IN VITRO ANTIBACTERIAL ACTIVITY OF UNIFLORAL HONEYS AGAINST HONEYBEE PATHOGENS *PAENIBACILLUS LARVAE* AND *ESCHERICHIA COLI*

Otilia Bobiş[1*], L.Al. Mărghitaş[1], D.S. Dezmirean[1], B. Gherman[1], F. Chirilă[1]

[1]*University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania*

Abstract

Different unifloral honeys from Romanian market were evaluated against two important honeybee pathogens: Paenibacillus larvae (causative agent of American foulbrood) and Escherichia coli (frequently found in honeybee digestive tract). Chemical composition as well as biologically active compounds from black locust (Robinia pseudoacacia), linden (Tillia spp.), canola (Brassica rapa), sunflower (Helianthus annuus) and heather honey (Calluna vulgaris) were determined in order to establish the authenticity in respect of botanical origin and quality parameters. Different concentrations of honey solutions as well as entire raw honey were evaluated for antimicrobial capacity using difuzimetric method. Minimum inhibitory concentration (MIC) was determined by succesive dillutions method and diferences between honey types were registered. Best results were obtained by using sunflower and heather honey, these two types presenting the highest diameter of inhibition for the two bacteries and the lowest minimum inhibitory concentration. Further in vivo studies are necessary to prove the efficacity of these honey types and their utilization in preventing bee diseases.

Key words: unifloral honey, antibacterial activity, *Paenibacillus larvae*, *Escherichia coli*

INTRODUCTION

Many literature studies demonstrated the antibacterial effects of honey [21, 22, 28, 9, 10] is due to its high osmolarity, low pH, hydrogen peroxide, phenolic compounds or other uncharacterized compounds.

Low water activity of honey is not a proper environment for majority of bacteria. The low pH, between 3 and 4.5 is also inhibitory to a wide range of bacterial strains. When diluted, honey may form hydrogen peroxide, a strong antibacterial substance. In diluted honey, the concentration of hydrogen peroxide is about 1000 times smaller than a 3% H_2O_2 solution. All thesae parameters together with other substances present in honey exert as a total, the antibacterial activity of honey.

Even in small amounts, the phenolic compounds present in honey, have a very important role. These compounds, secondary metabolites occuring in plants, end up in honey via nectar and pollen from the plants that bee use as raw material to produce honey. Phenolic compounds exhibit antimicrobial, anticarcinogenic, anti-inflamatory effects, acting like antioxidants [12, 1, 2].

It is known that honey contain both substututed benzoic acids and cinnamic acids, flavonols, flavones and flavanones [3].

Americal foulbrood is a dangerous bee disease worldwide spread, which destruct the brood, so affecting the bees in larval stage. The causative agent of this disease in *Paenibacillus larvae* [11], a gram positive spore forming bacteria. The multiplication of this bacteria occurs in two formes: vegetative and spores. Spores remain viable for long periods of time, even in difficult environmental conditions [16, 17]. Finding a proper antibacterial agent for this bacteria is difficult because it grows poorly in artificial media, as the best multiplication environment is bee haemolimph.

Escherichia coli, a common gram-negative bacteria, can be found in bee intestines and gut, eventhow didn't exert a

*Corresponding author: obobis@usamvcluj.ro
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real disease. High concentration of this bacteria may produce bee dihareea thow. E.coli represent o model organism for bacterial study, being very sensitive to antibiotics, but also receiving rapidly drug resistance due to overuse of antibiotics [13].

Honey is considered a natural product free from any contaminants, antibiotics, pesticides and so on. For this reason, using antibiotic treatment against the above bacteria, which may cause different diseases in bee colony is forbidden.

Many studies were made on different bee products that were used as „self medication" in bee colonies in the attempt to find natural remedies for different bee diseases [27, 20, 4, 26].

The purpose of this study was to investigate „in vitro" the effect of different honey types on *Paenibacillus larvae* and *Escherichia coli*, since the literature concerning the so called „self medication" is limited. This study is also a continuation of a previous study of our department and laboratory [4], bringing more answers regarding the antibacterial components from different honey types.

MATERIAL AND METHOD

Honey samples. Six types of Romanian nectar honeys (4 samples each) were collected directly from beekeepers, freshly harvested, untreated and unpatsturized.

The botanical origin of honey samples was proven by pollen and physico-chemical analysis. Each honey type was kept in closed containers in the dark at 4°C until analysis and testing.

Pollen analysis was performed after the method of Louvreaux [14], counting at least 500 pollen grains and expressing procentually the specific pollen.

