Study on Antimicrobial Activity in Different Types of Honey

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Abstract
Many times when it is studied the therapeutic effect of honey appears an important question regarded to which type of honey is suitable for medical purpose and which type has the best antibacterial effect. In this study were been analyzed six samples of honey from different botanical origins, including unifloral (Acacia honey, Lime honey, Cole honey), and multifloral types (forest honey from low altitude, from high altitude and field honey) obtained direct from beekeepers localized in unpolluted areas from the Western part of Romania. Was been evaluated the pH from honey samples, microbiological characteristics (standard plate count or SPC) and antibacterial activity (method of growth inhibition) in some pathogen microorganisms cultures from *Pseudomonas aeruginosa*, *Escherichia coli, Bacillus subtilis, Staphylococcus aureus*. Also it was performed a study for bacterial, fungal or yeast organisms detection in all six honey samples. The count of aerobic mesophilic bacteria, mold and yeast registered low values, being absent from honey samples pathogen bacteria like coliform bacteria, *Shigella*, *Salmonella, Proteus* and *Clostridium* species. After measuring of the size of zone diameter of inhibition (ZDI) for evaluate the honey antibacterial activity, the highest value was obtained in forest honey from high altitude (39 mm) and the lowest value in the case of Cole honey (11 mm) in the case of undiluted samples. All dilutions of honey samples registered lower values of ZDI than those undiluted. All these results, correlated with many others from many studies demonstrate once again that this natural and ancient product is so valuable by medical point of view and anytime can be used like alternative methods of treatments.

Key words: *unifloral honey, multifloral honey, microorganisms, antibacterial activity, medical purpose.*

1. Introduction

Honey is the fastest and strongest known natural energizer. It is the result of a huge work of the hive, that transforms sugars found on the flowers and leaves of plants into a paste slurry, with a highly complex chemical composition. A teaspoon of honey contains over 400 of organic substances, which could not be replicated with the sophisticated equipment of XXI century. The substances it contains are very easily assimilated by the body, which receives the honey not only the calories it needs, but also minerals, vitamins and enzymes. The Honey Research Unit at the University of Waikato, New Zealand biochemist Peter Molan has identified a type of honey with healing qualities very strong: antibacterial, antimicrobial, antiviral, antioxidant, antiseptic, anti-inflammatory and antifungal, making it extremely efficient in supporting the immune system and improving a wide variety of health problems. Manuka Honey is a special flower honey produced by bees from the flowers of Manuka (*Leptospermum scoparium*), a species of tea tree, existing only in some areas of New Zealand and Australia. Peter Molan classified Manuka honey depending on the power antibacterial factor entering UMF (Unique Manuka Factor). Honey UMF 10+ only classified

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and over, can be used effectively to solve health problems. Antimicrobial properties of Manuka honey occurs in species: *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter freundii*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Streptococcus faecalis*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Methicillin-resistant Staphylococcus aureus* (MRSA), vancocomycin-resistant *Enterococci*. To produce 500 g of honey bees traveling an average of 88.000 km and almost two million flowers pollinate [1, 2]. The antiseptic and healing action is due largely generated peroxide in honey, which is released gradually in the wound moist, decomposing into oxygen and water. The antimicrobial capacity of honey varies according to the type of honey. An *in vitro* study compared the antibacterial action of honey manuka honey polyphlore to that, the species of coagulase-positive staphylococci gold from infected wounds. The result showed that both types of honey were effective, although they acted differently: honey polyfloral by generating superior peroxide and manuka honey by nonperoxidic antibacterial activity. Manuka honey is obtained from a species of small shrub, named *Leptospermum scoparium*, which grows in New Zealand. Honey's anti-inflammatory action is achieved by stimulating the production of cytokines by monocytes (alfaTNF, IL 1beta, IL 6). Cytokines are recognized as mediators of immunity, inflammation, proliferation and differentiation of cell lines. Monocytes are the major blood cells reach the site of inflammation after infiltration of neutrophils and have a role in the process of phagocytosis [1,2].

Monofloral honey is made primarily from the nectar of one type of flower. Different monofloral honeys have a distinctive flavor and color because of differences between their principal nectar sources. To produce monofloral honey, beekeepers keep beehives in an area where the bees have access to only one type of flower. In practice, because of the difficulties in containing bees, a small proportion of any honey will be from additional nectar from other flower types. Polyfloral honey nectar is resulting in more flowers and because of that it is one of the most complete honeys. Polyfloral honey is generally reddish-yellow color and consistency fluid, viscous or crystallized. Because it includes nectar from dozens or hundreds of herbs, honey polyfloral borrow their therapeutic properties and thus is one of the most complex honey as therapeutic actions. Among the main properties of honey polyphlore are: disinfectant, antiseptic, sedative, diuretic, laxative.

