In vitro Study of Honey Antimicrobial Activity

Daniela Moţ

Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania" from Timişoara, Faculty of Animal Sciences and Biotechnologies, 300645Timişoara, 119 Calea Aradului, Romania

Abstract

From ancient times honey was renowned for healing properties both in internal consumption and of external applications. Samples of non-standardized honey obtained direct from beekeeper were used in a study using a method of growth inhibition in the culture medium of microorganisms. In this aim were been chosen a few of high pathogenic bacterial species implicated in severe infection both in animals and human: *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Enterococcus faecium and Pasteurella haemolytica.* The zone diameter of inhibition (ZDI) was the most developed with the highest value in the case of *Bacillus subtilis* (35 mm) and the lowest value was obtained in the case of *Salmonella typhimurium* (21 mm). Also in a diluted sample of honey was calculated the total number of bacteria colonies, then was inoculated material from a culture of *Staphylococcus aureus.* After bacteria inoculation was calculated again the total number of bacteria colonies and then the samples were been maintained in thermostat (37^{o}C) 12 hours and was repeated the counting of total number of bacteria colonies. In comparison with the value obtained at first counting of bacteria colonies after inoculating of *Staphylococcus aureus* culture (1326 colonies/milliliter), at the second counting the obtained value was nearly half (698 bacteria colonies/milliliter) from this one. All these demonstrate the antibacterial activity of honey apparently due to some bactericidal factors in its composition.

Key words: *antibacterial activity, growth inhibition, honey, microorganisms.*

1. Introduction

Honey, so appreciate in our times is as old as history is itself. Is hard to believe, but one of the earliest evidence of honey is on a rock painting dating back 8 000 years, found in Valencia, Spain, representing a honey seeker robbing a wild colony. Bees have lived long before man, because bees were found fossils of ancient tens of millions of years, and Homo sapiens has only 50,000 years. From ancient times human have eaten honey, bathed in it and fixed their wounds with it. Archaeologists discovered honey comb in Egypt buried with the pharaohs in their tombs and was so well preserved being still eatable. In the Old Testament, the land of Israel was often referred to as the "land flowing of milk and honey". God nourished Jacob with honey from the rock, and gave Israel fine flour, olive oil and honey. John the Baptist ate locusts and wild honey. Honey is mention in the scrolls of the Orient, the Talmud and Koran. Aristotle (384-322 BC) when discussing different honeys, referred to pale honey as being "good as a salve for sore eyes and wounds". The Romans used honey to heal their wounds after battles. Hannibal, a great warrior gave his army honey and vinegar as they crossed the Alps on elephants to battle Rome. During the 10 century, the Kings and Queens of England had fermented honey wine (Mead), the Edmeades family produced some of these. In our country was one of beekeeping activity, along with grazing and agriculture [1]. The beginnings of our Romanian civilization are related also by the honey. Thus, a traditional dish ceramic Cucuteni culture period 6000-5000 IC, there are graphical representations of bees "goddesses". Then Xenofon, general and historian (427-355 î. e. n.) highlights that food

