value = (base volume used for the sample - base volume used for control) ×10 [13]

2.1.4 Ash test

10 grams of honey was transferred to a ceramic dish, to which a few drops of olive oil was added. The solution was slowly heated to 600ºC in a furnace for 20 min and the weight of the white ash was measured. To determine the percent of mineral materials in the sample, the resulting weight difference was multiplied by 100.

2.1.5 Determining the HMF content

Hydroxymethyl furfural (HMF) was detected using a spectrophotometer. The samples were clarified with Carrez reagent, sodium bisulfate was added, and the samples' absorption were read at 285 and 335nm with water as blank [14].

2.2 Bacteria and Culture Conditions

The bacterial strains *Streptococcus mutans* PTCC 1683*, Lactobacillus casei* PTCC 1608*, Lactobacillus rhamnosus* PTCC1637 and *Lactobacillus plantarum* PTCC 1058 were obtained from the Iranian Research Organization for Science and Technology. Blood agar (BA)

medium was used to determine the zone of inhibition and Müller-Hintonbroth was used to prepare serial dilutions for MBC determination. All bacterial strains and materials used in this study were obtained from Merck.

2.3 Determination of the Zone of Inhibition

In order to study the antimicrobial effect of the honey samples, we used the disk diffusion method. After 18h in culture (using Mueller-Hinton broth), bacteria were prepared at a standard density (1×10^6 CFU ml^{-1}) of 0.5 McFarland and 500µL was plated on BA. The liquid was distributed on the plate surface using a sterile loop. We utilized blank disks (6mm diameter) containing 30µL of honey with the following concentrations: 9.3, 18.75, 37.5, 75, 150, 300, 600 and 1200ppm. The disks were placed on the inoculated BA plates and incubated at 37ºC for24, 48 and 72h. The diameter of each zone of inhibition was measured with callipers. All experimental conditions were performed in triplicate.

2.4 Determination of MIC and MBC Using Dilution of Wells

In order to determine the MIC of honey against *S. mutans, L. casei, L.Rhamnosus,* and *L. plantarum,* liquid cultures were used to prepared suspensions of each bacterial strain at standard turbidity of 0.5 McFarland. We prepared a serial dilution of milk vetch honey using Mueller-Hinton broth as the diluent. All dilutions were prepared as 1:1, which yielded a dilution series with concentrations of: 9.3, 18.75, 37.5, 75, 150, 300, 600 and 1200ppm prepared. All dilutions were inoculated with 1ml of overnight bacterial culture and incubated at 37ºC at 150 RPM for 24h. Following incubation, the minimum honey concentration demonstrating no bacterial growth was selected as the MIC. In order to assess MBC, 30µL of cultures that did not show bacteria growth were transferred to BA plates and incubated at 37ºC for 24h. The culture with the minimum concentration that showed no bacterial growth on BA plates was selected as the MBC.

2.5 Statistical Analysis

Data analysis was performed using SPSS version 20, ANOVA and Tukey's comparison procedure.

3. RESULTS AND DISCUSSION

3.1 Honey Analysis

We determined that the water content of the sample was about 18.5% which, in comparison to other reported values, demonstrates that the honey was fresh. Honey color is measured as optical density and it has been shown that color changes during storage at temperatures between 45 and 80ºC. An optical density of 0.2 or lower is one indication of fresh honey. Our honey sample had an optical density of 0.183, which is in range of fresh honey [15]. The pH value of our sample was 4.5, which isin the range of fresh honey. This demonstrates that the storage conditions did not affect pH. We performed the ash test according to the method presented by the Institute of Standard and Industrial Studies of Iran. The mineral materials content in the honey sample was 28%. HMF was determined as described in Materials and Methods. HMF is produced when some sugars in honey (i.e. glucose) break down under certain conditions, such as high temperature. Bradawl et al. showed that HMF content can

vary from 5 to >300mg/kg. The HMF content of our honey sample was 8mg/kg, demonstrating minimal sugar break down and indicating freshness (Table 1).

Table 1. chemical and biological properties of milk vetch flower honey

No	Physical and biological Properties	Measured Values
1	Water Content	18.5
2	Color	0.183
3	pH	4.5
4	Mineral	28%
5	HMF	8 mg/kg

3.2 Determination of the Zone of Inhibition

Using the disk diffusion test, we determined the zone of inhibition diameter of honey against *S. mutans, L. casei, L. rhamnosus* and *L. plantarum*at concentrations ranging from 9.3–1200 ppm. The results were analysed using SPSS version 20 software (IBM) with theone-way ANOVA method and the averages were compared using the Toki method. Table 2 presents the effects of different honey concentrations on the 4 bacterial species tested at 24, 48 and 72h. According to these results, a concentration of1200ppm has a significant inhibitory effect on all four species ($P<0.0001$).

