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Original article

Quality assessment, antimicrobial activity organic sunflower honey and use of Maldi-tof mass spectrometry for the identification bacteria isolated from honey

Saša Milosavljević [1], Tanja Zugić Petrović [2], Katarina Mladenović [2], Mirjana Grujović [2], Stefan Kolašinac[3], Dragan Orović [1]

[1] Department of Food Science and Technology, College of Agriculture and Food Technology, Ćirila and Metodija 1, 18400 Prokuplje, Serbia; [2] Department of Biology and Ecology, Faculty of Science, University of Kragujevac, RadojaDomanovića 12,34000 Kragujevac, Serbia; [3] Department of Botany, Faculty of Agriculture, University of Belgrade, Nemanjina 6, 11080 Zemun, Belgrade, Serbia.

Summary. The aim of this study was to investigate the physicochemical parameters of quality, microbiological safety and antimicrobial potential of four samples of organic sunflower honey from the Banat area (northeastern Serbia). Humidity, ash, pH, free acidity, electrical conductivity, hydroxymethylfurfural content, sugar content and diastase activity were measured. Microbiological analysis revealed the presence of total aerobic mesophilic bacteria, coliforms, aerobic endospores, lactic acid bacteria (LAB) and a good number of molds and yeasts. The isolate identification was carried out using Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The antimicrobial effect of organic sunflower honey was investigated on five ATCC strains of bacteria: Staphilococcus aureus ATCC 29213; Escherichia coli ATCC 25922; Salmonella enterica ATCC 10708; Yersinia enterocolitica ATCC 23715; and Bacillus subtilis ATCC 23857. The results of the study showed that all honey samples meet international quality standards for all physicochemical parameters. Microbiological analysis of Sunflower honey confirmed the total bacterial counts for all samples ranged from1.80 to 1.85 x10-2cfu/g-1, whereby no presence Clostridium spp., coliform bacteria, as well as molds was detected. Investigation of the antimicrobial activity of honey samples revealed that all bacteria showed clear zones of inhibition in honey concentrations of 40-100%, which is a satisfactory result for flower honey.

Key words: Antimicrobial activity, physicochemical properties, honey, microbiological safety.

Introduction

Honey is a sweet and aromatic substance produced by honeybees from the nectar of plant flowers and honeydew that is consumed as food of a high nutritional value (1). White, (1978) (2) indicates that honey is a mixture of carbohydrates (85-95%) of which are fructose and glucose), organic and amino acids, proteins, minerals, vitamins, and lipids, with the physio-chemical composition. Color, aroma, and taste of honey differ according to the micro-climatic conditions of the environment- weather, vegetation or plant species that bees feed on, processing, manipulation, packaging and storage time which directly affects the characteristics of honey (3-4). Useful characteristics of honey are rapid absorption, antioxidant and antimicrobial factors, exceptional therapeutic properties in colds, skin wounds and burns, various gastrointestinal diseases, and oncological conditions. Furthermore, the antioxidant and antimicrobial properties of honey are

due to the synergistic effect of its polyphenols, amino acids, vitamins, and enzymes (5). In recent years, a branch of alternative medicine called apitherapy has been developed, which offers treatments based on honey and other bee products against many diseases including bacterial infections. Honey can be contaminated just like any other natural food, e.g., heavy metals, pesticides, and antibiotics. Today, special attention is being paid to organic honey due to increasing pollution and numerous poisons, which has led to a certain demand for certified organic honey (6). Certified organic honey is described as lacking any chemical pollution including that related to honeybee migration in search of good blossoms not directly controlled by beekeepers. Chemical contamination during the final packaging process and storage process results in the deterioration of the quality of the products (7).

