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Glucose oxidase activity and hydrogen peroxide accumulation in Croatian honeys

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ABSTRACT

Ability of black locust (n = 7), chestnut (n = 3), lime (n = 5), mint (n = 3), and honeydew (n = 12) honeys to produce hydrogen peroxide upon dilution, as well as their glucose oxidase activity were investigated in the present study. The glucose oxidase (GOX) activity was determined by the standard horseradish peroxidase/*o*-dianisidin method, while hydrogen peroxide concentration in honey solutions of five different mass to volume ratios (1:1; 1:2; 1:4; 1:8; 1:16) by semi-quantitative method using MQuant™ peroxide test strips. The obtained results showed that chestnut, lime, honeydew and mint honeys exhibited high GOX activity (341.26 ± 128.78, 350.16 ± 124.91, 376.82 ± 69.02, 402.47 ± 60.99 μg H_2O_2/h g), while black locust honeys much lower GOX activity of 25.58 ± 21.87 μg H_2O_2/h g. The accumulation of hydrogen peroxide in serially diluted honeys has shown asymmetrical inverted U-shaped curve, where the increase in hydrogen peroxide accumulation with dilution reached a maximum point, after which its concentration rapidly declined. Hydrogen peroxide content in honey solutions of different mass to volume ratio varied from 0 to 294.1 μmol/L h. Lime and chestnut honeys generated the highest hydrogen peroxide content (264.71 ± 65.77, 245.10 ± 84.90 μmol/L h) on average, while black locust, mint and honeydew honeys at least two-fold lower amounts (113.40 ± 50.84, 127.45 ± 33.96, 112.75 ± 98.42 μmol/L h). Lack of correlation between glucose oxidase activity and hydrogen peroxide content indicates that the glucose oxidase activity does not present a reliable parameter for the prediction of hydrogen peroxide content produced in honey solutions.

Introduction

Since the ancient times, honey has been used as a topical antiseptic for the treatment and prevention of wound infections. Its antimicrobial activity has been attributed to hyper-osmolarity, low pH (acidity), and the presence of antimicrobial compounds such as hydrogen peroxide (H_2O_2), methylglyoxal, bee defensin-1, phenolic acids (syringic, ferulic, cinnamic, benzoic, and caffeic acids), and flavonoids (pinocembrin, chrysin, galangin, pinobanksin) (Bang et al., 2004; Brudzynski et al., 2017; Brudzynski 2006; Cooke et al., 2015; Gradvol et al., 2015; Kerkvliet, 1996; Kıvrak and Kıvrak, 2017; Kwakman and Zaat, 2012). However, it should be noted that antimicrobial activities of most honeys were found strongly dependent on the presence of hydrogen peroxide, with the exception of Manuka honey (Brudzynski, 2006; White et al., 1963).

H_2O_2 is a by-product of glucose oxidation to gluconic acid catalysed by the bee-derived enzyme glucose oxidase. In ripened undiluted honeys, glucose oxidase is inactive, but upon honey dilution, it regains its activity and produces hydrogen peroxide (Kerkvliet, 1996; Kwakman and Zaat, 2012). The efficiency of hydrogen peroxide production by glucose oxidase increases with honey dilution simply as a consequence of the facilitated access of the enzyme to its substrate, but after reaching a certain level of honey dilution, the amount of the

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produced hydrogen peroxide falls, due to the lowered substrate concentration, as well as a consequence of the disruption of honey two-phase colloidal state necessary for its production. According to Kerkvliet (1996), the presence of glucose oxidase does not always guarantee the liberation of H_2O_2 in diluted honey. There are several factors known to affect H_2O_2 accumulation in honey: a) inactivation of glucose oxidase by exposure to heat or light (Kerkvliet, 1996; Schepartz and Subers, 1964; White and Subers, 1964), b) glucose oxidase inhibition by its substrate (glucose) at high substrate concentrations (Schepartz and Subers, 1964), c) non-competitive inhibition of glucose oxidase by its product, hydrogen peroxide, at elevated glucose concentration (Tomotani et al., 2005), as well as d) decomposition of H_2O_2 by honey components (Kerkvliet, 1996; Kwakman and Zaat, 2012). Among the honey components reported to affect H_2O_2 accumulation by decomposing it in diluted honeys are enzyme catalase (Schepartz and Subers, 1966), ascorbic acid (vitamin C) (Kerkvliet, 1996), and in some instances ferrous ions (Kerkvliet, 1996). Nevertheless, the determination of hydrogen peroxide content in honey gives an indication of the antibacterial activity of honey (Brudzynski, 2006; Kerkvliet, 1996).