After determining that 1200 ppm was the most effective inhibitory concentration, we compared its effects on the four bacterial species tested (Fig. 1). As is clear from Fig. 1, honey has less effect on species *S. mutans* than the other three species at all-time points tested ($P<0.001$). The zone of inhibition of *L.casei* is slightly less than 11mm at 24h, which increased to approximately 12mm after 72h. *L. plantarum* and *L. rhamnosus* had the next highest zones of inhibition, which were ~10.5 and ~9 at 24h, respectively. This did not change after 72h. *Smutants* is the species with the smallest zone of inhibition, which was ~6.1 at 24h and to 6.5 after 72h. These data demonstrate that honey displays antibacterial properties against *L. plantarum, L. casei* and *L. rhamnosus*, however, *S. mutans* is more resistant to its antibacterial effects. The data presented in Figs. 2–4 further demonstrate this result.

Fig. 2 presents the antibacterial effects of increasing honey concentrations on zone of inhibition at 24h. There is an exponential increase in diameters between 0 and 75ppm. However, at higher concentrations, there is only a very slight increase in diameter, indicating that, although the higher concentrations have a larger zone of inhibition, honey at lower concentrations (below 75ppm) is more efficient as an antibacterial agent.

Table 2. The effects of different honey concentrations on four bacterial species

Honey (ppm)	*Lactobacillus casei* Mean ± SE			*Lactobasillus plantarum* Mean ± SE			*Lactobacillus rhamnosus* Mean ± SE			*Streptococcus mutans* Mean ± SE		
	24	48	72	24	48	72	24	48	72	24	48	72
9.3	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00 ±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
18.75	1.17±0.09^{b}	1.57±0.20^{b}	1.57±0.20^{b}	0.47±0.26^{a}	0.57±0.35^{a}	0.57±0.35^{a}	0.27±0.27^{a}	0.33±0.33^{a}	0.33±0.33^{a}	0.10±0.10^{a}	0.10±0.10^{a}	0.10±0.10^{a}
37.5	4.22±0.31^{c}	4.40±0.23^{c}	4.57±0.28^{c}	1.90±0.58^{a}	2.03±0.71^{a}	2.03±0.71^{a}	1.20±0.49^{ab}	1.33±0.56^{ab}	1.40±0.62^{ab}	0.67±0.33^{a}	0.77±0.39^{a}	0.77±0.39^{a}
75	5.80±0.42^{d}	6.27±0.30^{d}	6.37±0.20^{d}	5.00±0.53^{b}	5.13±0.52^{b}	5.27±0.40^{b}	3.93±1.17^{bc}	4.03±1.22^{bc}	4.03±1.22^{bc}	1.57±0.53^{ab}	1.73±0.63^{ab}	1.80±0.65^{ab}
150	6.30±0.15d^{e}	6.37±0.09^{d}	6.53±0.07^{d}	5.92±0.46^{b}	5.92±0.46^{b}	6.22±0.61^{b}	5.43±0.92^{c}	5.80±0.95^{cd}	5.90±1.00^{cd}	2.96±0.43^{bc}	3.17±0.17^{bc}	3.23±0.23^{b}
300	7.36±0.13^{e}	7.71±0.17^{e}	8.17±0.40^{e}	6.91±0.40^{bc}	7.04±0.38b^{c}	7.26±0.38^{bc}	5.67±0.33^{c}	5.88±0.29^{cd}	6.28±0.50^{cd}	3.83±0.17^{cd}	3.93±0.22^{cd}	4.03±0.29^{bc}
600	8.52±0.17^{f}	9.57±0.14^{f}	10.20±0.15^{f}	8.10±0.29^{c}	8.10±0.29^{c}	8.40±0.26^{c}	6.80±0.20^{cd}	7.10±0.15^{de}	7.43±0.23^{de}	5.33±0.67^{de}	5.65±0.64^{de}	5.85±0.73^{cd}
1200	10.6±0.32^{g}	11.2±0.32^{g}	11.77±0.19^{g}	10.2±0.39^{d}	10.3±0.43^{d}	11.05±0.33^{d}	9.14±0.52^{d}	9.90±0.21^{e}	10.2±0.36^{e}	6.27±0.64^{e}	6.73±0.67^{e}	7.04±0.84^{d}

