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ORIGINAL ARTICLE

TO COMPARE ANTIBACTERIAL EFFECT AND BIOCHEMICAL ANALYSIS OF NATURAL HONEY (FROM HONEYDEW) AND HONEY PRODUCT OF COLONY NUTRITION WITH SYRUP SUGAR

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Abstract: The aim of present research was to investigate antibacterial properties of honey (honey from the nectar of flowers) and honey from feeding colonies of bees manually with honey syrup by assuming that one hundred percent natural honey or honey from flowers syrup and honey from colonies fed with sugar prevent from the growth of gram-positive and gram-negative bacteria. In this study, during 3 months, biochemical analyzes (including measuring sucrose percentage, the hydroxyl-methyl furfural, pH, the diastase enzyme, amount of reductant sugar before hydrolysis, the amount of glucose or second sugar after hydrolysis, Brix value, moisture content and refractive index of 4 hives with including natural honey and honey from colonies feeding with sugar syrup with standard method No. 92 of Institute of Standards and Industrial Research of Iran were measured. Also, the test of determining the MIC and MBC were performed by microplate method in the presence of standard drug Gentamicin. The results demonstrated that the minimum inhibitory concentration (MIC) of natural honey in *Staphylococcu aureus*, *Streptoccouss pneumoniae*, *Bacillus cereusm*, *Escherichia coli*, *Pasteurella multocida* and *Pseudomonas aeruginosa* was 1/1000, 1/100, 1/1000, 100/100, 1/1 and 1/10 µg/ml, respectively. Also, MIC of honey from colonies fed with sugar syrup (with 4.5% sucrose) in *Staphylococcu aureus*, *Streptoccouss pneumoniae*, *Bacillus cereusm*, *Escherichia coli*, *Pastorella multicida* was 1/1000, 1/100, 1/100, 100/100 and 1/1 µg/ml, respectively but it was ineffective on *Pseudomonas aeruginosa*.

Keywords: Natural honey, Nutrition honey, Gram-positive and gram -negative bacteria

INTRODUCTION

The antimicrobial properties of medicinal plants and extracts have been considered from the last time and has been used these materials without the knowledge of microbes and only practical way to treat diseases. After the discovery of germs and antibiotics, researchers were considered antimicrobial properties of some organic compounds such as essential oils and extracts of plants. Today, the use of herbal products and extracts is increasing in the most advanced countries (Sato and Miyata, 2000). Today, with the emergence of various synthetic drugs and antibiotics, many previous incurable diseases are treated. Of course, Many synthetic drugs can not be used in weaker people. While getting new diseases and more resistant strains of bacteria are appeared (Pinto et al, 2009).

With the current situation, the need to use more the essential oils and natural extracts is felt to fight against pathogenic microbial species. Natural remedies have several crucial advantages compared to chemical synthetic drugs that can cite to cases such as having fewer side effects, the use of synthetic drugs in the sensitive individuals, low cost and availability. Honey has antibacterial properties that recently have been used to treat diseases. Therefore, the medical honey has been accepted as an antibacterial agent in the treatment of wounds, burns, superficial infection. Also, honey considers as an antiseptic and has different natural substances with inhibitory properties (Molan, 1992; Branski et al, 2009; Fazli et al, 2009).

Infectious diseases are a major cause of mortality in human, especially in the developing countries. In the recent years, drug resistant to disease has been increased, so, a strategic antimicrobial is needy and therefore, plants and plants productions such as honey are used for the treatment of infectious diseases. Honey has anti-bacterial activity against gram-positive and gram-negative bacteria (Blair and Carter, 2005; Ghaleb et al, 2011; Willix et al, 1992).

This study has been performed on the effect of natural honey and honey from colony fed by sugar syrup, and also comparing their effects on 3 gram-positive bacteria (*Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus cereus*) and 3 Gram-negative bacteria (*Escherichia coli*, *Pasteurella multocida*, *Pseudomonas aeruginosa*) and the results were compared with Gentamicin antibacterial drugs. By definition (according to National Committee for Clinical Laboratory Standards Manual (NCCLS)), MIC is the lowest concentration of drug that inhibit the growth of tested bacteria (Last concentration of staining that is not caused any resentment). Also, The MBC is defined as the minimum concentration of mentioned drug to reduce 99.9 percent of the initial density of bacteria (Qaiyami, 2007; NCCLS, 1997).