While Croatian honeys have been reported to exhibit good antibacterial activity against several foodborne pathogens (Gradvol et al., 2015), there are no data on their peroxide activity according to our knowledge. In this respect, the present study examined hydrogen peroxide accumulation in black locust, chestnut, lime, honeydew and mint honeys diluted at five different mass to volume ratios. Additionally, the determination of glucose oxidase activity in honeys was performed to assess potential correlation between glucose oxidase activity and accumulated H_2O_2. All this was done as a first step in the prediction of a potential antibacterial activity, as well as to assess the potential therapeutic effect of Croatian honeys in a treatment of wounds.

Materials and methods

Honey samples

A total of 30 honey samples: 7 of black locust (*Robinia pseudoaccacia* L.), 3 of chestnut (*Castanea sativa* Mill.), 5 of lime (*Tilia* spp.), 3 of mint (*Mentha* spp.) and 12 of honeydew honeys from the production season 2016 were collected from Croatian beekeepers. With the aim to determine the botanical origin, samples were subjected to a qualitative orienting melissopalynological analysis according to the method of Louveaux et al. (1978). The following physicochemical parameters were determined: moisture, electrical conductivity, HMF content and diastase activity according to the International Honey Commission methods (Bogdanov, 2009). Classification of samples was performed based on the data prescribed in the national regulations (Ministry of Agriculture, Fisheries and Rural Development, 2009; Ministry of Agriculture, 2015).

Determination of glucose oxidase

Glucose oxidase activity in honey was determined by horseradish peroxidase/*o*-dianisidin method as previously described by Flanjak et al. (2016a).

Estimation of hydrogen peroxide content

Hydrogen peroxide content in honey solutions of different mass to volume ratio (Table 1) was estimated by semi-quantitative method using MQuant™ peroxide test strips (Merck, Germany).

The analysis of peroxide accumulation was carried out as follows: to mass of 0.25, 0.5 or 1 g of honey in a 5 mL eppendorf tube appropriate volume of deionized water was added to achieve the desired mass to volume ratio (Table 1), and honey was dissolved by mixing on a vortex mixer Vibromix 10 (Tehtnica, Slovenia) approximately 1-3 minutes. Immediately afterwards, the pH of honey solution was measured by the pH meter HI2020 Edge® (Hanna Instruments, USA) and solution was incubated at 37 °C for 60 minutes in a water bath Memmert WNB 14 (Memmert GmbH, Germany). After 1 hour of incubation, samples were cooled in an ice-cold water bath for approximately 2 minutes, and hydrogen peroxide content was measured by dipping the MQuant test strip (no. 10011; Merck, Germany) into honey solution for 1 s. After 15 s of colour development, the obtained green colour was compared with the colour scale provided by the manufacturer, and the amount of accumulated hydrogen peroxide per hour was read off in mg H_2O_2/L. The obtained value, multiplied by thousand (conversion of mg to μg) and divided by M_w of hydrogen peroxide of 34.0147 (conversion of grams to moles), gave amount of accumulated hydrogen peroxide in μmol/L h.

Statistical analysis

Mean values and standard deviations were calculated, and minimum and maximum values were given using Microsoft® Excel® 2016 MSO. The differences between honey types for glucose oxidase activity were analysed by Kruskal-Wallis multiple comparison Z-value test using Dell Statistica (data analysis software system), version 12.

Table 1. Dilutions of honey samples

Mass to volume ratio	Honey mass (g)	Water volume (mL)	φ (mL/mL)	γ (g/mL)	Dilution*
1:1	1	1	0.43	0.58	2.35
1:2	1	2	0.27	0.37	3.70
1:4	1	4	0.16	0.21	6.40
1:8	0.5	4	0.08	0.11	11.80
1:16	0.25	4	0.04	0.06	22.60

* Dilution calculated on the basis of volume to volume ratio

Results and discussion

The present study investigated the ability of the selected Croatian honeys to accumulate hydrogen peroxide in water solutions upon dilution. Black locust *(n = 7*), chestnut (*n = 3*), lime (*n = 5*), honeydew (*n = 12*) and mint (*n = 3*) honeys were diluted by deionized water at five different mass to volume ratios (1:1; 1:2; 1:4; 1:8; 1:16) and after 60 minutes of incubation at 37 °C, the content of the produced hydrogen peroxide was measured by the MQuant peroxide test strips. However, since hydrogen peroxide formation in honey depends on the glucose oxidase (GOX) action (Brudzynski 2006; Brudzynski et al., 2011; Kerkvliet, 1996), it was necessary to determine the activity of this enzyme in honey samples before measuring the hydrogen peroxide content in honey solutions.