Honey contains certain microorganisms that can tolerate high concentrations of sugar and acidity, and which originate from the digestive system of honeybees, from pollen, dust, air and plants (8). The most common honey organisms are yeasts, moulds (*Penicillium* and *Aspergillus* genera), bacteria of *Enterobacteriaceae* family, and sporogenic bacteria, which defines the hygienic and sanitary status of honey (9). The presence of microorganisms in honey can sometimes affect the stability of a product and its hygienic quality. The results of many studies show that the microorganisms found in honey are not dangerous for human health, even in products from which *Aspergillus flavus* was isolated, the presence of aflatoxin was not detected because there were no favorable conditions for synthesis. In this paper, the organic sunflower honey (*Helianthus annuus Linn.)* from the Banat area (northeastern Serbia, an area known for sunflower cultivation), was studied for the first time. Sunflowers are one of the most important cultures containing oil since sunflower oil is rich in phenolic compounds (10). In apitherapy sunflower honey is used in diseases of the heart and blood vessels, diseases of the digestive system and kidneys, diseases of the lungs, whereas with children as a stimulant of the immune system). This study aimed to characterize organic sunflower honey through the analysis of physicochemical quality parameters (moisture, ash, pH, free acidity, electrical conductivity, hydroxymethylfurfural content, sugar content and diastase activity) and microbiological safety (presence of aerobic mesophilic bacteria, coliforms, aerobic endospores, lactic acid bacteria (LABs, molds and yeasts), as well as the antimicrobial ability of organic sunflower honey. This research could contribute to the valorization of sunflower honey, which would lead to standardization and increased production of this product.

Materials and Methods

Honey samples

Four samples of sunflower organic honey were taken from four households in the Banat area in production year 2018 and stored in a refrigerator at 4°C until further analysis.

Physicochemical analyzes

The honey samples were analyzed for: reducing sugars, sucrose, water, water-insoluble substances, free acids, hydroxymethylfurfural (HMF), minerals and electrical conductivity using harmonized European Commission methods (11) for honey. Diastasis activity was analyzed by AOAC method 958.09 (12).

Determination of water content. The water content in the honey was determined by measuring the refractive index using an Abbe refractometer (Abbe refractometer, Tokyo, Japan) at 20°C.

Determination of electrical conductivity. A sample quantity equivalent to 20 g of anhydrous honey was dissolved in distilled water. The prepared solution was quantitatively transferred to a 100 mL volumetric flask and refilled to the mark with distilled water. An aliquot of 40 mL of the sample solution was transferred into a beaker and thermostated in a water bath at 20°C.

Determination of free acid content. The prepared sample was triturated in the presence of phenolphthalein with a solution of 0.1 mol / l NaOH until light pink colour appeared. The free acid content, expressed in mEq of acid / kg of honey, was calculated using the following formula: Free acid content = mL 0.1 M NaOH × 10.

Determination of mineral content. 0.6-0.7 g of the sample was weighed and transferred to a

polytetrafluoroethylene (PTFE) cuvette for microwave digestion. 1 ml of 30% hydrogen peroxide and 7 ml of 65 % nitric acid were added. The sample was mineralized in an Ethos 1 microwave oven, Advanced Microwave Digestion System, Milestone, Italy. Rotor: HPR-1000 / 10S high pressure rotor. Upon completion of the digestion, the sample was cooled, transferred to a normal 50 ml vessel and supplemented with bidistilled water to a final volume. The content of the elements was determined by the instrument: Thermo Scientific iCAP 6500 Duo ICP (Thermo Fisher Scientific, Cambridge, UK). Multi Element Plasma Standard Solution 4, Specpure, 1 g / l for all elements was used as the reference standard. The results are expressed as percentage of minerals per kg of honey.

Determination of the content of reducing sugars. The principle of this method is based on the reduction of the Fehling's solution by titration with a solution of reduced sugars of honey using methylene blue as an indicator.

Determination of sucrose content. The principle of this method is based on sucrose hydrolysis, reduction of the Fehling's solution by titration with reduced sugars from the hydrolyzate of honey with methylene blue. Sucrose content is calculated as the difference between the amount of invert sugar after and before hydrolysis and the difference obtained is multiplied by a factor of 0.95.

Determination of insoluble substances content in water by gravimetric method. 20 g of the sample (to the nearest ± 10 mg) were weighed out and dissolved in a specified amount of distilled water at 80°C and mixed well. The prepared sample is first filtered through a dried and measured sintered funnel, with a pore size of 15 - 40 mm. The sediment was washed with boiling water (80°C) to release sugar, which was determined by a Mohr test. The funnel was dried at 135°C (drying time 1 hour), cooled and measured with accuracy of 0.1 mg.

Determination of HMF content. The principle of the method is based on the reaction of 5-hydroxymethylfurfurol with barbituric acid and p-toluidine to give a compound whose absorption maximum is in the UV-Vis range at 550 nm.

