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Comparative study of the chemical composition and biological potential of honey from different regions of Serbia

Svetlana Djogo Mracevic[a*], Marko Krstic[a], Aleksandar Lolic[b], Slavica Razic[a]

[a] Department of Analytical Chemistry, University of Belgrade - Faculty of Pharmacy, Vojvode Stepe 450, 11221 Belgrade, Serbia

[b] Department of Analytical Chemistry, University of Belgrade - Faculty of Chemistry, Studentski trg 12-16, 11000 Belgrade, Serbia

* Corresponding author E - mail address: svetlana.djogo@pharmacy.bg.ac.rs

1. **Introduction**

From ancient times honey has attracted a lot of attention for both its nutritional value as well for broad spectrum of therapeutic purposes. Some examples, about its usage in ancient Egyptian medicine could be found on papyri. Hippocrates and Celsius, both Greek philosophers, were convinced about the uses of honey in medicine and numerous formulations for treatment of different disorders were recorded. When one read about the honey and medicine in ancient Rome, also can find interesting facts, Pliny the Elder stated in his book "Historia naturalis" with "The Arabians are producing honey from the reed for medical use only" [1]. However, we shouldn't be seduced into thinking that the ancients had hard evidences about their exotic, fragrant remedies that would still cure.

Despite its long history it's well-known that just last decades brought a scientifically sounded knowledge of real value of honey. The curiosity with this substance, as a very complex mixture, is that many variables are responsible for its extraordinary nutritive value [2], but never being constant in both qualitative and quantitative composition. Geographical origin, biodiversity of flora, climate and weather conditions and various anthropogenic influences greatly affect honey's chemical composition and in that way modify its potential in the sense of low-to-high biological activity. There are a lot of reports about antioxidant, antimicrobial, antiviral, antiinflamatory, antimutagenic, cytostatic, and immune-suppressive potential of honey [3-5]. The major components of honey are sugars (fructose 25-45% and glucose 20-40%), but it contains around 200 compounds, including amino acids, enzymes, protein, vitamins, minerals, ash, organic acids and phenol and flavonoid compounds which greatly contribute to its biological activity [6].

Mineral content of honey is typically about 0.1-0.2% for the blossom honeys and around 1% in honeydew honeys, depending on its botanical, but also on geographical origin [2,7]. The most abundant element in honey is K, followed with other minerals such as P, Mg, Na, Fe and trace

elements as Mn, Cr, Se, Co, Zn, Cu, Ni, Pb, Cd, etc. Most of trace elements are considered essential to various life forms having a number of important functions in biochemical processes, as constituents of bioactive compounds [8], while in higher concentrations might become toxic. Moreover, it is well-known that honeys could be an excellent bioindicator of environmental pollution by tracing heavy metals such as As, Cd, Pb and Hg, in an area of about 7 km^2, which is considered to be the field of bee grazing [2].

The botanical origin of honeys is one of its main quality parameters. It has been reported that its composition and antioxidant capacity depend on geographical origin, floral source, seasonal and climate factors, as well as production process [4,9]. Therapeutics potential of honeys has been rediscovered in past few decades and resulted in an increased interest for its nutritive value as well as antioxidant and antimicrobial activities [10]. It is well-known that phenolic compounds are partly responsible for antimicrobial activity of honey. However, the concentration of hydrogen peroxide (H_2O_2), which is generated by glucose oxidase (GOX)-mediated conversion of glucose in honey, can contribute to the antibacterial properties of honey, as well [3]. This assumption is based on the oxidative damage and bacterial DNA degradation as bacteria-killing mechanism. Based on determination of its physicochemical properties as well as its antioxidant and antimicrobial potential significant potential of honey for human health has to be considered.

Antioxidant activity assays can be separated in two groups. The first group are hydrogen electron transfer (HAT) assays and in the second assays based on single electron transfer (ET). Assays involving HAT reactions are oxygen radical absorbance capacity (ORAC) or total radical trapping antioxidant parameter (TRAP) and among assays including ET reactions are Trolox equvivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant parameter (FRAP) and diphenyl-1-picrylhydrazyl (DPPH) assay [11,12]. Both HAT and ET assays measure the radical

scavenging capacity [13]. Since the antioxidant capacity varies largely on the sample origin, and the antioxidants content, each of the assays has its pros and cons. Gašić et al. in their work applied the modified DPPH assays for the determination of antioxidant activity of some Serbian honey samples [14]. Inhibition and antagonistic effects of honey, against almost 60 bacterial species, including aerobal, anaerobal, Gram positive and Gram negative strains, is reported in many studies [4, 9, 10, 15,16].

According to the Statistical Office of the Republic of Serbia (2019) the honey annual production in Serbia in last five years is from 5000 to 12000 t per year. The main types of honeys produced in Serbia are acacia, multifloral, sunflower and linden, and honeydew honey. There are also several certified brands of Serbian honeys present on the global market, all based on their geographical origin (Homoljski, Đerdapski, Vlasinski, Kačanski and Fruškogorski linden honey). The main objectives of this work were to evaluate the physicochemical characteristics and mineral content of seven different Serbian honeys, to determine antioxidant activity as well as the antibacterial and fungicidal potential against pathogenic bacterial strains (*Esherihia coli* and *Staphilococus aureus)* and fungi *Candida albicans*.