Determination of GOX in honey samples by the standard horseradish peroxidase/*o*-dianisidin method revealed that all examined samples possessed the glucose oxidase activity, and can thus be analysed for hydrogen peroxide accumulation.

Mean values of the GOX activity in the examined honeys are shown in Table 2. Chestnut, lime, honeydew and mint honeys possessed statistically similar ($p < 0.05$) high GOX activity (341.26 ± 128.78, 350.16 ± 124.91, 376.82 ± 69.02, 402.47 ± 60.99 μg H_2O_2/h g, respectively), while black locust honeys approximately 10-fold lower activity (25.58 ± 21.87 μg H_2O_2/h g) (Table 2). Flanjak et al. (2016a) have reported similar values for the GOX activity in chestnut, honeydew and black locust honeys.

Once the presence of endogenous the GOX activity in examined honeys was confirmed, the effect of honey dilution on hydrogen peroxide accumulation was investigated. The pH of honey solutions in deionized water was measured taking into account that hydrogen peroxide accumulation in honeys upon dilution depends on the GOX activity, which is in turn affected by the pH (Kerkvliet, 1996; Schepartz and Subers, 1964).

The effect of dilution ratio on the pH of honey solution is shown in Table 3. The pH of the examined honeys varied from 3.51 to 5.85, which is in agreement with previous reports on the pH range of various honeys (Kıvrak et al., 2017; Persano Oddo and Piro, 2004). Honey dilution caused an increase in the measured pH. However, the pH increase from the lowest to the highest dilution was slight and was ranging from 0.23 pH units for chestnut honey, to 0.39 pH units for black locust. Such slight changes in pH upon honey dilution were not surprising, since it is well known that honey acts as a buffer (Buba et al., 2013).

Table 2. Glucose oxidase activity of Croatian honeys

Honey type	Statistic	Glucose oxidase activity (μg H_2O_2/h g)
Black locust (*n*=7)	Mean ± SD	25.58 ± 21.87
	Min	6.32
	Max	62.26
Chestnut (*n*=3)	Mean ± SD	341.26 ± 128.78
	Min	195.81
	Max	440.80
Lime (*n*=5)	Mean ± SD	350.16 ± 124.91
	Min	235.51
	Max	547.56
Honeydew (*n*=12)	Mean ± SD	376.82 ± 69.02
	Min	297.15
	Max	525.62
Mint (*n*=3)	Mean ± SD	402.47 ± 60.99
	Min	345.15
	Max	466.57

Table 3. The pH of honey solutions of different dilution ratio

	Honey dilution				
	2.35	**3.70**	**6.40**	**11.80**	**22.60**
Honey type	**pH***				
Black locust	3.82 ± 0.16	3.86 ± 0.15	3.99 ± 0.15	4.06 ±0.14	4.21 ± 0.15
Chestnut	4.59 ± 0.58	4.63 ± 0.55	4.76 ± 0.60	4.79 ± 0.59	4.82 ± 0.62
Lime	3.87 ± 0.06	3.97 ± 0.03	4.05 ± 0.04	4.09 ± 0.03	4.12 ± 0.05
Honeydew	4.88 ± 0.35	4.95 ± 0.35	5.05 ± 0.37	5.12 ± 0.37	5.17 ± 0.39
Mint	3.82 ± 0.32	3.89 ± 0.32	3.96 ± 0.34	4.02 ± 0.32	4.09 ± 0.27

* Results present mean ± SD for each type of honey

Table 4. Hydrogen peroxide content in honey solutions of different dilution ratio