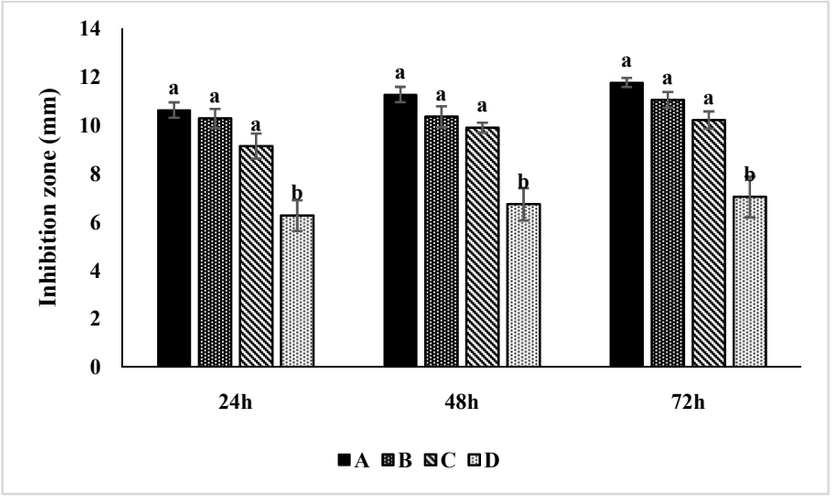

Fig. 1. Comparison of the effects of concentration 1200 of honey on four bacteria species. A: *L. casei, B: L. plantarum, C: L. rhamnosus*, D: *S. mutans*. means with different letters show significantly different between treatment groups (p<0.0001)

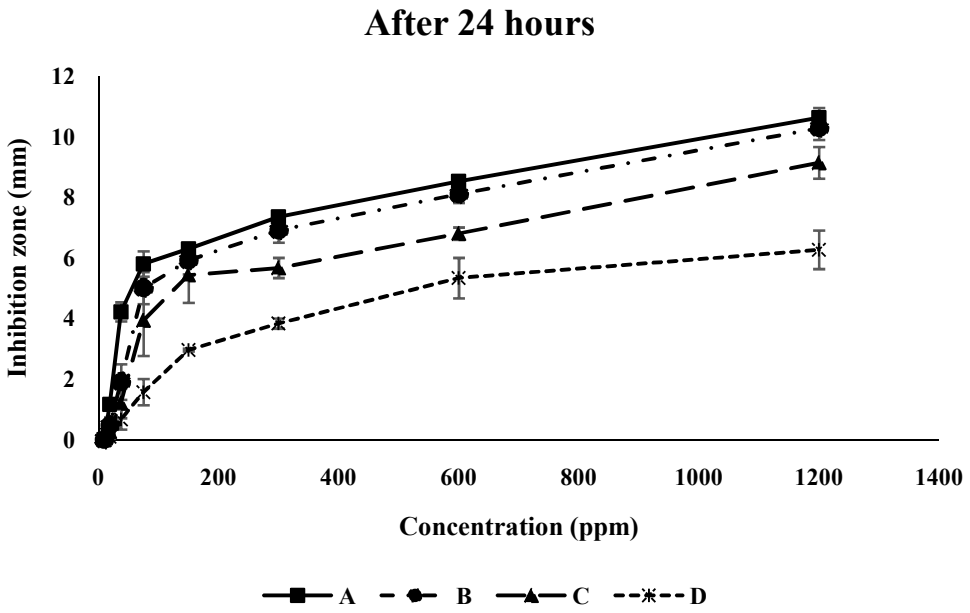

Fig. 2. The antibacterial trend of increasing honey concentration in four bacterial species after 24h. A: *L. casei, B: L. plantarum, C: L. rhamnosus*, D: *S. mutans*