2. **Material and methods**

2.1. *Honey samples*

A total of 20 samples of seven different honey types: medow (multifloral), linden (*Tilia europea*), oilseed rape (*Brassica napus*), sunflower (*Helianthus annuus*), acacia (*Robiniapseudoacacia*), honeydew and phacelia (*Phacelia*) were donated from selected beekeepers during the harvesting periods 2015 and 2017 from several region of the Republic of Serbia in the counties of Vojvodina, West and Central Serbia (Table 1, Figure 1). All honey

samples were kept at room temperature (20±5°C) in tightly closed plastic vessels and in the absence of light.

2.2. *Chemicals and reagents*

All solutions were prepared using analytical grade reagents and deionized water with resistivity of 18.2 $M\Omega\,cm^{-1}$ obtained by a Milli-Q system (Millipore, Bedford, USA). Nitric acid (65%) suprapure quality (HNO_3, G.R., Lach-ner s.r.o., Czech Republic) and H_2O_2 30% solution (Macron Fine Chemicals, Avantor Performance Materials, Poland) were used for sample digestion. Standard solutions for standard addition method and recovery tests were prepared by suitable dilution of the stock solutions containing 1000 $mg\,L^{-1}$ of each element. Working standards for external calibration were made by appropriate dilution of stock using a standard solution (Titrisol, Merck, Darmstadt, Germany) containing 1000 $mg\,L^{-1}$ of K, Fe, Mg, Mn, Na, Se, Si, Zn, Al, Cu, Ni, As, Pb, Cr, and Cd.

2.3. *Physicochemical parameters*

The following physicochemical parameters were determined according to the Harmonized Methods of the International Honey Commission (IHC) and AOAC Official methods of Analysis [17,18]: moisture content, pH, electrical conductivity (EC), free acidity (FA), lactonic acidity (LA), and total acidity (TA). Ash content was estimated by conductometry using the following equation [19,20]:

$$\text{Ash content (\%)} = 0.083 \times \text{conductivity} - 0.092 \quad \text{(eq. 1)}$$

2.4. *Determination of free radical scavenging activities of honey samples*

For estimation the ability of honey samples to neutralize DPPH radicals, the method previously described by Gašić et al. was applied [14]. Each honey sample (5 g) was mixed with 15 mL of ultrapure water, homogenized in ultrasonic bath for 15 min at room temperature, transferred to

50 mL volumetric flask, and filled up with deionized water. The solution was then filtered through 0.45μm PTFE membrane and analyzed for determination of radical scavenging activity (RSA).

The radical scavenging activity of the honey solutions was determined by a modified DPPH method [14,22,23]. 0.0078 g of DPPH was dissolved in 250 mL of methanol. The absorbance of freshly prepared DPPH methanolic solution was 0.871 at λ = 515 nm. Honey solutions (0.1 mL)s were mixed with 4 mL of DPPH solution, left in dark for 60 min (until stable absorption was obtained) and the decrease of absormance measured at λ = 515 nm. The prepared working standards were: 50, 100, 200, 300, 400 and 500 μg/mL. RSA was calculated as a percentage of DPPH decolorization using the equation:

$$\%RSA = (A_{DPPH} - As)*100/A_{DPPH} \quad \text{(eq. 2)}$$

where A_{DPPH} is the absorption of methanol solution of DPPH, As is the DPPH absorbance in the presence of honey solution. The assays were performed in triplicate and the results were expressed as mean values.

2.5. *Antimicrobial activity*

The antimicrobial activity of honey was tested against the strains of Gram negative bacteria (*E. coli* ATCC 25922), Gram positive bacteria (*S. aureus* ATCC 25923) and fungus (*C. albicans* ATCC 10231). Bacterial and fungal cell suspensions was prepared in Tripton soybean broth (TSB) and Sabouraud dextrose broth (SDB), respectively and adjusted to 10^5 - 10^7 CFU/mL.

Tested honey samples (10 g) were inoculated with prepared cell suspensions (1 mL) and left for 30 min at 25ºC. The samples were diluted with peptone water (90 mL) and 1 mL of the obtained mixture inoculated on selected media. After the incubation at 30 °C / 72 h for bacteria and 25 °C / 120 h for *C. albicans*, the activity was measured. Control cultures of bacterial and *C. albicans*

suspension were inoculated on sterile TSB and SDB under the same conditions as treated samples [24-26].

2.6. *Sample preparation for mineral component analysis*

Microwave acid assisted digestion of the samples (0.5 g) was performed by addition of 4 mL HNO_3 (65%) and 1 mL H_2O_2 (30%) in quartz glass vessels (microwave digestion system - CEM Mars 5, USA). Temperature program was applied as follows: (1) 10 min at 200 ºC, pressure increase to 40 PSI, 2) hold time of 5 min at 200 ºC with pressure of 40 PSI, and (3) 5 min for cooling.

After digestion, samples were diluted with deionized water to a total volume of 50 mL and filtrated through 0,45 μm PTFE membrane filter. The blank passed the same procedure for quality control of the used reagents. All procedures were performed in triplicate, including the blank solutions.

External calibration was performed by measuring of 5 working standards. The correlation coefficients were > 0.99 in all cases.

2.7. *Determination of the mineral content*

For elements determination inductively coupled plasma optical emission spectrometer (Thermo scientific iCAP 6000 series ICP-Spectrometer, USA) was used. Instrumental operation conditions of ICP-OES were as follows: RF frequency 27.12 MHz; operating power, 1150 W; peristaltic pump rate: 50 rpm; plasma argon flow rate 0.5 L/min; argon carrier flow rate 0.5 L/min; sample flow rate 0.02 mL/min.