	Honey dilution				
	2.35	**3.70**	**6.40**	**11.80**	**22.60**
Honey sample	**Hydrogen peroxide content (μmol/L h)**				
Black locust 1	88.24	147.1	147.1	58.82	14.71
Black locust 2	88.24	147.1	147.1	88.24	29.41
Black locust 3	29.41	147.1	147.1	88.24	29.41
Black locust 4	88.24	147.1	88.24	58.82	14.71
Black locust 5	58.82	88.24	147.1	58.82	14.71
Black locust 6	58.82	88.24	88.24	29.41	14.71
Black locust 7	0	14.71	14.71	14.71	0
Chestnut 1	14.71	14.71	58.82	147.1	58.82
Chestnut 2	88.24	147.1	294.1	294.1	88.24
Chestnut 3	88.24	147.1	294.1	294.1	88.24
Lime 1	294.1	294.1	294.1	294.1	88.24
Lime 2	147.1	294.1	294.1	294.1	88.24
Lime 3	147.1	294.1	294.1	294.1	88.24
Lime 4	294.1	294.1	294.1	294.1	88.24
Lime 5	58.82	147.1	147.1	147.1	88.24
Honeydew 1	0	14.71	58.82	147.1	88.24
Honeydew 2	58.82	147.1	147.1	294.1	88.24
Honeydew 3	0	0	58.82	147.1	58.82
Honeydew 4	0	0	0	29.41	88.24
Honeydew 5	58.82	147.1	294.1	294.1	88.24
Honeydew 6	0	14.71	29.41	88.24	88.24
Honeydew 7	0	0	14.71	29.41	88.24
Honeydew 8	0	0	58.82	147.1	88.24
Honeydew 9	0	0	14.71	58.82	58.82
Honeydew 10	0	0	14.71	88.24	88.24
Honeydew 11	0	0	0	14.71	58.82
Honeydew 12	0	0	0	14.71	58.82
Mint 1	0	0	58.82	147.1	88.24
Mint 2	0	0	58.82	147.1	58.82
Mint 3	0	0	58.82	88.24	58.82

Determination of hydrogen peroxide content in honey solutions of different mass to volume ratio revealed that H_2O_2 content in honey samples varied from 0 to 294.1 μmol/L h (Table 4). The content of hydrogen peroxide differed among samples of the same botanical origin, as well as between samples of the different botanical origin, and was found to be dependent on honey dilution. Serially diluted honeys have shown asymmetrical inverted U-shaped curve of H_2O_2 accumulation, where the increase in hydrogen peroxide accumulation with dilution reached a maximum point, after which its concentration rapidly declined (Table 4, Fig. 1). Lime and chestnut honeys exhibited the highest generation of hydrogen peroxide content, while black locust, mint and honeydew honeys generally produced lower amount of hydrogen peroxide.

The highest production of hydrogen peroxide in water solutions of black locust honey

(up to 147.1 μmol/L h) was achieved at mass to volume ratio between 1:2 to 1:4 (dilutions 3.7 and 6.4), with hydrogen peroxide concentration variations among samples between 14.71 and 147.1 μmol/L h, and mean value of 113.40 ± 50.84 μmol/L h. Mint honeys generated averagely 127.45 ± 33.96 μmol/L h of hydrogen peroxide at mass to volume ratio of 1:8 (dilution 11.8) with a range from 88.24 to 147.1 μmol/L h, while honeydew honeys exhibited the highest hydrogen peroxide producing ability at dilutions corresponding to mass to volume ratio of 1:8 or 1:16 (dilutions 11.8 and 22.6). At present dilutions, honeydew honeys produced hydrogen peroxide in range from 14.71 to 294.1 μmol/L h, with average values of 112.75 ± 98.42 μmol/L h for 11.8 dilution, and of 78.43 ± 14.48 μmol/L h for 22.6 dilution. Among the examined, lime honeys were found to be the greatest producers of hydrogen peroxide with the average value of 264.71 ± 65.77 μmol/L h in wide range of honey dilutions (from 3.7 to 11.8; or mass to volume ratio from 1:2 to 1:8).