MATERIALS AND METHODS

Honey sample preparation: in this study, honey samples were collected from a beehive at the university of Marand that had been fed only from sugar syrup in spring of 2012 and other beehive that that had been fed by the solution of 15% sugar syrup. Then, the samples were transferred to the microbiology laboratory, faculty of veterinary medicine, university of Urmia, as soon as possible and were stored at 4 ° C. In this study, the extract of each honey was extracted using a special fabric filter for honey based on standard method. 2.5 grams of it were mixed into the tube with 10 ml physiological saline and then, were put it on Shaker (150 rpm). In addition, the honey samples were sterilized by using 0.22 micron sterile syringe filter and were diluted to concentrations of 1/10, 1/100, 1/1000, 1/10000 µg/ml.

Preparation of bacterial strains: The bacteria were used in this study including; I) Gram-positive bacteria including *Staphylococcus aureus* (PTCC 29213), *Streptococcus pneumoniae* (PTCC 1240), *Bacillus cereus* (PTCC?) and II) Gram-negative bacteria Escherichia coli (PTCC 1533), *Pasteurella multocida* (PTCC?) and *Pseudomonas aeruginosa* (PTCC 1310) that were provided from bacteria set of veterinary medicine faculty, university of Urmia. Mentioned bacteria were cultured in Mueller-Hinton broth (Merck-Germany) incubated at 37 ° C for one day and after grow they were used for testing. Analysis of biochemical parameters of natural honey and nutritional honey was measured by standard method of INSO 92 (Swiss Bee Research Center, 1999).

Antimicrobial susceptibility testing: the MIC and MBC test were performed by microplate method (according to National Committee guideline of Clinical Laboratory Standards Guide (NCCLS) and was evaluated in the presence of standard drug Gentamicin (*NCCLS, 1997*).

Broth Micro-dilution method: this method was used for determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In this method, the dilutions of natural honey and sugar syrup honey was prepared to 100 Lambda separately in each well containing Mueller-Hinton liquid medium (to 800 lambda) in 48-well microplate (8 rows x 6 columns) . In each of the micro plate rows, bacteria and in each of column, selected drug dilutions and drug Gentamicin, and water was added to control positive and negative control in the final two columns. In the next phase, studied bacteria suspension equal to the half pipe McFarland (1-2×10^8) was regulated in physiological serum and fixed concentrations as 100 Lambda was added by sampler to each well and micro plates were incubated for 24 hours at 37 ° C. After incubation, the results of each well were recorded. The experiment was carried out in three replications. Data analysis was performed using SPSS software version 20.

RESULTS

The results of the broth micro dilution test namely minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of natural honey and honey from from colonies fed with sugar syrup showed that natural honey was effective on all studied bacteria except *Pseudomonas aeruginosa.* Also, nutrient honey with 4.5% sucrose (sucrose in standard values) was effective on all studied bacteria except *Pseudomonas aeruginosa*. More details about the treatments can be seen in Table 1. Biochemical analysis of natural honey and other sugar honey showed that the amounts of sugar, honey and other honey, sucrose, first glucose or sugars before hydrolysis, second glucose or sugar after the hydrolysis, hydroxyl methyl furfural factor or HMF, diastase enzyme concentration, moisture content, Brix, refractive index or refraction coefficient, pH, the acidity of

the honey were completely different in these honey. The results of the analysis are shown in Table 2.

Table 1. Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) of natural honey, honey from hives fed with sugar syrup (honey with 5.4% sucrose) (µg /ml).

Treatment	*Staphylococcu aureus*		*streptoccouss pneumoniae*		*Bacillus cereusm*		*Escherichia coli*		*Pastorella multicida*		*Pseudomonas aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Natural honey	1/1000	1/100	1/100	1/10	1/1000	1/100	1/100	1/10	1/100	1/10	1/10	–
Honey with 4.5% sucrose	1/1000	1/100	1/100	1/10	1/100	1/100	1/100	1/10	1/100	1/10	–	–

Table 2. Biochemical analysis of natural honey and honey from colonies fed with sugar syrup.

No.	Biochemical analysis	Natural honey (%)	Honey with processed sucrose of 1-5% (%)	No.	Biochemical analysis	natural honey
1	Sucrose percentage (gr%)	2.5	4.5	6	Brix	84
2	reductant sugar (gr%)	77.18	75.89	7	refractive index	1.5003
3	Second sugar (gr%)	79.68	80.39	8	pH	3.90
4	HMF (mg/Kg)	2.25	3.75	9	diastase enzyme concentration	14.65
5	moisture content	14.80	15.30			

DISCUSSION

Honey is the release of trees sugar, flowers and plants nectar that collect by honey beekeepers, modify and store within the cells of the frame. Honey contains various combinations of herbal compounds and phenolic and flavonoids content that can be assigned to the highest antioxidant activity and are factor that have strong antioxidant properties. Herbal and chemicals compounds in honey can be limited to phenolic acids and polyphenol compounds. Various polyphenols found in honey that have been reported.