For purpose of method validation specificity, linearity, working range, accuracy, precision, LOD, and LOQ were tested. Basic calibration was performed by the measurement of 5 calibration solutions within a concentration range of 0.01 - 2.0 mg/L and the obtained correlation coefficients > 0.99 confirmed a good linearity. The limits of detection (LOD) and quantification (LOQ) were calculated based on *3SD/m* and *10SD/m*, respectively, where *m* is the slope of the calibration curves and *SD* is standard deviation of 10 consecutive measurements of the blank, multiplied by the dilution factor used for sample preparation (Table S1). Accuracy of method was tested by recovery experiments using certificated reference material of fish protein (*DORM-4, National Research Council, Canada)* and cooking chocolate (*Standard Reference Material® 2384, National Institute of Standards& Technology, USA).* The obtained recovery values were in the range from 71% to 127%.

2.8**.** *Chemometric analysis*

Dataset composed of 20 samples (rows) and 16 variables (columns) was subjected to multivariate analysis run by IBM SPSS 20.0.0 for Windows software package.

The results underwent the cluster analysis which comprises an unsupervised procedure that involves measuring the distance between objects to be clustered. The Ward's method as the amalgamation rule and squared Euclidean distance as metric were used.

3. **Results and discussion**

3.1. *Characterization of honey samples*

Physicochemical parameters of honey are good indicators of its quality and useful tool for the botanical discrimination of unifloral honey [21]. Moisture content, electrical conductivity, pH., free lactonic and total acidity for investigated samples are given in Table 2.

Moisture content is an important parameter responsible for stability and resistance to spoilage by yeast fermentation [27]. According to EU Directive (110/2001) it should not be higher than 20%. Moisture content of honeys in this study varied between 15.7 and 17.3%, with no significant differences between honeys samples ($p<0.05$) [28]. According to the literature, this value is related to the harvest season, the level of honey's maturity temperature and relative environmental humidity during producing of honey [21,29]. The obtained results are similar to previously reported for Serbian honeys [30, 32].

Electrical conductivity is closely connected to its ionic and organic acids content and usually is used in routine quality control instead of the ash content as an alternative [29]. Two samples of honeydew (HD1 and HD2), phacelia (PH1) and multifloral honey (MF3) have high EC between 0.70 and 0.95 mS/cm, Sakac *et al*. [30] reported EC in Serbian honeys (acacia, multifloral, and sunflower) 0.25, 0.71, and 0.73 mS/cm, respectively. Electrical conductivity in various honey samples was reported to be in the range of 0.27-2.49 mS/cm [32-35].

Total ash content of all honey samples varies from 0.05 to 0.50%. This value is expected to be in positive correlation with the mineral content of honeys, and in that way considered, a good criterion for tracing the botanic origin [27]. According to the literature, light-coloured and blossom honeys usually have a lower ash content than dark-coloured and honeydew and their blends [20,27,36]. Obtained results showed significant correlation ($p \leq 0.05$) between total mineral and ash content. Generally darker honey samples like honeydew, phacelia, and linden,

have higher total mineral and ash content, while acacia has the lowest mineral and ash content (67.90-94.89 mg/kg and 0.02 to 0.09%) as previously reported for Serbian honeys [30,37]

Honey has predominantly acidic nature, mostly due to the content of gluconic acid, formed as result of activity of glucose oxidase (GOx), during ripping and conversion of glucose. In the present study pH is in range between 3.41 (OR4) to 4.54 (HD1), and within the standard limit (pH 3.40-6.10) [29,32,33,35,37]. The values of free, lactonic and total acidity ranged from 10.12 (acacia, AC1) to 45.75 (sunflower, SF1), from 0.25 (multifloral, MF4) to 8.37 (linden, TI4), and from 11.63 (acacia AC2) to 46.85 meq/kg (sunflower, SF1), respectively. According to EU Council Directive [28], the upper limit for free acidity is 50.00 meq/kg, and this value is important parameter of honey deterioration. High free acidity may indicate sugar fermentation into organic acids as well as geographical origin and harvesting season [39,40]. In this study free and total acidity were much lower than 50.00 meq/kg for all samples, confirming their good quality.

3.1. *Radical scavenging activity*

Free radicals play an important role in the development of degenerative and chronic diseases such as cancer, atherosclerosis and diabetes mellitus, therefore is desirable to have antioxidant compounds included in a daily diet and prevent damages caused by free radicals.

DPPH (*2.2-diphenyl-1-picrylhydrazyl*) is stable, free radical which has been commonly used for testing the free radical scavenging ability of various substances and compounds [41,42]. Antioxidans are capable to neutralize it [33] and cause the color change from purple to yellow. The reduction capability of DPPH was determined by the decrease the absorbance at 517 nm.