The obtained data on hydrogen peroxide level in Croatian honeys are in accordance with the report of Brudzynski (2006), who found H_2O_2 concentration at 8-fold dilution of Canadian honeys in range from 29.4 to 238.5 μmol/L h, but are lower from those reported by Bang et al. (2004), Brudzynski et al. (2011) and Cooke et al. (2015) who reported up to 2.68 mmol/L h of the H_2O_2 produced. The most probable reasons for such differences in hydrogen peroxide content in honeys of various reports are: a) botanical origin of honey, b) method of the H_2O_2 determination, as well as c) the solvent used. Namely, majority of research on hydrogen peroxide accumulation in different honeys were performed with honeys of botanical origin different from those examined in our study. The only exception is the report of Brudzynski (2006), who among 42 honey samples, examined one sample of black locust honey. Furthermore, the H_2O_2 content accumulation in honeys was measured by different methods: fluorescent assay (Amplex Red Hydrogen Peroxide/Peroxidase Assay kit) (Brudzynski, 2006; Brudzynski et al., 2011; Brudzynski et al., 2017), by measurement of oxygen concentration (decomposition of H_2O_2 by exogenously added catalase) (Bang et al., 2004), while only Cooke et al. (2015) measured hydrogen peroxide content in honeys using MQuant test strips, but examined special types of honey. According to our knowledge, there is only one other report on the H_2O_2 content in honeys where MQuant test strips were used (Kerkvliet, 1996). However, author did not show H_2O_2 content in honeys of different botanical origin, but gave the range of detected hydrogen peroxide content in various honey samples. As already mentioned, Bang et al. (2004) detected higher amounts of the produced H_2O_2 in honeys than found in the present study. Apart from the aforementioned possible differences regarding botanical origin of the examined samples and different method for H_2O_2 determination used, the increased level of hydrogen peroxide could also be attributed to the fact that authors used buffered solutions of honey. Namely, it is well known that the GOX activity is pH dependent, showing maximum activity at pH 6.1 (Schepartz and Subers, 1964). Thus, at the optimal pH for the GOX activity, it should be expected that enzyme produces more H_2O_2. Kerkvliet (1996) reported that buffered honey solutions at pH = 6.5 produced twice-higher amount of H_2O_2 than water solutions.

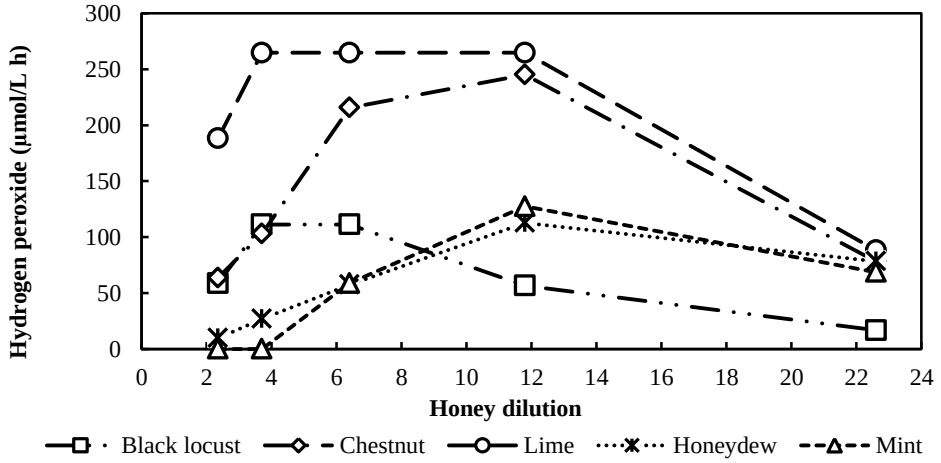

Fig. 1. Hydrogen peroxide accumulation in honeys of the different botanical origin as affected by dilution. Mean values of hydrogen peroxide content for each type of honey of the different botanical origin are shown. Standard deviations were omitted due to the great variability between samples of honey of the same botanical origin.