Phisico-chemical analysis. Water content, pH and acidity, HMF content, diastazic index, main sugars, were determined following the methods from International Honey Commision, methods also validated in APHIS Laboratory, USAMV Cluj-Napoca.

Bacterial strains. P. larvae was isolated from an infected honeybee colony during 2010, transferred into test tubes containing Brain Heart Infusion (BHI) supplemented with thiamine (vitamin B1) (BHIT) and kept in the collection of the Department of Microbiology (USAVM, Cluj-Napoca, Romania). In order to confirm the strain identity of the suspected *P. larvae* isolate, a fragment of 16S rDNA was amplified using two primer pairs (GM3F/GM4R and Pl5/Pl4) [11, 24]. *P. larvae* DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN). PCR reactions were carried out using the MyTaq™ HS DNA Polymerase kit (Bioline) according to the recommendations of the supplier. The PCR products were run out on an agarose gel to confirm the correct sizes of the DNA bands.

For „in vitro" tests on *Escherichia coli* an international reference standard strain was used (FV 755 serogroup O139, Spain). *Antibacterial activity* was tested with difuzimetric method, similar with antibiogram test. Sterile Petri dishes of 9 mm diameter were used, by pouring on a plane surface, close to a fire source, sterile Mueller-Hinton agar medium or BHI medium. After solidification, using a stainless steel cylinder (5 mm diameter), wholes were made in the agar, disposed in radial model. The plates was seeded with bacterial cultures and in every whole 20µl honey sample was placed. Petri dishes were incubated at 37ºC for 24 hours for *E.coli* and 48-72 hours for *P.larvae*.

Minimum inhibitory concentration of honey samples was tested using 96 wells plates, 200 µl volume each whole, where 100 µl Mueller-Hinton medium was placed in the first 10 wholes. 100 µl of tested product was placed also in the first well, mixed and 100 µl of the mixture placed in the second well, continuing until the 10th well, where 100 µl mixture is discharged. Every well is afterward seeded with 10 µl bacterial culture, including well nr.12 (reference). The plate is thermostated at 37ºC and incubated 24 hours for *E. coli* and 48-72 hours for *Paenibacillus larvae*.

RESULTS AND DISCUSSIONS

Melisopalinological characterization of honey samples show that the samples were of declared origin, having specific pollen in the

amount suggested by regluations and literature studiers for every type of honey used in this study [23, 25].

Physico-chemical analysis ranged in the limits of standard regulations of every honey type (Table 1). Small water content (16.0 – 20.0%), with high content for heather honey (standard limit 23%), small HMF content (0.59 – 15.85 mg/kg) excepting again heather honey, show freshness and unheating of the samples. Diastazic activity, the amount of amilase in honey, present rather high values, again confirming the authenticity and freshness of honey. Similar results were obtained for other Romanian honeys [5, 8, 15, 18, 19], or for different European honeys [23, 29].

Sugar spectrum show as princiapl saccharides glucose and fructose, with the highest content of fructose in black locust honey and the lowest in linden honey (Fig. 1). Lowest glucose content was registered in black locust honey and highest amount in canola honey. As it can be seen from Fig. 1, the amount of fructose and glucose is about the same in linden, canola and sunflower honeys, explaining in part the rapid cristalization of these types of honey (F/G ratio being most of the time subunitary). All the sugars found in our samples lies within the limits of European honeys and also other Romanian honeys.

Table 1 Phisico-chemical characteristics of different Romanian honey types

Rank	Honey type	Water (%)	Diastazic activity (Schade U/g)	HMF (mg/kg)	pH	Free acidity (meq/g)	Total acidity (meq/g)
1	Black locust (*Robinia pseudoacacia*)	16.0	28.49	2.69	4.03	6.471	12.409
2	Linden (*Tillia sp.*)	18.7	14.4	15.85	4.17	16.794	30.788
3	Canola (*Brassica rapa*)	18.2	17.19	0.59	4.22	6.835	8.442
4	Sun flower (*Helianthus annuus*)	18.1	22.55	8.52	3.75	25.725	47.615
5	Heather (*Calluna vulgaris*)	20.0	37.68	36.17	4.30	20.914	32.785