There are a wide variety of types of honey, depending on the consistency (liquid or crystalline), on the color (colorless, white, pale yellow, golden, green, brown or red) or on the plants visited by the bees.

Acacia honey (from *Robinia pseudoacacia)* still remains liquid naturally very rich in fructose. Acacia honey is easily recognized by color (almost colorless, light golden yellow), taste (sweet, specific) and unmistakable consistency (uniform, fluid or viscous). Acacia honey has a higher content of fructose compared to other varieties of honey. Because of this, honey locust is the only natural honey crystallizes from Romania (in fact it may partially crystallize in about two years). Acacia honey is easily assimilated and give much energy, nutrients and bioactive substances.

Lime honey (from *Tilia cordata)* is one of the most popular varieties of honey. It has a yellow-orange-red and contains apart monosaccharides, oils rich in *farnesol*, a substance who print characteristic smell and taste, an unmistakable fragrance and flavor, lime flowers. Lime honey consistency is uniform, fluid, viscous or crystallized. Fresh crystallized honey has a lighter color. Lime honey is an excellent natural sedative and nervous and is recommended for nervous system disorders. It is also successfully used for colds, coughs and other respiratory disorders (bronchitis, laryngitis).

Cole honey (from *Brassica rapa oleifera*) is honey exciting, easily distinguishable from the other varieties of honey, primarily by white color, sometimes white-gray-yellow, creamy consistency and because "like sherbet." Cole honey has a specific odor, slightly unpleasant flavor of cabbage, but has a pleasant taste and less sweet than other types of honey. Cole honey crystallization occurs very rapidly due to high glucose (dextrose), fructose is much higher than (levulose). For this reason, cole honey found only in crystallized form, whatever time of year. Cole honey is indicated in diseases of the respiratory tract, bones, kidneys, pancreas and spleen. Also protects the liver and regenerate the epidermis.

Forest honey is the only natural honey in our country which is produced by bees from the nectar of flowers. Forest honey is also called in from Romanian *mana* honey because it occurs in forest areas which can be both coniferous (pine, spruce, pine, etc.) and hardwood (maple, oak, etc.). This may be of plant origin, when bees collect the sap directly from the leaf or young shoots, or may be animal when interpose some small insects, aphids from the family. They feed on tree sap they suck from the crust, sequester and eliminate nitrogenous substances excretion particular high sugar content. Bees harvest these sugars from the leaves populated by aphids and processes resulting honey hand. Dark *mana* honey (dark brown) is due to high content of minerals. It has a specific taste, slightly caramelized and a high viscosity. Forest honey has a lighter color in crystallized form. Depending on its composition (low glucose), there are situations in which hand honey may not crystallize. This honey contains organic acids, bioflavonoids, vitamin C and B group vitamins, much *inhibin* (a bactericidal substance) and enzymes. As mentioned above, it is much richer in minerals (calcium, magnesium, iron, potassium, phosphorus, selenium) than floral honey. Forest honey has the following properties: immunostimulant, antioxidant, anti-inflammatory and blood cleanser. Forest honey is recommended for osteoporosis, to restore the immune system weakened, in the case of infectious diseases, diseases of the large intestine, anemia and gastro-intestinal disturbances, lack of vitamins and minerals and elimination of toxins from the organism. Forest honey from high altitude include honey pine and other coniferous trees-is very rare, collection efficiency of bees being unique. Exceptional properties over lungs and respiratory system, benefiting properties anti-infectious, expectorants, cough suppressants and when it is consumed with honeycomb, bronchodilators.