One of the important factors that clearly affected generation of H_2O_2 in the examined honeys was honey dilution. While black locust honeys exhibited the highest H_2O_2 production ability at dilutions between 3.7 and 6.4 (corresponding to mass to volume ratio of 1:2 to 1:4, or 16 to 27 % of volume to volume ratio), lime honeys generated hydrogen peroxide to the highest possible extent in wider dilution range from 3.7 to 11.8 (corresponding to mass to volume ratio from 1:2 to 1:8, or 8 to 27 % of volume to volume ratio). The optimal dilution for chestnut honeys was found to be between 6.4 and 11.8, mint honeys 11.8, while honeydew honeys exhibited maximum H_2O_2 generation either at 11.8 or 22.6 dilution (corresponding to mass to volume ratio from 1:8 to 1:16, or 4 to 8 % of volume to volume ratio) (Table 4, Fig. 1). The observed data on optimal honey dilutions necessary for maximum H_2O_2 generation are opposite from those reported by Bang et al. (2004), who found a maximal level of hydrogen peroxide in honeys diluted between 30 and 50 % volume to volume ratio, but congruent with reports of Brudzynski (2006) and Brudzynski et al. (2011, 2017), who found that maximal production of H_2O_2 in the examined honeys requires dilutions between 4 to 16-fold what corresponds from 6.25 to 25 % of volume to volume ratio. According to Brudzynski et al. (2017), dilution dependent inverted asymmetrical U-shaped curve of the H_2O_2 production/accumulation in honeys could arise from the fact that honey macromolecules such as proteins (GOX, among others) and polyphenols form colloidal particles in viscous solution of reducing sugars, glucose and fructose. This two-phase state of honey structure seems to be a key determinant for the hydrogen peroxide production. Namely, a rapid decrease in the H_2O_2 production coincides with the phase transition point, when decreased concentration of glucose and fructose upon dilution (that impart viscosity) creates unstable conditions that lead to degradation of colloidal particles. Moreover, chemical composition, specifically concentration of macromolecules such as polyphenols, seems essential for the formation of colloidal particles. Darker honeys of higher polyphenol concentration form micron-size particles and two-phase state conformation that supports the H_2O_2 production, while light honeys with low concentration of polyphenols show the diluted phase conformation not supportive for the H_2O_2 production (Brudzynski et al., 2017). Thus, it seems quite possible that the observed differences in H_2O_2 production ability of the examined honeys upon dilution in our research reflect differences in honey colloidal two-phase state stability.

The most striking fact observed in the present study is the lack of correlation between glucose oxidase activity and hydrogen peroxide concentration in the examined honeys (data not shown). Although chestnut, lime, mint and honeydew honeys showed the same level of the GOX activity (Table 2), they differed in their H_2O_2 production ability (Table 4, Fig. 1). Lime and chestnut honeys produced the highest amount of hydrogen peroxide, while mint and honeydew honeys generated much lower content of H_2O_2 similar to those produced by black locust honeys, which showed at least 10-fold lower GOX activity. Just for illustration, black locust honeys 1, 4 and 5 with the low GOX activity of 18.50, 23.40 and 11.67 μg H_2O_2/h g produced maximally 147.1 μmol/L h of H_2O_2, while honeydew honeys 4, 6, 7, 9-12 with the GOX activity ranging from 317.74 to 525.62 of μg H_2O_2/h g, produced maximally 58.82 or 88.24 μmol/L h. This indicates that the GOX activity does not present a reliable parameter for the prediction of H_2O_2 content in honeys, and *vice versa*. However, it should be noted that the GOX activity and H_2O_2 accumulation were not tested under the same conditions. The GOX activity in the examined honeys was measured at optimal conditions (glucose concentration of 1.5 mol/L, pH of sodium phosphate buffer of 6.1, and optimal temperature of 37 °C) for maximal velocity (V_m) determination (Schepartz and Subers, 1964), while hydrogen peroxide accumulation in water solutions of quite lower pH ranging from 3.51 to 5.85 (Table 3), and at lower glucose concentrations. Based on the average values of glucose content in honeys reported by Persano Oddo and Piro (2004), glucose concentration in water solutions of the examined honeys ranged from approximately 1 mol/L (1:1 mass to volume ratio of honey) to 0.1 mol/L (1:16 mass to volume ratio), what obviously led to the lower enzyme activity and subsequently lower H_2O_2 production. In addition, at the pH range of water honey solutions (Table 3), the GOX activity should be quite lower, in the range from 0 to 20 % of its maximal activity (Schepartz and Subers, 1964), thus producing lower amount of hydrogen peroxide. According to our knowledge, there are no available literature reports that correlate evolved H_2O_2 concentration with the glucose oxidase activity in honey. The only correlation reported is those by Bucekova et al. (2014), who found that the amount of H_2O_2 produced in honey solution could be correlated with the GOX levels. However, in this research authors examined the GOX levels in honey by immunobloting technique using a polyclonal antibody against the GOX, while hydrogen peroxide content in honeys

diluted to 40 % of volume to volume ratio was determined after four hours of incubation at 37 °C.