Determination of diastasis activity. The principle of this method is based on the hydrolysis of a 1% solution of starch by an enzyme from 1 g of honey for one hour at a temperature of 40°C.

Isolation and identification of microorganisms from honey samples

To determine the presence of total aerobic mesophilic bacteria, coliforms, aerobic endospores, lactic acid bacteria (LAB) and the total number of molds and yeasts, 10 g of each honey sample was homogenized in 90 ml of distilled water with peptone (Torlak, Belgrade, Serbia). Decimal dilutions were made in the same dilute. Isolation of aerobic mesophilic bacteria was performed using Standard Plate Count (SPC) at 30°C incubation for 48 h. To determine the presence of clostridium, aliquots of 10, 5, 1 and 0.1 ml of the initial suspension were added to an empty tube, heat-treated at 80°C for 5 minutes and covered with sulfite-polymyxin-sulfadiazine (SPS, HiMedia, Mumbai, India). agar, the tubes were incubated at 37°C for 5 days. Analysis of total coliforms bacteria was carried out on violet red were glucose agar (VRBG, HiMedia, Mumbai, India) incubated at 35°C for 24-48 h. Isolation of endospore aerobic bacteria was performed by diluting the suspension of the honey sample first by heating in boiling water for 2 minutes to remove all vegetative forms, and then the samples prepared were poured onto nutrient agar (HA, Torlak, Belgrade, Serbia) and then incubated at 30°C for 48 h. LAB isolation conducted on De Man Rogosa Sharpe agar (MRS, Torlak, Belgrade, Serbia) at 30°C for 48 h. The presence of molds and yeasts was determined using Sabouraud maltose agar (SMA, Torlak, Belgrade, Serbia), incubation was carried out at 25°C for 5-7 days. After isolation pure isolates were subjected to Gram staining, preliminary determination was carried out using standard morphological and bohemian methods according to Naseer et al., (2015) (13). The isolate identification was performed using Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS). Samples were prepared according to the manufacturer's instructions.

After completion of incubation, a small number of individual colonies of tested bacteria was applied with a sterile stick directly to a steel plate in 96 spots in the form of a thin film. The film-coated plate was

allowed to dry at room temperature for about 1 min, and then 1 µl of VITEK MS-CHCA matrix, (BioMérieux), was applied to the plate. After adjustment and calibration of the apparatus according to the manufacturer's instructions, bacterial isolates prepared in this way were then subjected to MALDI TOF mass spectrometry. *Escherichia coli* strain ATCC 8739 was used to calibrate the apparatus. During the mass spectrometry process itself, under a laser effect on a thin film of bacteria and matrix, proteins are ionized and separated in an electric field, and then directed to a vacuum tube and separated according to mass and charge. In this way, the proteins arrive at the detector in sequences that are inversely proportional to their mass, creating a protein profile (mass spectral fingerprinting). The major peaks belong to ribosomal and other predominantly represented proteins, such as HSP (heat shock proteins), DNA binding proteins, and RNA chaperones. Then, the obtained profile is analyzed and compared with the database, which enables precise identification of microorganisms. The VITEK MS V2.0 Knowledge Base - Industry Use database was used to read the results.

Mass spectra ranging from 2000 to 20,000 Da were obtained in a linear, extraction mode, with positive polarity. The resulting Spectrum was introduced into Biotyper software (BrukerDaltonics, Bremen, Germany), which is equipped with a nitrogen laser (337 nm) using Flexcontrol software ver. 3.1 (Bruker Daltonics). MALDI-TOF identification was performed using the corresponding values to match manufacturer-suggested results ≥ 2.00 (14).

Antimicrobial activity of honey samples

Five ATCC strains of bacteria were used in the study: *Staphilococcus aureus* ATCC 29213; *E coli* ATCC 25922; *Salmonella enterica* ATCC 10708; *Yersinia enterocolitica* ATCC 23715; and B*acillus subtilis* ATCC 23857. The antimicrobial activity of organic sunflower honey was determined by a modified method of Matzen et al. (2018) (15). The preparation of the samples involved dissolving the honey in sterile distilled water under aseptic conditions to a final concentration of 80, 60, 40 and 20% (w / v). Positive (first) and negative control (second) were control discs (6 mm) impregnated with 1 µg penicillin (Jugocillin, Galenika, Belgrade, Serbia) and discs impregnated with sterilized distilled water. The incubation was carried out at 37°C for 24 h. Antimicrobial activity was detected based on the appearance of a light zone around the discs as a consequence of growth inhibition of the susceptible strain and was defined as a growth inhibition zone.