2. Materials and methods

Six samples of crude honey from *Apis mellifera* were been collected during June-July 2015 from apiaries in Timiş and Caraş-Severin district, in different regions, mountain and plain. Samples of honey had different botanical origins, including unifloral (acacia honey, lime honey, cole honey), and multifloral types (forest honey from low altitude, from high altitude and field honey) obtained direct from beekeepers localized in unpolluted areas. All samples were collected with sterile syringes and kept in a cooling bag until arriving to laboratory of microbiology, than were been stored in refrigerator until analysis. The pH was measured with special indicator paper pH Box from Merck, in solutions containing 10 g honey in 75 ml of distilled water. For microbiological determination, 10 grams from each sample was mixed with 90 ml of saline water to prepare the initial dilution which was used like stock dilution for further serial dilutions for standard plate counting (SPC). Serial dilutions (10^{-1} to 10^{-3}) the samples in saline water were plated on WL-nutrient agar from Merck Germany). The plates were than incubated at 30˚C for 48 hours. After 48 hours were counted the bacterial colonies or colony-forming units (CFU) using the LKB 2002 apparatus produced by Pol-Eko Aparatura SP-J from Poland. For Bacillus species identification was heat the initial dilution at 80˚C for 10 minutes and cooled rapidly in iced water. After that, the initial dilution was poured on plates and incubated at 30˚C for 48 hours. For coliform counts was used Deoxycholate Citrate Lactose Agar (DCLA) from Difco (USA) and plates were than incubated at 37˚C for 24 hours. For identify yeasts was used CM0545 Oxytetracycline-Glucose Yeast Extract Agar from Oxoid (Great Britain) and for moulds was used CM0041 Sabouraud Dextrose Agar from Oxoid (Great Britain). For emphasize the antibacterial activity was used the method of growth inhibition in some pathogen microorganisms cultures from *Pseudomonas aeruginosa ATTC 27853*, *Escherichia coli A TTC 10536*, *Bacillus subtilis ATCC 6051*, *Staphylococcus aureus ATTC 6538 P*, obtained from the microbiology laboratory collection. All these bacterial species were inoculated in usually liquid culture. The method of diffusion is a qualitative *in vitro* test for detecting the susceptibility of bacteria to antimicrobial substances [4]. For each bacterial species were prepared Petri dishes with Mueller Hinton Agar. Every Petri dish was inoculated in flood with a sterile pipette with each of the above enumerated species of bacteria. 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 was been poured in one sample of studied honey, undiluted and 75%, 50% diluted in distilled water and in the other concave, considered blind test (M), only distilled water. All inoculated Petri dishes remained at room temperature 30 minutes, for a preliminary diffusion, after that were incubated at 37^{o} C for 24 hours [3].

3. Results and discussion

Table 1 shows the values of pH measured in all six studied samples of honey. The lower pH value was obtained in Acacia honey (3.0) and the highest value of pH in the case of forest honey of high altitude (4.5). This low acidity (average of pH 3.8) inhibits the growth of many species of pathogenic microorganisms [4-6]. The organic acids are responsible for acidity of honey and contribute largely to its characteristics and taste. In table 2 is presented the results of microbiological determinations of studied samples of honey. All honey samples had low levels of microbiological contamination. The standard plate count (SPC) registered low values in almost samples, with a maximum number (100 cfu/g) in the case of cole honey, followed by acacia honey (30 cfu/g) and lime honey (10 cfu/g). In majority of samples were found spores of *Bacillus*, with maximum value in cole honey (30 cfu/g) followed by acacia honey, lime honey and field multifloral honey (10 cfu/g). Coliforms were not detected in any studied sample, yeasts were detected in small number in Cole honey and Field multifloral honey (10 cfu/g), while molds were detected in acacia and cole honey (10 cfu/g).

Table 1. The results of pH measurement

Type of honey	pH
Acacia honey	3.0
Lime honey	4.0
Cole honey	4.0
Field multifloral honey	3.5
Forest honey low altitude	4.0
Forest honey high altitude	4.5
Average	3.8

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Table 2. The results of microbiological tests

Type of honey	Counts of microorganisms				
	SPC	*Bacillus sp*	TC	Moulds	Yeasts
Acacia honey	30	10	<10	10	<10
Lime honey	10	10	<10	<10	<10
Cole honey	100	30	<10	10	10
Field multifloral honey	10	10	<10	<10	10
Forest honey low altitude	<10	<10	<10	<10	<10
Forest honey high altitude	<10	<10	<10	<10	<10

In tables 3, 4 and 5 are shown the results of growth inhibition test in some pathogen microorganisms cultures from *Pseudomonas aeruginosa ATTC 27853, Escherichia coli ATTC*

10536, Bacillus subtilis ATCC 6051, Staphylococcus aureus ATTC 6538 P with undiluted, 75% and 50% diluted honey.

Table 3. The results of agar diffusion technique with undiluted honey

Bacteria species	Diameter of inhibition area (mm)					
	Acacia honey	Lime honey	Cole honey	Field honey	Forest honey I	Forest honeyII
P. aeruginosa	19	17	11	16	20	23
E. coli	17	21	14	14	19	22
B. subtilis	17	25	16	18	23	29
S. aureus	19	29	18	24	33	39

Table 4. The results of agar diffusion technique with 75% diluted honey

Bacteria species	Diameter of inhibition area (mm)					
	Acacia honey	Lime honey	Cole honey	Field honey	Forest honey I	Forest honeyII
P. aeruginosa	15	13	8	12	17	18
E. coli	12	17	11	10	13	20
B. subtilis	10	21	12	13	19	26
S. aureus	16	26	14	19	29	35

Table 5. The results of agar diffusion technique with 50% diluted honey

Bacteria species	Diameter of inhibition area (mm)					
	Acacia honey	Lime honey	Cole honey	Field honey	Forest honey I	Forest honeyII
P. aeruginosa	9	9	3	9	15	17
E. coli	7	11	5	8	12	18
B. subtilis	5	19	8	11	16	24
S. aureus	10	22	9	17	26	31