* Moţ, D., Phone 0256 277 192, Fax 0256 277 110, Email dana_tm@animalsci-tm.ro

Getae consisted primarily of honey, vegetables, milk or preparation and very little meat. Romania produces an average of 20,000 to 22,000 tons of honey annually, ranking fourth in Europe. Romania produces mainly acacia honey, lime honey and poly-flora honey. In 2011 it produced 21,000 tonnes provided by private producers, which own 1.3m bee families. The Romanian producers are very happy with the quality of their honey and there are good chances the production will rise due to EU funding, organic farming measures, new technologies and treatments introduced, as well as due to the very favourable natural conditions. Considering that honey is both a nutrient a drug and an ointment, basing on the ancient experience, in our days appeared an alternative medicine branch, called apitherapy. At present a number of honeys are sold with standardized levels of antibacterial activity. The *Leptospermum scoparium* honey, or Manuka honey, derived from Manuka tree (*Leptospermum scoparium*) the best known of the honeys, has been reported to have an inhibitory effect on around 60 species of bacteria, including aerobes and anaerobes, Gram-positives and Gram-negatives [2]. This honey is a promising functional food for treatments of wounds or stomach ulcer. Tan and al. [3] reported that Tualang honey has variable but broad-spectrum activities against many different kinds of wound and enteric bacteria. There also many reports of honey being very effective as dressing of wounds, burns, skin ulcers and inflammations [4]. The composition of honey is the answer to all healing properties. Honey contains water, carbohydrates, fructose, glucose, maltose, sucrose, proteins, amino-acids, vitamins and minerals. Vitamins contained in honey are: thiamine, riboflavin, niacin, panthotenic acid, pyridoxine (B_6) and ascorbic acid (C). Honey contains also minerals like: calcium, copper, iron magnesium, manganese, phosphorus, potassium, sodium and zinc [5]. The present paper aims to highlight the antibacterial properties of honey and that the importance of her medical care for humans and animals with application might happen when.

2. Materials and methods

To emphasize the antibacterial properties of honey was selected a sample of polyfloral honey from a beekeeper which has installed beehives 130 kilometres from Timişoara. For study were been chosen seven species of pathogenic bacteria that cause serious diseases in humans and animals taken from the Microbiology laboratory collection: *Bacillus subtilis ATCC 6051, Staphylococcus aureus ATTC 6538 P, Escherichia coli ATTC 10536, Pseudomonas aeruginosa ATTC 27853, Salmonella typhimurium LT2, Enterococcus faecium TX16 and Pasteurella haemolytica type A (Mannheimia haemolytica 1999)* inoculated in usually liquid culture medium. The applied method was the diffusion method, a qualitative *in vitro* test for detecting the susceptibility of bacteria to antimicrobial substances [6]. For each bacterial species were prepared Petri dishes with Mueller Hinton Agar. Every Petri dish inoculated in flood with a sterile pipette the studied species of bacteria, so that were been finally obtained seven Petri dishes. Then, equal concaves were practiced with a matrix with 4 mm diameter, two in each Petri dish with agar gel and the agar discs were removed with a needle. In each Petri dish, in the concave practiced in was been poured in one sample of studied honey 5% diluted in distilled water and in the other concave, considered blind test (M), only distilled water. All seven inoculated Petri dishes remained at room temperature 30 minutes, for a preliminary diffusion, after that were incubated at 37 ^{o} C for 24 hours. In a 1/1 diluted with distilled water sample of honey was been calculated the colony-forming units (CFU), also named aerobic plate count, on a nutrient agar poured in thin layer on Petri dishes. For this counting was used from serial dilutions of honey the 10^{-1} dilution, based on the idea that the bacterial load in this case is reduced. The Petri dishes, two for a sample, were been flooding inoculated with sample of honey in 10^{-1} dilution. The Petri dishes were been incubated at 37 ^{o} C for 24 hours. Counting of the bacterial colonies or colony-forming units (CFU) was performed using the Nitech LKB 2002 apparatus. Then, a sample from the same studied honey 1/1 diluted with distilled water was been inoculated with a usually liquid culture of *Staphylococcus aureus ATTC 6538P* strain from laboratory collection. After bacterial culture inoculation was performed the counting of CFU, in the same way, this time using another dilution, of 10^{-3} in the view of avoiding the obtaining on the culture medium of confluent colonies which make it impossible

counting. The same honey sample inoculated with *Staphylococcus aureus* was then incubated 12 hours in thermostat at 37° C. Thereafter was performed again a CFU counting, at the same dilution of 10^{-3}, in the view of comparison with the first counting of honey inoculated sample.