The free radical scavenging capacity (%RSA) varies significantly among the honey samples (Table 2). The highest antioxidant activity was observed with two samples of honeydew honeys, HD1 and HD2, with 79.10 and 75.89% RSA, respectively. The lowest activity was observed in the acacia honey samples, AC1, AC2, and AC3 with 23.77, 22.96, and 24.57% RSC. Estevinho *et al.*, 2008 demonstrated that dark honeys, as honeydew [43], have DPPH inhibition values above 70%, and light, as acacia, below 40%. Wilczynska *et al.* [44] found that the antioxidant activity of some Polish honeydew honey samples was 72-83% while for acacia samples was 25-36% RSA, what is in accordance with results in this study. Gul and Pehlivan [4] investigated antioxidant activity of monofloral honey samples from different regions of Turkey, and found that antioxidant activity of sunflower honey was 19.24% RSA. Sunflower honeys in this study showed higher activity 33.18% (SF1) and 40.18% (SF2), which can be explained by different geographical origin. According to the literature, antioxidant activity of honeys is generally associated with significant content of phenolic compounds [45]. Besides, it widely varies with different floral types, geographical and botanical origin, climate, production and storage conditions [46]. It is important for this study that %RSC cannot be predicted only on the basis of total phenolic and flavonoide compounds [42]. Other components such as peptides, organic acids, enzymes and minerals like Se, Fe, Mn, Cu, and Zn, as cofactors in antioxidant enzymes, have a significant influence [45].

3.3. *Multi-elemental composition*

The mineral content of honey is dependent on its botanical and geographical origin (including both, pedologic and climatic conditions). The content of macro and micro elements is mostly related to the composition of the soil where the plants grow, as well as the type of plant from which the honeybees collected their food [39]. It was reported that light colored honeys have

lower mineral content than dark colored [36]. On the other hand, increased concentration of specific trace and toxic elements in honey could be a result of industrial contamination and production process as well. Due to its acidic nature, honey can cause the corrosion of metals like Al, Cd, Cr, Fe, and Pb and migrate from the material of the containers for the collection, production and disposal of honey. In addition, sugar cakes and syrups, as bees' food, can also be a significant source of Mn, Fe, Cd and Pb [9]. Taking into account all afore mentioned makes quantification of metals extremely important for both human health and environmental biomonitoring [2,39]. Twenty samples of different unifloral and multifloral honey samples, collected from four different regions of Serbia were analyzed to estimate concentrations of K, Fe, Mg, Mn, Na, Se, Si, Zn, Al, Cu, Ni, As, Pb, Cr, and Cd. Metals concentrations were determined by ICP OES and presented in Table 3.

The most abundant elements were K, Mg, and Na as major elements. Potassium concentration ranged from 46.35 mg/kg (acacia honey, AC1) to 466.69 mg/kg (multifloral honey, MF3). Generally, oilseed and acacia honeys have lower concentration of K than honeydew, multifloral, and linden honeys. It is noteworthy for samples with higher concentration of K to have higher electrical conductivity. Besides that, K concentration in multifloral honeys varies from 106.49 mg/kg (MF1) to 466.69 mg/kg (MF3) which could be connected with their botanical and geographical origin. According to the reported data, potassium is the most abundant element founded in various honey samples from Serbia [30], Croatia [2,40], Yemen [41], Anatolia, Turkey [42], and Morocco [36].

Magnesium and Na concentrations were in the ranges between 5.71 to 72.31 mg/kg, and 6.75 to 160.04 mg/kg, respectively. Those concentrations vary significantly even in the samples of the same botanical and geographical origin, but collected in two different years (2015 and 2017)

indicating the influence of different harvest conditions. In comparison with other studies, the values of Mg and Na were similar to those reported for honey collected from the Croatia [8,47] and Hungary [48]. Lower concentrations of Mg (2.5 - 30.8 mg/kg) were reported by Sakac *et al.* [30], and higher in Yemen (52.42-161.54 mg/kg) and Greek honeys (0.19-170.3 mg/kg) [7,48].

Trace elements such as Fe, Zn, Cu, Mn, and Se are essential for basic physiological processes, and have nutritional benefits when used as allowable daily intake. The concentration of Fe measured in honey samples vary from 0.79 (multifloral, MF1) to 5.99 mg/kg, (linden, TI2). These results are in agreement with iron concentration in honey samples from Serbia [30]. Authors found 0.79 mg/kg Fe in multifloral honeys from the same region of Serbia, and 0.89 and 1.24 mg/kg of Fe in acacia and sunflower honeys, respectively. Similar results (0.39-9.33 mg/kg) were reported by Louppis *et al*. [7], for Greek honeys, and for Croatian honeys 0.36 – 7.55 mg/kg and 1.03 – 15.3 mg/kg [8,47]. Higher ranges were obtained in Romania 11.53 – 54.78 mg/kg [51] and honeys from United Arab Emirates 1.15 – 110.79 mg/kg [33]. The obtained zinc concentrations in studied samples were within a range of Serbian regulative standards and vary from 0.36 (Oilseed rape, OR4) to 7.68 mg/kg (multifloral honey, MF4), with one exception of sample TI4, where 20.36 mg/kg was determined. These results were also in agreement with previously published results for Serbian honeys [30]: 0.78 mg/kg compared to 0.83 mg/kg in this study (acacia honey), 1.84 mg/kg compared to 2.6 mg/kg (multifloral honey), and 1.73 mg/kg compared to 2.8 mg/kg (sunflower honey) from the same region of Serbia. Zinc concentration in Hungarian honeys was 3.35 and 2.02 mg/kg for blossom and flower honeys, respectively [50]. In Greek honeys, Zn concentrations were in the range of 0.34-6.99 [7] and in Croatian 1.33-3.02 and 0.515-14.2 mg/kg [8,47]. El-Haskony *et al.* [34] reported Zn concentration range of 1.41-4.26 mg/kg for Moroccan honeys. Similar results for Zn (0.74 – 8.01 mg/kg) were published by

Oroian *et al.* [51]. The influencing sources of Zn could be galvanized recipients in honey harvesting and storage conditions [51]. On the other hand, the influence of floral type cannot be ruled out [52].