In 1992, researchers such as Rodriguez and pajero reported that there are ingredients in honey such as glucose sugars and fructose and other sugars such as sucrose and Maltose; also, other minor amounts of organic acids such as lactones, the amino acids, minerals and vitamins including B1, B2 and acid nicotinic, enzymes, pollen and wax and pigments.

Carbohydrates are found abundant in natural diet as the main source of Honey bee's energy. It is possible to convert these carbohydrates into fat and stored in the body. Differences in the use of carbohydrates between larvae and young bees may be necessary due to the removal or absence of enzymes. Young bees can use from glucose, fructose and sucrose and maltose and trehalose while they can not use national Rhamanose, xylose and arabinose, galactose, mannose, lactose and raffinose, inulin and dextrin. Bees can be fed with various and complementary food. Hidack in 1970 stated that in early spring when pollen or nectar can be used before or during other seasons of the year with low amounts of complementary feeding could help to colony survival and stability or to increase production and bee population. In the present study, levels of glucose, fructose and sucrose were measured in each honey after feeding colonies with the syrup (about natural honey) and with sugar syrup (in the case of honey from colonies fed with sugar syrup). The

results revealed that mentioned sugars were existed in both types of honey with different values. Also, fructose in honey natural was more than honey from the feeding colonies with sugar syrup. Moreover, sucrose amount in natural Honey (2%) was less than the honey from colonies feeding with sugar syrup (4%).

In this study, natural honey from plant syrup and honey from colonies fed with sugar syrup were used. The required honey in this study was prepared in the spring and summer from hives of Marand, East Azarbaijan Province, Iran. Mussa et al. In a study on the physical, chemical and microbiological properties of 3 samples of natural honey, honey produced by bees with complementary feeding with sugar syrup found out that natural honey had moisture content less than the produced honey with complementary feeding of bee. The same results were obtained in this study as it was 14.8% in natural honey and 15.3 in honey from colonies feeding with sugar syrup. Regard to recent studies, natural honey has also the highest amount of diastase enzyme and honey from colonies feeding with sugar syrup had the lowest enzyme which can be due to thermal process and denaturation of the natural enzymes in honey. In this research, natural honey had the largest amount of diastase enzyme as well (which has a direct impact on antimicrobial activity) (14.65 unit) and honey from colonies feeding with sugar syrup had the least amount of diastase enzyme (13.98 unit), respectively.

As can be seen in Table 2, there was difference between the groups of honey in the amount of HMF. The result was very close in natural honey samples and the second group (sugar). The cause of high HMF in nutritional honey with sugar was to apply the thermal process on the syrup (sucrose), i.e. temperature of 88 °C for 2 hours.

Donors reported in 1977 that since the ratio of fructose to glucose in honey is different from different regions and different sources, during the formation of HMF that is the result of acid hydrolysis and de-hydration of six-carbon sugars, fructose decomposes faster and reacts quickly compared to other sugars (theory of Custer, Lee, Wangi about the stability of sugar molecules, 1990). In 1997, Bath & Singh stated that the chemical properties of honey, such as PH and acidity of the amount of minerals had affect on forming HMF (Rubén et al, 2013).

Based on Bath survey, HMF of most samples (including samples of honey, and honey containing sugar) was less than defined range i.e. less than 40 mg/Kg except honey from tropical countries or regions that in this situation, the amount of HMF should not be more than 80 mg/Kg. In present research, HMF content of honey and sugar honey was less than 40 mg/eq and the data of both groups were close together.

Usually, the percentage of sucrose in natural honey sample does not exceed from 8%. In Bath & Singh surveys, amount of second group sucrose (sugar) was equal to 4.5%, which is much closer to the standard range and its cause can be due to complementary feeding of bees with sugar syrup (sucrose). In the present study, sucrose percentage of natural honey was 2% and the sucrose percentage of colonies fed with sugar syrup was 4.5%.

In other research of Mussa, microbial total counting of honey was too low range of 1×10^3 to 8×10^3 cfu/g, and it was not observed coliform bacteria in any sample except for SSH of honey (sucrose syrup honey) with frequency of 1×10^3 cfu/g. In total, natural honey and honey type of SSH had very close microbial properties (Mandal et al, 2012). In the present study, also natural honey and honey fed with sugar syrup (with 4.5% sucrose) had similar anti-bacterial activity (inhibitory effects).