3. Results and discussion

In Table 1 are presented the results of agar diffusion technique using the seven bacterial species mentioned. After 24 hours of incubation at 37 ° C, around every bacterial species inoculated in culture medium formed inhibition areas by variable diameters. The lowest zone diameter of inhibition (ZDI) was formed around *Salmonella typhimurium* at 1/1 dilution (21 mm), which shows that this bacteria manifested the maximal resistance to honey. The largest zone diameter of inhibition (ZDI) developed around *Bacillus subtilis* (35mm) at 1/1 dilution (Table 1) which manifested the greatest sensitivity to honey, followed by *Escherichia coli* (31 mm) (Table 3), *Staphylococcus aureus* (29 mm) (Table 2), *Pseudomonas aeruginosa* (27 mm) (Table 4), *Enterococcus faecium.* (26 mm) (Table 6), *Pasteurella haemolytica* (23 mm) (Table 7) and *Salmonella typhimurium* (21 mm) (Table 5), all of them at 1/1 dilution.

Table 1. The results of agar diffusion technique with *Bacillus subtilis*

Dilution	Diameter of inhibition area (mm)	M
1/1	35	0
1/2	25	0
1/4	19	0
1/8	14	0
1/16	11	0
1/32	4	0

Table 2. The results of agar diffusion technique with *Staphylococcus aureus*

Dilution	Diameter of inhibition area (mm)	M
1/1	2 9	0
1/2	21	0
1/4	16	0
1/8	9	0
1/16	3	0
1/32	0	0

Table 3. The results of agar diffusion technique with *Escherichia coli*

Dilution	Diameter of inhibition area (mm)	M
1/1	31	0
1/2	27	0
1/4	21	0
1/8	15	0
1/16	9	0
1/32	3	0

Table 4. The results of agar diffusion technique with *Pseudomonas aeruginosa*

Dilution	Diameter of inhibition area (mm)	M
1/1	27	0
1/2	20	0
1/4	13	0
1/8	8	0
1/16	2	0
1/32	0	0

Table 5. The results of agar diffusion technique with *Salmonella typhimurium*

Dilution	Diameter of inhibition area (mm)	M
1/1	21	0
1/2	17	0
1/4	11	0
1/8	8	0
1/16	2.4	0
1/32	0	0

Table 6. The results of agar diffusion technique with *Enterococcus faecium*

Dilution	Diameter of inhibition area (mm)	M
1/1	26	0
1/2	22	0
1/4	16.8	0
1/8	11	0
1/16	4	0
1/32	0	0

Table 7. The results of agar diffusion technique with *Pasteurella haemolytica*

Dilution	Diameter of inhibition area (mm)	M
1/1	23	0
1/2	17	0
1/4	13	0
1/8	5	0
1/16	2.7	0
1/32	0	0

Table 8. The results of CFU counting

Dilution	Number of CFU	Mean
10^{-1}	13	
10^{-1}	25	19
10^{-3}	1621	
10^{-3}	1031	1326
10^{-3}	733	
10^{-3}	663	698