Copper was measured in four samples (TI2, OR3, PH1, and HD2), in quantities 0.71, 1.60, 0.62, and 0.53 mg/kg, respectively. Copper has important role in living organisms, especially in metabolism because it is an active site in many metaloenzymes [53]. The excess of copper deposits in organism causing neurological symptoms [54]. Increasing concentration of Cu could be the result of industrial contamination, the use of pesticides, but also the use of copper-based cookware which are usually used in households [55]. Mohammed *et al.* [48], Louppis *et al.* [7], and Czipa *et al.* [50] have reported similar results of 0.31, 0.77, and 0.27 mg/kg in Yemeni, Greek and Hungarian honeys, respectively. The concentration of Cu found in Croatian honeys were in the ranges 0.14-1.39 [8] and 56.2-588 µg/kg [47], and in Romanian 64.419 – 549.132 µg/kg [51].

Manganese content was found in the range from 0.21 mg/kg for linden honey (TI4) to 7.96 mg/kg for honeydew honey (HD2). Its concentration in honey could vary depending on the soil type and can be an indicator of geographical origin [48,49,56]. However, its content is similar for majority of Croatian (0.131 – 27.2 mg/kg), Hungarian (1.1 mg/kg), and Romanian (0.5 – 8.6 mg/kg) honeys [47,50,51].

Concentration of Al in honey was measured in the range of 0.29 - 4.77 mg/kg what is in agreement with data (0.741-27.60 mg/kg) reported by Lovakovic *et al.* [47]. These results are generally lower than those reported by Bilandzic *et al.* [8] (1.21-20.5 mg/kg) and higher than results reported by Karabagias *et al.* [40] (0.91mg/kg). In the recent years increased attention for

screening the aluminium concentration was correlated with its toxic impact in human pathologies, such as Parkinson's and Alzheimer's disease and renal osteodystrophy [57]. Silicon content is in range from 1.21 to 7.84 mg/kg, and similar than reported for different Egiptyan. Marocco and Spanish honeys, but lower than in Greece honeys [40].

Nickel was detected only in three samples HD1, AC2, and AC3 (honeydew and acacia) in quantities 0.08, 0.05, and 0.05 mg/kg, respectively. In general, it can be explained with lower exposition to the sources of anthropogenic pollution and safe distance of hives from industrial plants. Nickel normally occurs in low levels in the environment, but if presents in a higher amounts, this element can cause enzyme inactivation, pulmonary health effects and tumors. In previous reports from Croatia, the concentration of Ni varied in different type of honeys. Bilandzic *et al.* [8] reported the interval of 0.75 - to 285 μg/kg for Ni content in bearberry and honeydew honey, and Lovakovic *et al.* [47] 9.08- 674 μg/kg for strawberry and honeydew honey. It is noteworthy to emphasize the higher concentrations of Ni in honeydew honeys in both studies. In Moroccan honeys, Ni was measured in the range from 0.02 to 0.15 mg/kg, and in Romanian from 101.18 to 570.01 μg/kg [34]. Much higher concentration of Ni were found in honeys from Czech Republic [58] and United Arab Emirates [41], *i.e.* up to 1.53 mg/kg and 7.78 mg/kg, respectively.

It has been reported that honey can be used as indicator of heavy metal contamination, especially lead and cadmium [59]. Both metals are highly toxic because once they enter the food chain they cause both acute and chronic poisoning, and exposure to these metals effects on the kidney, liver, heart, vascular and immune system, causes chromosome aberration, skin allergy, cancer and birth defects [60]. The maximum level of Cd and Pb in honeys (0.1 mg/kg) was set by European Commission Regulation [61]. Cadmium was detected in three samples, linden honey, (TI2) and

acacia honey, (AC2) in concentration 0.02 mg/kg and in multifloral honey (MF2) 0.15 mg/kg. The most common sources of Cd contamination are phosphate fertilizers. sewage sludge, smelting, mining or traffic. The honey with the highest Cd content (MF2) was collected from region which is a part of National park, excluding aforementioned sources. However, it can be explained by presence of St John's-worth, widespread in this region and recognized as bioaccumulator of Cd [62]. The following ranges were reported for Croatian [8,47] and Romanian [51] honeys: 0.45-6.62 µg/kg, 0.239-2.53 µg/kg, and 0.5-11.60 µg/kg, respectively.

Lead was detected in one sample,honeydew honey (HD1) with concentration of 0.42 mg/kg, while concentrations of As, Se, and Cr were below the detection limits in all samples.

3.4. *Antibacterial activity*

The results of the *in vitro* study of antibacterial activity of different honey samples are presented in Table 4.

All honeys tested in this study showed antibacterial activity against Gram positive and Gram negative bacteria, but significant differences were observed. Generally, stronger activity against *E. coli* than is in accordance with the earlier studies [10,15]. The highest activity of inhibition of bacterial growth against *E. coli* was observed with samples TI1, TI2, MF2, and AC2 as follows: 92%, 83%, 80.6%, and 82.1%, respectively. The minor activity against *E. coli* showed two samples, honeydew HD1 (5%) and multifloral honey MF1 (10%). On the other side, these two samples exhibited maximum activity against *S. aureus*. *i.e.* 67.7% (HD1) and 48.4% (MF1). The same was observed with other samples as well: the higher activity against *E. coli* and lower against *S. aureus*. The representative example of this observation is sample TI4 (linden honey) with 71.4% activity against *E.coli* and with no activity against *S. aureus*. The most plausible

hypothesis can lie in different mechanisms encountered with microbiological activity of different strains.