Statistical analysis

The obtained results were expressed as mean values of triplicate measurements ± standard deviation (SD). Analysis of variance (ANOVA) was performed to determine the existence of statistically significant differences in physicochemical and microbiological parameters between honeys. The ANOVA was followed by Tukey post-hoc test for multiple comparisons ($p<0.05$). Data were analyzed with SPSS 26.0 (SPSS, Inc., Chicago, IL) software.

Results and Discussion

Table 1 contains the results of the physicochemical analysis of the organic samples of sunflower honey. As expected, ANOVA showed that there were no statistically significant differences among honeys in physicochemical and microbiological parameters (p value >0.05) (table 1 and 2). In the honey samples, the moisture content (M %) ranged from 16.59 to 16.62% with an average value of 16.6 ± 0.0%, which is in accordance with the legal maximum limit of 20% for the moisture content in honey.Results show a very small difference in the water content of these samples, which may be due to the identical geographic area dominated by identical micro-climatic conditions as well as the very similar movement patterns of bees and the hives they use. Makarewicz et al. (2017) (16) in their work represent a slightly higher percentage of water for sunflower honey, which had a value of 17.3 ± 0.2, noting that according to Council Directive (2001) (17) such values mean that the water content should not exceed 20 per cent. Investigating the physical and chemical properties of Romanian sunflower honey Oroian et al. (2017) (18) produce remarkably similar results when it comes to the percentage of water, which ranged from 15.80-19.60,

pointing out that the moisture content of honey is very important information that can alert the manufacturer to how to keep and store the product. During storage, high moisture content can lead to fermentation caused by the action of osmotolerant yeast, it can also accelerate crystallization in some types of honey (19). Researchers looking at organic honey have noted that factors such as harvest season, maturity in the hive and other environmental factors affect the water content of the product (19 - 22). Knowledge of the water content of honey is important for improving its conservation and storage as well as preventing mold growth on its surface. Another important parameter that defines the quality of honey is certainly hydroxymethylfurfural (HMF). The HMF content for the analyzed sunflower honey ranged from 8.18 to 8.25 These results, with samples 1, 3 and 4 having almost identical HMF values. are very close to the minimum on the Gothe scale of 8 while the maximum HMF content is 40 mg / kg on the same scale. The results obtained are very similar to those presented by the authors of Kádár et al. (2010) (23) for sunflower honey from Spain, while HMF results for honey from the geographical area of the Czech Republic and Romania were slightly higher than 20 mg / kg. Vranic et al. (2017) (24) warn of the fact that high HMF content may indicate counterfeiting of honey by the addition of inverted sugar syrup, as a consequence of heating the sugar in the presence of acid to sucrose inversion. Diastase activity is another parameter that defines the quality of honey, the obtained results of analyzed honey samples had values for diastase activity from 10.8 (in samples 1 i 2) to 10.82 (in sample 4), which is within the required standard for this parameter. Data on diastase activity suggest amylase enzyme activity, as an indicator of honey freshness, indicates errors caused by heat and poor storage of honey, so it has nothing to do with the floral type of honey (25). Free acidity is inked with the natural presence of organic acids in honey, which remain in equilibrium with internal esters, lactones and some inorganic ions such as: phosphates, sulfates and chlorides (26). Free acidity in sunflower honey samples varied from 23.9 (for the sample 4) to 24.2 (for samples 1 i 3), statistically with no significant difference among analyzed samples. The slightly lower results for free acidity in sunflower honey samples were presented by the authors of Oroian et al. (2017) (18) moving in the range of 6.30-16.80. Such significant variations in free acidity results can be attributed to the different sampling season (26). Variations in the chemical composition of sunflower honey are not infrequent, so the author Dinkov (2014) (27), exploring honey from the Balkan Mountains in Bulgaria, notes that variations in the chemical composition of sunflower honey are the most common, be it changes in water content (33.33%). total acidity (6.67%), reducing sugars, sucrose or diastase activity. The sugar content of honey is defined through the content of glucose and fructose, i.e., sucrose. The analysis of sunflower honey showed results that ranged from 61 to 61.3, with a mean value of 61.15 ± 0.12 for glucose and fructose. The sucrose value was less than 0.5% (mean value 0.485 ± 0.0), which is in accordance with the Rules on the quality of honey and other bee products (Official Gazette of the RS No.101 / 15). The amount of sucrose in honey is usually directly related to the percentage of pollen in sunflower. Ash is one of the quality parameters related

Table 1. Physicochemical parameters of honey samples.