Dilutions other values are becoming smaller, some at 1/32 dilution, even zero, without any inhibition zones development. In this test none of the bacterial species studied showed no resistance to honey. In the case of blind tests with distilled water all obtained values were zero. Honey antibacterial activity has been the subject of study for many researchers which were mainly interested by the minimum inhibitory concentration (MIC) reflecting the quantity needed for bacterial inhibition. Following the *in vitro* methods, many bacteria mostly multi-drug resistant (MDR) causing severe human and animal infections that were found susceptible to honeys [7,8]. Mandal S. and al. [9] explored time-kill activity of autoclaved honey against *E. coli, P. aeruginosa* and *S. typhi* in order to establish the potential efficacy of such local honey (not studied before) collected from a village of the West Bengal state, India, demonstrating a strong antibacterial effect. Another study on antibiotic resistant isolates of some MDR bacteria species observed that all these were killed within 24 hours, become necessary to establish various local honeys based upon kill kinetics and their effective *in vivo* applications against MDR infection [10]. The antibacterial role of honey is attributed to its properties correlated with its high osmolarity, acidity (low pH) and content of hydrogen peroxide (H_2O_2) and non-peroxide components, to the presence of phytochemical components like methylglyoxal (MGO). The antimicrobial agents in honey are predominantly hydrogen peroxide, of which the concentration is determined by relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower pollen. Most types of honey generate H_2O_2 when diluted, because of the activation of the enzyme glucose oxidase that oxidizes glucose to gluconic acid and H_2O_2, which thus attributes the antimicrobial activity [11]. But, in some cases, the peroxide activity in honey can be destroyed easily by heat or the presence of catalase. Honey is characteristically acidic, with pH between 3.2 and 4.5, which is low enough to be inhibitory to several bacterial pathogens and in undiluted honey the acidity is a significant antibacterial factor. The antibacterial property of honey is derived also from the osmotic effect of its high sugar content and low moisture content, along with its acidic properties of gluconic acid and antiseptic properties of its H_2O_2. In the last table (Table 8) are shown results of CFU counting in honey before and after *Staphylococcus aureus* liquid usually culture inoculation. Before inoculation honey 1/1 diluted with distilled water, in CFU counting an average of 19 bacterial colonies/ millilitre, a reduced number demonstrating that honey is not contaminated. Generally, in honey like primary source of microbial contamination can include pollen, the digestive tract of honeybees, dirt, dust, air and flowers. Secondary sources of microbial contamination in honey are human, equipments, containers and wind. Shortly after *Staphylococcus aureus* inoculation, in CFU counting were been identified an average of 1326 colonies/millilitre. The numbers of colonies was almost half of the initial, with 698 colonies/millilitre, demonstrating that honey, through its components has the capability to destroy bacteria [12]. Many other studies demonstrated that if honey is diluted with water, it supports the growth of non-pathogenic bacterial strains and killing of dangerous strains. It was also demonstrated that undiluted honey has been found to stop the growth of bacterial and even fungal species.

Antibacterial activity of honey varies between different types of honey. It has been observed that exists different types of honey and a method has been used to determine the „inhibine number" of honey as a measure of their antibacterial activity [13].Was demonstrated that honey contains an antimicrobial peptide, named bee defensin-1 responsible of substantially contribution to the bactericidal activity. Bee defensin-1 was previously isolated from royal jelly [14] the major food source for bee queen larvae (and then referred to as royalisin) and was identified in honeybee hemolymph. Royal jelly is produced by young worker bees and contains their hypoparyngeal and mandibular gland secretions. Since it was been found bee defensin-1 in honey, this suggests that after transition in

hypopharingeal gland function of the worker bees with age, the gland still produce bee defensin-1. This peptide contributes to protection of both royal jelly and honey against microbial spoilage. The clearing of infection seen when honey is applied to a wound may reflect more than just antibacterial properties. A research shows that the proliferation of peripheral blood B-lymphocytes and T-lymphocytes in cell culture is stimulated by honey and phagocytes are activated by honey, monocytes too, releasing cytokines, tumour necrosis factor(TNF-alpha), interleukins (IL-1 and IL-6) which activate the immune response to infection [15].

4. Conclusions

Honey, a remarkable natural product of bees, with so many health beneficial actions, including antibacterial effect was used from 1/1 to 1/32 dilutions with distilled water against seven bacterial species responsible of severe infections in man and animals. The obtained results emphasized that no one of studied bacteria manifested resistance to honey, on the contrary, all studied bacteria species manifested in varying degrees sensitivity to honey, at small dilutions. The largest zone diameter of inhibition (ZDI) developed through agar diffusion technique and the great sensitivity to honey manifested *Bacillus subtilis* (35mm) at 1/1 dilution which manifested the greatest sensitivity to honey, followed by *Escherichia coli* (31 mm), *Staphylococcus aureus* (29 mm), *Pseudomonas aeruginosa* (27 mm), *Enterococcus faecium.* (26 mm), *Pasteurella haemolytica* (23 mm) and *Salmonella typhimurium* (21 mm). All these results shows that honey can be successful include for alternative and natural treatment in wounds, burns, skin infections, or in internally administrations in ulcer treatments and many other diseases. When in 1/1 diluted honey was been inoculated an usually liquid culture of *Staphylococcus aureus*, initially in CFU counting was obtained an average number of 1326 colonies/milliliter. After 24 hours of incubation this number was reduced almost at half value, demonstrating the antibacterial effect of honey due to its precious component substances. All these results emphasized the medicinal importance of honey, a possible therapeutic potential especially today, when many resistant pathogens develop and spread and the effectiveness of the antibiotics is diminished. Honey or isolated components thereof may serve as new agents in prevention and treating infections, in particular those caused by antibiotic-resistant bacteria.