The activity of the examined honey samples against *C. albicans* was almost negligible. The fungus growth inhibition was above 10% only with six honeys while the rest of samples no inhibition activity was observed. The obtained results are in accordance with data reported, even for other types of honeys [15].

4. Chemometric evaluation

Having in mind a large dataset, multivariate analysis appeared as a good choice for extraction additional information, hidden in the multidimensional mathematical space.

In order to identify relatively similar *i.e.* groups of objects (samples) in the space of measured features (variables) a hierarchical agglomerative cluster analysis (HCA) was performed. Ward's method, as an amalgamation rule, was applied on standardized concentrations of the elements and the Euclidian distance as a measure of the proximity between samples. Well differentiated corresponding dendrogram is shown in Fig. 2. At the distance of 9 three clusters exhibit obviously the homogeneity, as the feature responsible for those groupings referring to the geographical and botanical origin. Only one exception (honey sample originated from region of Backa) was observed. **The first cluster** is composed mainly of samples from region of Banat (SF2, MF4, OR4, SF1) and two multifloral samples from region of Zlatibor (MF1 i MF2). Beside the geographical origin the obtained grouping can be the result of botanical origin as well, since 3 out of 4 multifloral samples were clustered. **The second cluster** comprises all linden and acacia honey samples, pointing out the botanical origin as dominant feature for clustering. This is additionally supported if we firstly, cut the distance at 7, when two sub-clusters of linden and

acacia honey samples are clearly differentiated. Then, in the second step, at the distance of 5 linden samples were differentiated further according to their geographical origin, *i.e.* the samples TI1, TI2, and TI3 creates one sub-cluster, leaving out TI5 as a sample from region on Backa. **The third cluster is composed of** both honeydew honey samples (HD1 and HD1) originated from a region of Zlatibor.

Based on above observations, it is important to stress that both botanical and geographic origin have to be considered.

4. Conclusion

The physicochemical properties, mineral content, antioxidant and antimicrobial activities of different Serbian honey types were estimated and discussed. Significant differences were found in the element concentration between honeys of different botanical and geographical origin, as well as harvest conditions. The concentrations of heavy metals were within the allowed limits. A high concentration of essential macro and micro-nutrients go into favor of high nutritional value of analyzed honeys, according to the Euroepan Commission Regulation. The presented results showed that these honeys have significant antioxidant activity (DPPH assay) and antibacterial potential against *E. coli* and *S. aureus*. These results offer a new perspective for the application of honey and its products for certain medical treatments (healing of decubitus wounds, diabetic foot, or post-traumatic wounds, prevention of infections, increase of immunity, etc.), as complementary to classical medicine.

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Table 1 Botanical and geographical origin of honeys from different region of Serbia

Sample No	Botanical Origin	Location, year of harvest	Region, County
TI1	Linden (*Tilia europea*)	Erdevik, 2015	Srem, Vojvodina
TI2	Linden (*Tilia europea*)	Erdevik, 2017	Srem, Vojvodina
TI3	Linden (*Tilia europea*)	Ljuba, 2017	Srem, Vojvodina
TI4	Linden (*Tilia europea*)	Zrenjanin, 2017	Banat, Vojvodina
OR1	Rapeseed (*Brassica napus*)	Kanjiža, 2017	Banat, Vojvodina
OR2	Rapeseed (*Brassica napus*)	Sremska Mitrovica, 2017	Srem, Vojvodina
OR3	Rapeseed (*Brassica napus*)	Karavukovo, 2017	Bačka, Vojvodina
OR4	Rapeseed (*Brassica napus*)	Zlatica, 2017	Banat, Vojvodina
MF1	Multifloral	Semegnjevo, 2017	Zlatibor, West Serbia
MF2	Multifloral	Šljivovica, 2017	Zlatibor, West Serbia
MF3	Multifloral	Jablanica, 2017	Zlatibor, West Serbia
MF4	Multifloral	Banatski Despotovac, 2017	Banat, Vojvodina
HD1	Honeydew	Kremna, 2017	Zlatibor, West Serbia
HD2	Honeydew	Nova Varoš, 2017	Zlatibor, West Serbia
SF1	Sunflower (*Helianthus annuus*)	Alibunar, 2017	Banat, Vojvodina
SF2	Sunflower (*Helianthus annuus*)	Zrenjanin, 2017	Banat, Vojvodina
PH1	Phacelia (*Phacelia*)	Mihajlovo, 2017	Banat, Vojvodina
AC1	Acacia (*Robinia pseudoacacia*)	Jabučje 2015	Šumadija, Central Serbia
AC2	Acacia (*Robinia pseudoacacia*)	Jabučje 2017	Šumadija, Central Serbia
AC3	Acacia (*Robinia pseudoacacia*)	Kragujevac, 2017	Šumadija, Central Serbia