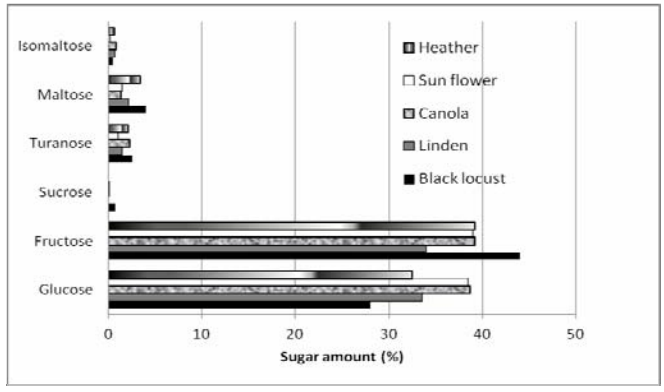

Fig. 1 Sugar spectrum for different Romanian unifloral honeys

Regarding the chemical composition of honeys, we know that the main components of honey (sugars) did not have antibacterial activity (Bobiş unpublished data). Free and total acidity is given by the presence of organic acids, and organic acids as single substances do have some antibacterial activity. In a previous study [7], several organic acids were determined in different types of honey: gluconic, oxalic, acetic, citric, ascorbic, tartaric and malic. From these acids the highest antibacterial activity

was exerted by formic and oxalic (acids that are present also in different preparations used by the beekeepers in honey colony management), and the following antibacterial activity was demonstrated by citric and malic acids. These two acids were found in heather and sunflower honey in higher amounts.

Total polyphenolic content is also high in these types of honey [18, 6] and is good correlated with antibacterial activity. These findings are important steps in recognizing that secondary metabolites from honey as well as other minoritar components play an important role in the antibacterial activity of honey.

Regarding the antimicrobial activity, we can report after the determinations that the present honey types posses antimicrobial activity towards *Paenibacillus larvae* and *Escherichia coli* (Table 2). Black locust and linden honey did not exert antibacterial activity against the two bacterial strains. The rest of 3 honeys (canola, sunflower and heather), have different antibacterial potential, as shown in Tabel 2. It can be seen that the two honey types with higher acidity (sunflower and heather), were registered with the highest antibacterial activity (Table 2).

Table 2 Antibacterial activity and minimum inhibitory concentration of different Romanian honey types

Honey type	Antibacterial activity (mm inhibition diameter)		Minimum inhibitory concentration (% honey concentration)	
	Escherichia coli FV 7550139	*Paenibacillus larvae* 9820	*Escherichia coli* FV 7550139	*Paenibacillus larvae* 9820
Black locust (*Robinia pseudoacacia*)	0	0	40	10
Linden (*Tillia sp.*)	0	0	40	10
Sun flower (*Helianthus annuus*)	12	26	20	5
Canola (*Brassicarapa*)	10	14	40	5
Heather (*Calluna vulgaris*)	12	26	20	5

Antibacterial activity of different types of Romanian honeys was measured on undiluted honey. Minimum inhibitory concentration of honey was tested on several dilutions of honey. First experiment was made with honey solution of 20% and 50% concentration (water dilutions)[4]. No antibacterial activity was observed, all wells of the plate being populated with bacteria. In the second experiment, 80% honey solution in water was made and used for minimum inhibitory concentration determination. As described in Material and methods, in the first well of the plate the dilution of honey is already reduced twice (100 µl of tested product and 100 µl of Mueler Hinton media). As it can be seen in Tabel 2, for black locust and linden honey the smallest concentration that reduce the bacterial growth of *Paenibacillus larvae* was redistered at the concentration of 10% (3rd well of the plate). For sunflower, canola and heather honey the concentration where the inhidition is produced is smaller (as expected), is 5%. These studies confirm us that honey really posses antibacterial activity against the causative agent of American foulbrood disease, and further studies, on different location honey types will be needed. Surprisingly sunflower honey show a very interesting bahavoiur and will have our attention in the future.

It is known that *E.coli* is usually a resistant bacteria, especially on natural antibacterial substances, due to the specified attribute, that is becoming more and more resistant to antibiotics due to overusing of antibiotics. Our results are yet encouraging, and need to be carried on. We observed bacterial growth in the second well of the plate for black locust,

linden and canola honey and for sunflower and heather, the inhidition was absent in the first two wells (corresponding to 40 and 20% honey concentration).

Spectrophotometric determinations are needed, to see what is really happening in dinamics when mixing bacteria with culture medium and tested honey, together with collecting honey samples from different locations from Romania.

CONCLUSIONS

Physico-chemical analysis of tested honey samples show authentic honeys, having these parameters in accordance with internal and international regulations on quality.

Antibacterial activity of honey was demonstrated on two bacterial strains, important in bee disease evaluation. *Paenibacillus larvae* was demonstrated to be susceptible at tested honeys, especially at sunflower and heather honey. This is a start point for further research actions in self treatment of bee colonies, fighting against American foulbrood disease.

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