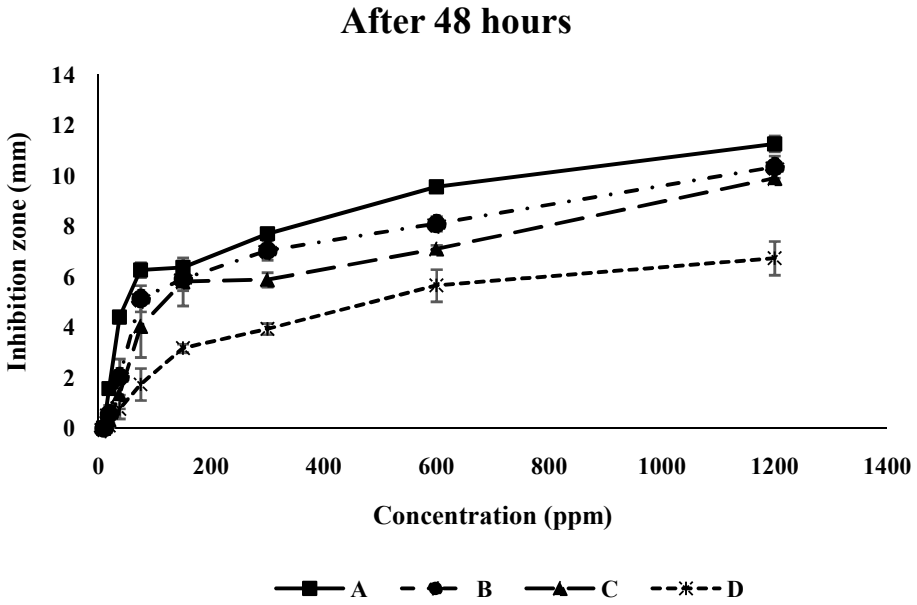

Fig. 3. The antibacterial trend of increasing honey concentration in four bacterial species after 48h. A: *L. casei, B: L. plantarum, C: L. rhamnosus*, D: *S. mutans*

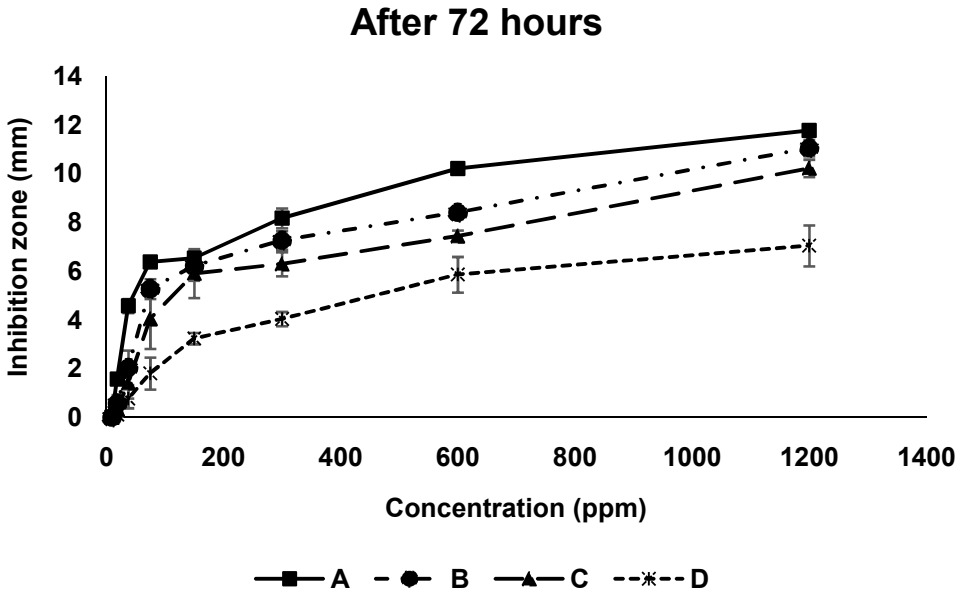

Fig. 4. The antibacterial trend of increasing honey concentration in four bacterial species after 72h. A: *L. casei, B: L. plantarum, C: L. rhamnosus*, D: *S. mutans*

To summarize, Table 3 shows the MIC and MBC for honey against the four bacterial species used in this study. *S. mutans* was demonstrated to be the most resistant species to honey.

Table 3. MIC and MBC of the honey on *S. mutans*, *L. casei*, *L. rhamnosus* and *L. plantarum*

NO	Bacteria	MIC (µg/ml)	MBC(µg/ml)
1	*Streptococcus mutans*	75	100
2	*Lactobacillus casei*	75	100
3	*Lactobasillus rhamnosus*	100	150
4	*Lactobasillus plantarum*	100	150

There are no previous reports demonstrating microbial resistance to honey, suggesting it can be used as an antimicrobial agent against a large number of bacteria without the emergence of bacterial resistance. In this study, we investigated the physiochemical properties and antibacterial activity of milk vetch honey against four bacterial species known to be the main causes of mouth and tooth disease. Our results suggest that the antibacterial potency of milk vetch honey is comparable to that of other types of honey. It has been reported that different types of honey have very different antibacterial potencies, which can vary much as 100-fold [16]. The flower from which bees produce honey is a critical parameter in determining the honey's properties. The MIC and MBC values have shown that there is a strong relationship between bacterial species and the antibacterial activity of honey. Our results demonstrate that concentrations below 37.5ppm are optimal for inhibiting *S. mutans, L. casei, L. rhamnosus* and *L. plantarum*. Manuka honey has been widely investigated and displays strong antibacterial properties. It is now available for use as an antibiotic [6,17]. In comparison, milk vetch honey showed similar antibacterial activity. Badet