Wide-ranging studies have been performed on the effects of anti-bacterial effects of honey. A bactericidal mechanism (removal or killing bacteria) is involved in the mechanism of cell division. MIC (minimum inhibitory concentration) for *Staphylococcus aureus* covered by national bee is 126.23 to 185.70 mg/ml. Also, Honey is effective against negative coagulase *Staphylococcus aureus*, applied effects of raw honey on infected wounds with acute inflammatory signs and symptoms is therefore leads to the reduction of signs. Antibiotic potential is comparable to other topical antibiotics. Honey activities associated with persistent infection result in reducing inflammation, discharge of pus and removal of red (from inflammation), simultaneous eradication of bacterial infections. When honey combines and used with antibiotics such as Gentamicin, antibacterial activity against *Staphylococcus aureus* increases to 22%. Also, honey is effective in Killing hard growth bacteria such as *Pseudomonas aeruginosa* (PA) and also, could lead to new treatments for the disease of refractory chronic nasal sinus swelling. Daily use of honey reduces the risk of chronic infections caused by microorganisms, as well.

Blair and Cooper showed that honey has anti-bacterial activity against gram-positive and gram-negative bacteria (Aguiar et al, 2014; Hidalgo et al, 2011; Masura et al, 2013). French et al. showed that raw honey can inhibit more fungi and bacteria isolated from infected wounds and infection of the surgical except *Pseudomonas aeruginosa* (Terrab et al, 2004; Moira et al, 2008; Othman, 2012; Hasan et al, 2012). In present study, the results showed that natural honey (from bees fed with plant syrup) is effective on gram-positive bacteria. As, the minimum inhibitory concentration (MIC) of natural honey was 1/1000, 1/100 and 1/1000 µg/ml dilution for *Staphylococcus aureus*, *Streptococcus pneumonia* and *Bacillus cereus*. Also, natural honey was effective on gram-negative bacteria, except *Pseudomonas aeruginosa*. As, MBC (MBC) for *E. coli* and *Pasteurella multocida* was 1/100 and 1/100 µg/ml dilution, respectively.

Honey is full of flavonoids. Flavonoid has created a lot of research interest due to anti-cancer and anti-bacterial properties. It is assumed that flavonoids show different routes, including the release of tumor necrosis factor alpha (TNF-α), Inhibition of cell proliferation, induction of programmed cell death, cell cycle stimulation and inhibition of oxidative lipoprotein. It is thought that these important properties of honey are due to the main compounds such as cresyn and other compounds of flavonoids. These characteristics are related to different plant flora that each one of the flora source show different active ingredients (Mandal et al, 2012).

Sugar syrup with physical properties as honey, can not control any bacteria and fungi that this issue proved that non-processed honey is superior in antimicrobial activity than hypertonique sugar solution (Rubén et al, 2013). In this study, honey bee feeding just with sugar syrup had inhibitory effects on gram-positive bacteria. As, the minimum inhibitory concentration (MIC) of honey with 4.5% sucrose for *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus cereus* was the dilution of 1/1000, 1/100, 1/100 µg/ml.

Also, in the mentioned survey, honey from colonies fed with sugar syrup had only inhibitory effect on gram-negative bacteria, except *Pseudomonas aeruginosa* and had no inhibitory effect. As, the minimum inhibitory concentration (MIC) of honey with 4.5% sucrose was 1/100 and 1/100 µg/ml for Escherichia coli, *Pasteurella multocida*, respectively.

In this study, Gentamicin 80 standard drug was used as positive control that the result of the treatments is comparable to this drug. Other studies have shown that when honey combined and used with antibiotics such as Gentamicin, the antibacterial activity against *Staphylococcus aureus* increases to 22% (Serrano et al, 2004).

enzymes in honey, including phosphatase and inhibine have bactericide property and prevents from the growth of bacteria in the honey. Honey enzymes gradually decreases with increasing temperature and may be eliminated (White et al, 1963). Acid in honey is likely affective to prevent the growth of microbes. The most acid that exist in honey is gluconic acid that produces from the effect of glucose oxidase on glucose production. Glucose oxidase, causes glucose oxidation to gluconic acid and hydrogen peroxides that prevents from during ripening corruption (Serrano et al, 2004). Catalase is the enzyme in honey that has plant source that regulates glucose oxidase activity that has germicidal activity (Lavie, 1968).

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