References

1. Hilda M. Ransome, The sacred bee in ancient times and folklore, Ed. Dover Books on Anthropology and Folklore, 2004, 19-91.
2. Molan P.,C., The antibacterial nature of honey . The nature of antibacterial activity, Bee World, 1992, 73, 5-28.
3. Tan H., T., Rahman R., A., Gan S., H., Halim A., S., Hassan S., A., Sulaiman S., A. et al., The antibacterial properties of Malaysian tualang honey against wound and enteric microorganisms in comparison to manuka honey, BMC Complement Alternat. Med., 2009, 9, 34 doi: 10.1186/1472-6882-9-34.
4. Lusby P., E., Coombes Al., Wilkinson J., M., Bactericidal activity of different honeys against pathogenic bacteria, Arch. Med. Res., 2005,36, 464-467.
5. David V. Ball, The chemical composition of honey, J. Chem. Educ., 2007, 84 (10), 1643.
6. Târziu E., Cumpănăşoiu C., Monica Şereş, Gros R., Bacteriologie specială-Practicum-, Ed. Waldpress, 2010, Timişoara.
7. Cooper R., A., Molan P., C., Handing K., G., Honey and Gram positive cocci of clinical significance in wounds, J. Appl. Microbiol., 2002, 93, 857-863.
8. Simon A., Traynor K., Santos K., Blaser G., Bode U., Molan P., Medical honey for wound care – still the 'Latest resort', Evid. Based Complement Alternat. Med., 2008, doi:10.1093/ecam/nem175.
9. Manisha Deb. Shyamapada Mandal, Honey: its medicinal property and antibacterial activity, Asian Pac. J., Trop. Biomed., 2011, 1(2), 154-160.
10.Kwakman PHS, Johannes P.,C., Van der Akker, Ahmet G., Aslami H., Binnekade J.,M., et al., Medical-grade honey kills antibiotic-resistant bacteria in vitro and eradicates skin colonization, Clin. Infect., Dis., 2008: 46 DOI:10.1086/58792.
11.Van der A.,J., Van der Worm E., Van Ufford H., C., Halkes S.,b., Hoekstra M.,J., Beukelman C.,J., An in vitro examination of the antioxidant and anti-inflamatory properties of buckwheat honey, J. Wound Care, 2008, 17, 172-178.
12.Taormina P., J., Niemira B., A., Beuchat L., R., Inhibitory activity of honey against foodborne pathogens as influenced by the presence of hydrogen peroxide and level of antioxidant power, Int. J. Food Microbiology, 2001, 69, 217-225.

13.Jeddar A., Kharsany A., Ramsaroop U.,G., Bhamjee A., Hafejee I.,E., Moosa A., The antibacterial action of honey, South Afri Med J., 1985, 67, 257-258.
14.Fujiwara S., Imai J., Fujiwara M., Yaeshima T., Kawashima T., and Kobayashi ., A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin, J. Biol. Chem., 1990, 265, 11333-11337.
15.Tonks A., Cooper R.,A., Price A.,J., Molan P.,C., Jones K.,P., Stimulation of tnf-alfpha release in monocytes by honey. Cytokine., 2001, 14(4), 240-242.