et al. showed that Manuka honey has antibacterial activity against *Streptococcus and Lactobacillus* bacteria with MIC and MBC values of 100 and 200µg/mL, respectively [18], whereas we demonstrated MIC and MBC values between 75 and 150µg/mL, respectively. As shown in Figs. 2–4, milk vetch honey had significant antibacterial activity against the four species tested at two different concentrations: Those>300µg/ml and those<75µg/ml. There was no considerable antimicrobial activity of honey samples at concentrations between these two values. Our statistical analysis also demonstrates that lower concentrations are more efficient, suggesting that the antibacterial activity of this honey sample may be related to components other than its sugar content. To the best of our knowledge, no data is available as to what causes this interesting effect. In many types of honey, there is a linear relationship between concentration and its antibacterial potency. However, in some types, according to the plant derived, hydrogen peroxide and non-peroxide factors this central rule Therefore, additional studies will need to be performed to answer these questions.

4. CONCLUSION

In this study the antibacterial properties of milk vetch honey against four oral bacteria investigated and it declared that this type of honey has two different points of inhibitory effect. According to our results it seems that the cause of inhibitory and bactericidal effect of honey is its high sugar concentration, while in compare the reason for low concentrations inhibitory effect is its specific components.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Gurib-Fakim A. Medicinal plants: Traditions of yesterday and drugs of tomorrow. Mol Asp Med. 2006;27(1):73-93.
2. Zeina B, Othman O, Al-Assad S. Effect of honey versus thyme on Rubella virus survival in vitro. J Altern Complement Med. 1996;2(3):345-348.
3. Kwakman PH, Zaat SA. Antibacterial components of honey. IUBMB life. 2012;64(1):48-55.
4. Kacaniova M, Bobkova A, Bobko M, Hleba L, Rovna K, Vukovic N, et al. Antimicrobial and antiradical activity of Slovakian honeydew honey samples. J Microbiol Biotechnol Food Sci. 2011;1(3):354-368.
5. Molan PC. The antibacterial activity of honey: 1. The nature of the antibacterial activity. Bee World. 1992;73(1):5-28.
6. Cooper R, Molan P, Harding K. Antibacterial activity of honey against strains of Staphylococcus aureus from infected wounds. J R Soc Med. 1999;92(6):283.
7. Allen K, Molan P, Reid G. A survey of the antibacterial activity of some New Zealand honeys. J. Pharm. Sci. 1991;43(12):817-822.
8. Mohapatra DP, Thakur V, Brar SK. Antibacterial Efficacy of Raw and Processed Honey. Biotechnol Res Int. 2011;6:1-6. DOI: 10.4061/2011/917505.
9. Ryan KJ, Ray CG. Sherris medical microbiology. McGraw Hill Medical. 2010;4:488-490.
10. Dicks LM, Silvester M, Lawson PA, Collins MD. Lactobacillus fornicalis sp. nov., isolated from the posterior fornix of the human vagina. Int J Syst Evol Microbiol. 2000;50(3):1253-1258.

11. Badawy OF, Shafii SS, Tharwat EE, Kamal AM. Antibacterial activity of bee honey and its therapeutic usefulness against Escherichia coli O157: H7 and Salmonella typhimurium infection. Rev Sci Tech. 2004;23(3):1011-22.
12. White JW. Honey. Advances in food research, Adv Food Res. 1978;24:287–374.
13. Acquarone C, Buera P, Elizalde B. Pattern of pH and electrical conductivity upon honey dilution as a complementary tool for discriminating geographical origin of honeys. Food Chem. 2007;101(2):695-703.
14. Zappalà M, Fallico B, Arena E, Verzera A. Methods for the determination of HMF in honey: A comparison. Food control. 2005;16(3):273-277.
15. Wooton M, Edwards RA, Farajiharemi R. Effect of accelerated storage conditions on the chemical composition and properties of Australian honeys. J Apicult Res. 1976;17(3):167-172.
16. Cooper R, Halas E, Molan P. The efficacy of honey in inhibiting strains of Pseudomonas aeruginosa from infected burns. J Burn Care Res. 2002;23(6):366-370.
17. Brudzynski K, Abubaker K, Miotto D. Unraveling a mechanism of honey antibacterial action: Polyphenol/H_2O_2-induced oxidative effect on bacterial cell growth and on DNA degradation. Food Chem. 2012;2(15):329–336.
18. Badet C, Quero F. The *In vitro* effect of manuka honeys on growth and adherence of oral bacteria. Anaerobe. 2011;17(1):19-22.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=561&id=32&aid=4910