The actual content of H_2O_2 in honeys presents the net rate of its production by glucose oxidase and its decomposition by the action of catalase, and/or antioxidant action of ascorbic acid, as well as some other endogenous compounds which might act as antioxidants (Brudzynski 2006; Cooke et al., 2015; Kerkvliet, 1996; Kwakman and Zaat 2012; Schepartz and Subers, 1964; White and Subers, 1963). Thus, it seems quite possible that the presence of abovementioned H_2O_2 decomposing molecules in honey caused the lack of correlation between H_2O_2 levels in the examined honeys and the GOX activity. However, the actual activity of catalase or amount of vitamin C were not examined in the present study, so their possible impact on the hydrogen peroxide level can only be supposed on the basis of various literature reports. Catalase is an antioxidant enzyme which decomposes hydrogen peroxide to water and molecular oxygen, and whose presence in honeys could be mainly attributed to the presence of pollen grains (Sánchez et al., 2005). Honeys of various botanical origin were found to possess some catalase activity (Huidobro et al., 1995; Sánchez et al., 2005; Schepartz and Subers, 1966), and negative correlation between its activity and the H_2O_2 accumulation in honeys was reported by Schepartz and Subers (1966). Huidobro et al. (1995) stated that honeydew honeys should exhibit greater catalase activity than chestnut ones, while Schepartz and Subers (1966) found that mint honey does not show catalase activity. Greater activity of catalase in honeydew than in chestnut honeys could explain why honeydew honeys in the present study exhibited much lower H_2O_2 accumulation than chestnut ones (Table 4, Fig. 1), although both types of honey had similar GOX activity (Table 2). However, what is the real effect of catalase on the H_2O_2 decomposition, still remains to be elucidated, especially considering the effect of low pH on the significantly decreased catalase activity (Alptekin et al., 2008). Vitamin C (ascorbic acid) is an antioxidant compound whose concentration in honeys of different botanical origin ranges from zero (Gheldof et al., 2002) up to 4250 mg/kg of honey (Chis et al., 2016; Ciulu et al., 2011; Kadri et al., 2017; Kerkvliet, 1996; León-Ruiz et al., 2011; Perna et al., 2013). While the highest vitamin C concentration was found in thyme honeys (Kerkvliet, 1996; León-Ruiz et al., 2011), black locust honeys were reported to have from 0 to 1.3 ± 0.2 mg/kg (Ciulu et al., 2011; Gheldof et al., 2002), lime honeys less than 0.1 mg/kg (Ciulu et al., 2011), chestnut honeys from 0 up to 200 mg/kg (León-Ruiz et al., 2011; Perna et al., 2013), while honeydew honeys from 0 up to 250 mg/kg (Chis et al., 2016; Kerkvliet, 1996; León-Ruiz et al., 2011). According to Kerkvliet (1996), the amount of vitamin C greater than 150 mg/kg of honey interferes with the peroxide formed, so it seems quite possible that high vitamin C content in honeydew honeys caused lesser amount of the H_2O_2 produced. Some other compounds of honey such as polyphenols could contribute to hydrogen peroxide decomposition through their antioxidant activity. Flanjak et al. (2016b) reported great correlation between polyphenol content in Croatian honeys and its antioxidant capacity determined by FRAP (ferric reducing antioxidant power) assay. However, clear connection between polyphenol antioxidant capacity and hydrogen peroxide accumulation in honey remains to be elucidated.

Conclusion

Investigation of ability of the selected Croatian honeys to accumulate hydrogen peroxide in water solutions upon dilution revealed that the H_2O_2 production in honeys significantly varies between samples of the same botanical origin, among samples of different botanical origin and is dependent on the dilution ratio. In general, lime and chestnut honeys were found as the highest H_2O_2 producers, while black locust, mint and honeydew honeys produced approximately 2-fold lower amount of hydrogen peroxide. Honey dilutions of 8- or 16-fold were found most prominent for the greatest H_2O_2 production. Comparison of glucose oxidase activity and hydrogen peroxide content revealed the lack of correlation between these two parameters, what indicates that glucose oxidase activity does not present a reliable parameter for the prediction of hydrogen peroxide content produced in honey solutions.

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