Parameters	Honey 1	Honey 2	Honey 3	Honey 4	*p* value
Moisture (% w/w)	16.6±0.0	16.59±0.0	16.62±0.3	16.59±0.0	1.00
HMF (after White),	8.2±0.01	8.18±0.0	8.25±0.0	8.22±0.1	0.418
Diastase activity	10.8±0.2	10.8±0.1	10.82±0.0	10.81±0.1	1.0
Free acidity (meq/kg)	24.2±0.2	24.0±0.1	24.2±0.2	23.9±0.8	0.778
Glucose and fructose content (%)	61.2±1.6	61.1±2.2	61±1.7	61.3±0.4	0.996
Sucrose (%)	0.48±0.0	0.49±0.0	0.49±0.01	0.48±0.0	0.052
Ash (%)	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	1.00

Mean ± SD (minimum–maximum).

to the botanical and geographical origin of honey samples. The determination of ash represents the residual mineral in honey after incineration. As the samples of the investigated honey were taken from the same geographical area, it is not surprising that the result obtained for them had a value of 0.01% for all samples. Pita-Calvo and Va´zquez (2017) (28) point out that the ash content is a quality criterion for botanical and geographical origin of honey. Ash content of honey is usually small and depends on the nectar composition of the dominant plants in the honey composition. Ash content of honey is generally small and depends on the nectar composition of the dominant plants in the honey composition.

Microbiological analysis

The microbiota found in honey may originate from bees, pollen, or may be due to poor hygiene conditions when handling the product. The honey samples tested showed very similar microbial contamination (no statistically significant differences) with only the total number of bacteria moving in range for all honey samples from 1.80-1.85 $x10^{-2}$cfu/g^{-1} (Table 2). Microbiological analysis of sunflower honey did not detect the presence of *Clostridium* spp, coliform bacteria, or mold. The microbiota that was isolated from all the samples tested consisted of: *Bacillus* spp. ranging from 0.49 -0.52 $x10^{-2}$cfu/g^{-1}, *Saccharomyces* spp., were detected only in the first two specimens, while in strain 4 two strains of *Exiguobacterium*spp. which have successfully grown on HA. MALDI TOF were confirmed in preliminary identification. *B. pumilus*, *Exiguobacteriumacetylicum*, while yeasts were identified as *Saccharomyces cerevisiae*. Pajor et al. (2018) (29) found that 80.4% of isolated strains from honey samples belonged to the genus *Bacillus*. *Exiguobacteriumacetylicum*bacteria make up the gut microbiota of bees (*Apis mellifera*) (Khan et al., 2017) (30). Frazie and Westhoff (1994) (31) note that the yeast in honey comes primarily from nectar and from the intestinal content of the honeybees. A study of 200 Swiss honey samples revealed the presence of *S. cerevisiae* whereby the cells of the detected yeast could not reproduce in a high sugar environment (32). Popa et al., (2009) (33) note that in all 10 Transylvania honey samples, the dominant microbiota is mold, while the yeasts are not isolated.