Table 2 The physicochemical parameters of analyzed honey samples

Sample	Moisture content (%)	pH	Free acidity (meq/kg)	Total acidity (meq/kg)	Total ash (%)	EC (mS cm^{-1})	RSA (%)
TI1	17.3 ± 0.1	4.05 ± 0.02	21. 25 ± 0.18	22.38 ± 0.12	0.26	0.43 ± 0.03	30.42 ± 0.56
TI2	16.2 ± 0.23	4.25 ± 0.03	18.75 ± 0.28	19.75 ± 0.23	0.30	0.47 ± 0.01	37.08 ± 1.32
TI3	15.9 ± 0.18	4.32 ± 0.03	17.00 ± 0.31	19.75 ± 0.20	0.46	0.67 ± 0.06	31.00 ± 0.97
TI4	17.1 ± 0.15	4.08 ± 0.02	21.75 ± 0.36	30.13 ± 0.30	0.23	0.39 ± 0.05	24.34 ± 0.29
OR1	15.8 ± 0.21	3.64 ± 0.01	22.50 ± 0.07	24.75 ± 0.17	0.05	0.17 ± 0.02	28.24 ± 1.67
OR2	16.4 ± 0.05	4.05 ± 0.02	19.00 ± 0.25	19.88 ± 0.34	0.14	0.28 ± 0.03	34.67 ± 1.34
OR3	16.5 ± 0.17	4.07 ± 0.04	13.62 ± 0.19	15.13 ± 0.21	0.08	0.21 ± 0.01	29.62 ±0.24
OR4	17.1 ± 0.23	3.41 ± 0.01	36.75 ± 0.41	37.50 ± 0.08	0.08	0.21 ± 0.04	30.20 ± 0.28
MF1	15.8 ± 0.19	3.68 ± 0.02	35.12 ± 0.30	38.63 ± 0.17	0.32	0.05 ± 0.01	38.12 ± 0.46
MF2	16.4 ± 0.25	3.87 ± 0.02	27.00 ± 0.23	29.40 ± 0.36	0.20	0.35 ± 0.02	46.27 ± 0.57
MF3	16.8 ± 0.30	4.41 ± 0.03	27.00 ± 0.36	30.20 ±0.35	0.70	0.95 ± 0.02	57.29 ± 0.21
MF4	16.5 ± 0.21	3.81 ± 0.02	28.25 ± 0.28	28.50 ± 0.20	0.29	0.46 ± 0.03	36.97 ± 0.69
HD1	17.1 ± 0.27	4.55 ± 0.01	23.37 ±0.35	26.25± 0.46	0.55	0.77 ± 0.04	79.10 ±0.47
HD2	16.9 ± 0.16	4.33 ± 0.03	32.50 ± 0.07	33.25 ± 0.21	0.49	0.7 ± 0.03	75.89 ± 0.76
SF1	15.9 ± 0.15	3.43 ± 0.03	45.75 ±0.44	46.85 ± 0.34	0.26	0.42 ± 0.06	33.18 ± 1.13
SF2	16.5 ±0.13	3.77 ± 0.02	33.12 ±0.36	35.38 ± 0.29	0.29	0.46 ± 0.01	40.18 ± 1.54
PH1	16.3 ± 0.24	4.34 ± 0.04	36.37 ± 0.39	40.25 ± 0.47	0.53	0.75 ± 0.02	79.45 ± 1.27
AC1	16.7 ± 0.36	3.67 ± 0.01	20.25 ±0.24	23.35 ± 0.13	0.02	0.13 ± 0.03	23.77 ± 0.09
AC2	16.8 ± 0.15	4.09 ± 0.02	10.12 ± 0.11	11.63± 0.21	0.09	0.22 ± 0.04	22.96 ± 0 .35
AC3	17.0 ± 0.13	3.47 ± 0.03	24.62 ±0.25	26.25 ± 0.17	0.07	0.2 ±0.02	24.57 ± 0.42

* All results are expressed as means of triplicate ± standard deviation

Table 3 Concentrations of major and minor elements in honeys from different region of Serbia (mg/kg) *