The results obtained in the growth inhibition test with pathogen microorganisms showed that in the case of using undiluted honey, the zone diameter of inhibition (ZDI) had higher values than the case of using 70% and 50% diluted honey. These results are in concordance with studies of Tasneem and Aruna [5,6,] who in 2013 observed in crude honey from a region in India that maximum inhibition was obtained in a similar test with undiluted honey and there was a progressive increase in inhibition at higher concentration of honey. When honey was used undiluted, the maximum ZDI for all four pathogen bacteria was obtained in the case of forest honey from high altitude, followed by forest honey from low altitude, lime honey and with equal results acacia honey and field honey. When honey was used in 75% dilution, maximum results were been obtained in the case of forest honey from high altitude, than lime honey, acacia honey and field honey with equal results, forest honey from low altitude, than, with minimum ZDI, cole honey. In the case of using honey 50% dilution the maximum results registered forest honey from high altitude, than forest honey from low altitude, lime honey, field honey, Acacia honey and cole honey. All these results show that in the studied types of honey had founded a low number of microorganisms. Also the obtained values of pH demonstrated that all types of studied honey represent a hostile medium for microorganism development, with a good stability and microbiological quality. Honey has a low pH primarily due to the conversion of glucose into hydrogen peroxide and gluconic acid by glucose oxidase. This low pH might also contribute to the bactericidal activity of honey, demonstrated by the titration of the pH of 10-40% honey solutions from 3.4-3.5 to 7.0 combined with neutralization of other bactericidal factors (H_2O_2, MGO and bee defensin-1) reduced the bactericidal activity of honey to the same level of a honey-equivalent sugar solution [6]. Was demonstrated that honey have an inhibitory effect for around 60 species of bacteria, aerobes and anaerobes, Gram-positives and Gram-negatives [7,8,9,10] and also an antifungal action for some yeasts, molds and dermatophytes [Brady]. Honey high antibacterial activity resulted from it osmotic effect, acidity, phytochemical factors and hydrogen peroxide [Jeddar]. Studies made on undiluted honey demonstrated that this can stop the growth of *Candida spp*, while *Clostridum oedemantiens, Streptococcus pyogenes* remained resistant. Some species of *Aspergillus* did not produce *aflatoxin* in various dilutions of honey and honey also has been found to stop the growth of *Salmonella, Escherichia coli, Aspergillus niger* and *Penicillium chrysogenum* [10-13]. Obi et al [10,11] reported the inhibitory effect of pure honey against local isolates of bacteria agents of diarrhoea**.** At concentration of 50% and above, honey excellently inhibited the growth of *Escherichia coli, Vibrio cholerae, Yersinia enterocolitica, Plesiomonas shigelloides, Aeromonas hydrophila, Shigella boydi, Clostridium jejun* and *Salmonella typhi.* It seems that antimicrobial ability of honey varies depending on the type of honey. Polyfloral honey has antibacterial action by generating more of the upper through peroxide, other types of honey through antibacterial non-peroxidic activity, equally effective in the fight against bacteria mainly, with small differences [14]. High values obtained in the antibacterial effect of forest honey can be explained by the fact that, besides the other usual components found usually in honey, it has a greater amount of bactericidal inhibine. As a method for measure the antibacterial activity of honey has been determined the „inhibine number" [15-18].

4. Conclusions

The obtained pH values in all six types of studied honey, between 3 and 4.5 defines honey like characteristically acidic, with values low enough to inhibit many animals and human pathogens. In undiluted honey the acidity it has proved to be a significant antibacterial factor. All analyzed six samples of honey are characterized by a low count of microorganisms, which proved that in these types of honey exist hostile conditions for the growth and surviving of microorganisms, what proves that studied types of honey had a good microbiological quality. The appreciable obtained values of ZDI in the growth inhibition test with pathogen microorganisms denote the fact that antibacterial activity varies with the type of honey. Honey can represent an alternative antimicrobial in times when the overwhelming use of antibiotics had resulted in widespread resistance, medicine needs effective strategies to save lives. No microbial resistance against honey has been observed, making it attractive as a treatment for wound infections. Honey possesses several antimicrobial properties and can act via various mechanisms of action. There are many different types of honey from around the world, made from different floral sources with variable mechanisms of action. The antimicrobial potency and medical applications of honey are tremendous as it has demonstrated inhibitory effects against a number of pathogenic bacteria.

This experiment demonstrated that honey from certain plants has an increased antibacterial activity. This conclusion can be important when honey is used like an alternative in various medical treatments applied to people and animals, with a suggestion: is very important that when honey is used as an antimicrobial agent to be selected from honeys that have been assayed in the laboratory for antimicrobial activity. It is also important that the type of honey used like antibacterial agent to be stored at low temperature and not exposed to light, so that none of the glucose oxidase activity is lost although honey will stop the growth of bacteria because of high sugar content.

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