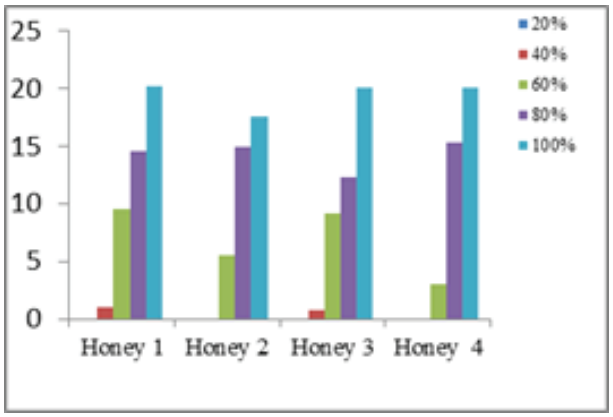
Antimicrobial activity

The antimicrobial activity of honey has long been known, and many researchers have studied and confirmed this activity of honey against a wide variety of human pathogens and bacteria of food spoilage. In the work of Wen et al. (2017) (34) point out that the animicrobic activity of honey comes from its osmotic properties, its high sugar content, its low pH, the presence of H_2O_2, phenolic compounds and the cationic antimicrobial peptide. Results of research on antimicrobial activity of sunflower honey samples according to *St.aureus* ATCC 29213, showed inhibition at concentrations of 60-100% in all tested samples, while in the sample of honey 3 and 1 at a concentration of 40%, the sizes of the inhibition zone were negligible according to the bacterium under study and ranged from 0.8-1 mm (Figure 1). However, in the case of *E. coli* ATCC 25922, the honey samples tested showed no inhibition at concentrations of 20-60%, while concentrations of 80-100% showed

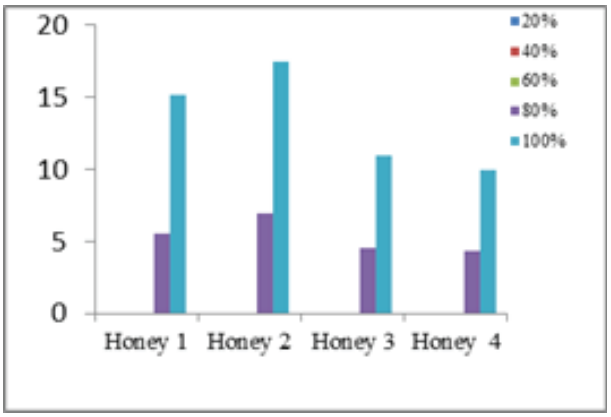
Table 2. Microbiological quality of fresh and stored honey [cfu/g^{-1}].

Total plate count	Honey 1	Honey 2	Honey 3	Honey 4	*p* value
Total bacterial count	$1.82x10^{-2}$±0.01	$1.85x10^{-2}$±0.01	$1.80x10^{-2}$±0.02	$1.85\ x10^{-2}$±0.04	0.154
Total coliforms count	-	-	-	-	-
Bacillus spp.	$0.51x10^{-2}$±0.03	$0.50x10^{-2}$±0.02	$0.49x10^{-2}$±0.00	$0.52\ x10^{-2}$±0.01	0.453
*Clostridium*spp	-	-	-	-	-
Lactic acid bacteria	-	-	-	-	-
The total fungi and yeasts	$0.28x10^{-2}$±0.01	$0.30x10^{-2}$±0.03	-	-	-

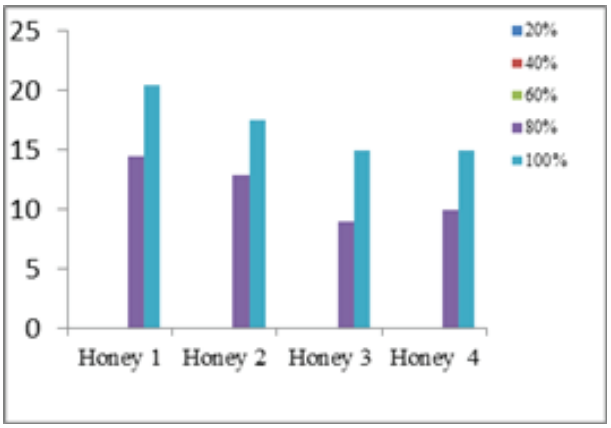
Mean ± SD (minimum–maximum).