Sample	K	Mg	Mn	Na	Si	Zn	Fe	Al	Se	As	Cd	Cr	Cu	Ni	Pb	Total mineral content
TI1	195.25 ± 1.67	25.82 ± 0.66	1.12±0.15	9.39±0.27	3.39±0.14	1.12±0.12	3.57±0.12	2.71±0.17	ND	ND	ND	ND	ND	ND	ND	239.66
TI2	258.55 ± 1.54	27.23 ± 0.23	0.53±0.04	29.32±0.59	3.38±0.18	1.06±0.10	5.99±0.34	0.29±0.02	ND	ND	0.02±0.004	ND	0.71± 0.07	ND	ND	326.79
TI3	401.16 ± 1.89	20.81 ± 0.42	0.34±0.02	8.38±0.19	2.90±0.12	1.43±0.15	2.25±0.09	3.41±0.24	ND	ND	ND	ND	ND	ND	ND	437.27
TI4	107.08 ± 1.10	13.85 ± 0.21	0.21±0.02	21.06±0.41	1.58±0.09	20.36±0.19	5.51±0.46	2.58±0.32	ND	ND	ND	ND	ND	ND	ND	169.65
OR1	47.70 ± 0.68	19.02 ± 0.26	0.34±0.03	9.71±0.26	1.76±0.10	0.45±0.04	2.70±0.06	3.41±0.42	ND	ND	ND	ND	ND	ND	ND	81.68
OR2	90.22 ± 1.32	20.16 ± 0.44	0.37±0.04	9.25±0.27	1.82±0.16	0.78±0.09	1.02±0.09	2.33±0.21	ND	ND	ND	ND	ND	ND	ND	123.62
OR3	53.75 ± 0.65	72.31 ± 0.89	0.72±0.08	160.04±1.05	1.94±0.14	1.27±0.12	5.59±0.28	4.56±0.54	ND	ND	ND	ND	1.60±0.11	ND	ND	297.21
OR4	64.33 ± 1.22	16.22 ± 0.15	0.27±0.01	12.96±0.20	5.82±0.42	0.36±0.04	0.91±0.03	2.74±0.27	ND	ND	ND	ND	ND	ND	ND	100.87
MF1	106.49 ± 1.12	17.71 ± 0.18	0.88±0.09	26.25±0.51	1.55±0.15	1.18±0.14	0.79±0.04	4.47±0.31	ND	ND	ND	ND	ND	ND	ND	154.85
MF2	164.24 ± 1.32	32.91 ± 0.54	1.04±0.12	15.80±0.31	2.35±0.20	0.77±0.08	3.54±0.15	2.82±0.28	ND	ND	0.15±0.02	ND	ND	ND	ND	220.65
MF3	466.69 ±1.94	61.26 ± 0.97	2.05±0.19	10.95±0.15	6.91±0.19	0.77±0.05	0.94±0.06	2.98±0.24	ND	ND	ND	ND	ND	ND	ND	549.57
MF4	162.31 ± 1.52	41.59 ± 0.57	0.31±0.04	14.25±0.22	2.34±0.11	7.68±0.58	2.09±0.14	3.10±0.41	ND	ND	ND	ND	ND	ND	ND	230.57
HD1	352.87 ± 1.74	63.60 ± 0.24	1.89±0.24	12.90±0.19	7.84±0.53	0.65±0.02	3.95±0.31	ND	ND	ND	ND	ND	ND	0.08±0.006	0.42±0.08	444.2
HD2	312.91 ± 1.76	62.63 ± 0.21	7.96±0.92	36.67±0.48	2.50±0.12	2.42±0.25	2.46±0.37	3.47±0.55	ND	ND	ND	ND	0.53±0.06	ND	ND	427.55
SF1	113.47 ± 1.14	33.01 ± 0.17	1.17±0.19	7.77±0.17	2.10±0.16	3.14±0.42	2.83±0.29	2.39±0.24	ND	ND	ND	ND	ND	ND	ND	163.49
SF2	133.39 ± 1.24	39.16 ± 0.51	0.25±0.02	18.76±0.33	2.90±0.18	2.46±0.31	3.09±0.32	3.50±0.32	ND	ND	ND	ND	ND	ND	ND	200.01
PH1	335.37 ± 1.67	39.17 ± 0.52	0.27±0.03	12.34±0.23	1.98±0.08	3.01±0.24	1.85±0.17	3.02±0.29	ND	ND	ND	ND	0.62±0.04	ND	ND	393.99
AC1	46.35 ± 0.65	5.71 ± 0.08	0.54±0.07	6.75±0.11	4.53±0.32	0.38±0.02	3.64±0.49	2.09±0.22	ND	ND	ND	ND	ND	ND	ND	67.9
AC2	63.49 ± 2.36	12.04 ± 0.24	0.54±0.06	12.18±0.21	1.58±0.07	1.08±0.09	3.91±0.36	2.26±0.27	ND	ND	0.02± 0.003	ND	ND	0.05±0.004	ND	94.89
AC3	56.46 ± 1.33	9.26 ± 0.21	0.66±0.05	12.52±0.27	1.21±0.09	1.03±0.08	3.16±0.28	2.60±0.29	ND	ND	ND	ND	ND	0.05±0.006	ND	84.35

*All results are expressed as means of triplicate ± standard deviation

ND = not determinated

Table 4 Antimicrobal activity of honeys expressed as % of inhibitory

Sample	*E. coli*	*S. aureus*	*C. albicans*
TI1	92.2±1.1	30.9±1.2	44.8±2.5
TI2	83.6±2.3	20.9±0.6	0.01±0.00
TI3	46.1±0.8	39.7±2.3	9.3±0.4
TI4	71.4±1.7	0.01±0.00	0.01±0.00
OR1	17.9±0.4	19.2±1.2	0.01±0.00
OR2	73.6±1.5	20.0±2.8	0.01±0.00
OR3	63.1±3.1	17.6±1.7	10.3±1.2
OR4	38.8±2.5	16.7±0.5	0.01±0.00
MF1	10.0±0.6	48.4±0.7	34.1±1.6
MF2	80.6±0.4	44.9±3.4	4.6±1.2
MF3	59.7±4.1	30.8±2.1	2.3±0.8
MF4	42.9±1.3	18.4±1.9	0.01±0.00
HD1	5.0±0.3	67.7±0.7	12.2±0.4
HD2	40.3±2.6	39.7±1.4	0.01±0.00
SF1	69.5±3.1	6.7±0.4	3.4±0.2
SF2	79.1±2.7	39.7±1.8	13.9±0.2
PH1	21.4±2.3	17.1±1.4	0.01±0.00
AC1	70.0±1.5	21.8±3.1	0.01±0.00
AC2	82.1±2.1	28.2±1.2	7.0±0.0
AC3	68.1±1.6	63.0±1.1	13.8±1.7

* All results are expressed as means of triplicate ± standard deviation

Figure captions

Figure 1 Map of Serbia indicating the geographic regions of honey samples

Figure 2 Dendogram for the classification of honeys collected from 5 different regions of Serbia

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