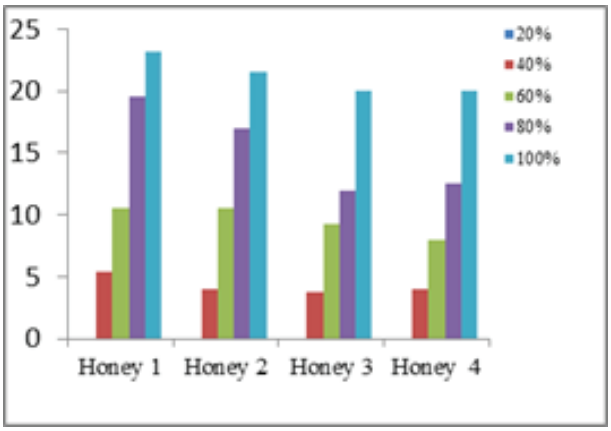
St.aureus ATCC 29213



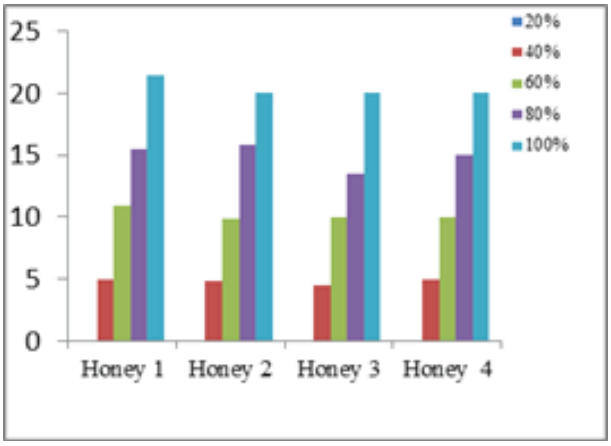
E. coli ATCC 25922



Sl. enterica ATCC 10708



Y.enterocolitica ATCC 23715



B. subtilis ATCC 23857

Figure1. The antimicrobial activity of organic sunflower honey against pathogenic bacteria (honey concentration: 20% - blue stripes; 40% - red stripes; 60% green stripes; 80% - purple stripes; 100% light blue stripes)

significant antibacterial activity, with sample 2 having the highest inhibition zone of 17.5 mm, which is consistent with the results of Junie et al. (2016) (35) who showed that thetested *E. coli* strains were sensitive to seven honey samples whose zones of inhibition ranged from 13-14 mm. Makarewicz et al., (2017) (36) found that sunflower honey at concentrations of 75% showed remarkable antimicrobial activity against *E. coli, B. subtilis, Micrococcus luteus* and *Proteusmyxofaciens* wherein the inhibition zones range between 10,7 and 34,3 mm. The results of the animicrobic activity to *Sl. enterica* ATCC 10708, are very similar to the results obtained for *E. coli*. The best results to *Sl. enterica* showed a sample of honey 1 at a concentration of 80-100%, while other samples at the same concentration showed slightly lower antimicrobial activity. *Y.enterocolitica* ATCC 23715 was inhibited by all tested honey samples at concentrations of 40-100%, with honey sample 1 showing the highest inhibition zones ranging from 5.4-23.2 mm. The antimicrobial activities of the studied honey samples according to *B. subtilis* ATCC 23857, ranged in concentrations of 40-100%, sample 1 showed the highest inhibition zones of 5-21.5 mm. Investigation of the antimicrobial activity of organic sunflower honey samples revealed that all bacteria showed clear zones of inhibition in response to all honey samples, satisfactory results for flower honey and similar results from other authors. Junie et al. (2016) (35) in the study of antimicrobial activity of seven honey samples from the territory of Romania results in the inhibition of all honey samples for bacterial growth, where sunflower honey samples gave zones of 14-18.5 mm in diameter. Likewise, *St aureus, Streptococcus pyogenes*, and *Serratia marcessens* significantly inhibited sunflower honey at 100 per cent concentration (37). Olakunle et al. (2013) (15) while investigating antimicrobial activity of pure honey against isolated wounds, came to the results that showed all isolated microorganism shad a considerable stronger inhibition zones.

Conclusion

The quality control results of organic sunflower honey samples represent a good ratio between physicochemical and microbiological parameters and can meet international quality standards. An important aspect of the research is certainly also the antimicrobial activity, which has been proven in the research with the inhibition of pathogens and ranged at concentrations of sunflower honey samples of 40-100%. Given that honey is nowadays also used as an additive in some food products, special attention is also given to its inhibition of food spoilers. The research opens a new field of complete determination of organic honey that is gaining in importance as a functional food.

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Correspondence:
Tanja Žugić-Petrović
Faculty of Science, University of Kragujevac,
Radoja Domanovića 12,34000 Kragujevac,Serbia;
Phone: +381643563298
Email:tanja.zugicpetrovic@yahoo.com