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The effect of the preparation method on the physical and chemical characteristics of propolis tinctures

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SUMMARY

The effect of the preparation method was examined with regards to the physical and chemical characteristics of the propolis tincture, namely the extraction time and the ethanol content of the extraction solvent to the dry matter, polyphenol, flavonoid, phosphorus, calcium and copper concentration, respectively. The dry matter, the polyphenol and the flavonoid content were the lowest in the water extract of the propolis; however, significant increase was noticed depending on the extraction time. Significantly higher concentrations were found in 50 V/V% tinctures. The highest dry matter and flavonoid contents were analysed in 100 V/V% tinctures, whereas highest polyphenol content was found in 80 V/V% tinctures. However, the differences were not significant in several cases between latter tinctures. Moreover, the increase was not determined in some cases depending on the extraction time. Phosphorus and calcium concentrations were decreased depending on the increasing ethanol content, whereas copper concentration was increased up to 80 V/V%. Higher increase was found in the case of 0 and 50 V/V% extracts than in 80 and 100 V/V% tinctures. Moreover, in latter cases, no significant differences were found on several occasions, depending on the extraction time. There was no connection between the flavonoids and the calcium as well as the phosphorus content, whereas flavonoids may be made complex with copper. However, the amount of the possible complex was negligible.

Keywords*: propolis, tincture, polyphenol, flavonoid, elemental content*

INTRODUCTION

Propolis or bee glue is a product of *Apis mellifera* L bees, which is collected from buds, leaves and bark of trees and processed by them (Valencia et al. 2012). In particular, it is used as an adhesive to cover the walls and fill the gaps in the hive. Moreover, antimicrobial effects are also substantial, additionally it used to embalm killed invaders which they cannot transport out from the hive (Halmágyi and Keresztesi 1991). Propolis has several beneficial biological effects which have been used in folk medicine for thousands of years, but nowadays is again an increasingly popular product (Castaldo and Capasso 2002). We can meet propolis in nutritional supplements, ointments, toothpastes or mixed with honey, but maybe the alcoholic extract of raw propolis is the most popular, namely the tincture (Net1). Tincture can be a home-made product, however there is no unified recipe for the process. There are possible methods on the internet (Net2, Net3, Net4), which offer different ethanol concentrations and extraction time, so they give free-hand to the makers. The methods are broadly agreed with some steps. The chopped raw propolis should extract by 5 times higher solution, 60–95 V/V% ethanol in most of the cases. The solution with the raw propolis should mix 1 or 2 times per day till 2–3 weeks. The process is finished when the solution is browned and the raw propolis become bright. After this, solution should sieved or the supernatant should be removed.

This paper highlights effect of the different processing methods on the physical and chemical parameters of the propolis tincture. The time of the extraction (1 hour, 1 day, 1 week, 1 month) and the ethanol content of the extraction solvent (0, 50, 80, 100 V/V%) is considered into dry matter, total phenolic and flavonoid contents, as well as calcium, phosphorus and copper concentrations of the tinctures.

MATERIALS AND METHODS

High purity deionized water (18.2 MΩ cm) was used from Milli-Q system (Bedford, MA, USA). Calibration standard is made from 1000 mg/l monoelemental standards (Scharlau, Barcelona, Spain). Analytical grade nitric acid (65%) and hydrogen-peroxide (30%) were from Scharlab S.L. (Sentmenat, Spain). Tinctures were made from absolute ethanol (≥99.8 V/V%) (VWR International, Fontenay-sous-Bois, France).

Preparation of propolis tinctures

Raw propolis were mixed from about 30 samples in equal ratio collected from Hungarian counties in 2014. The mixture represents an average propolis sample of Hungary. Approximately 0.5 g homogenized propolis were weighted into centrifuge tubes. Then 5 ml extraction solvent was added to the propolis, 0, 50, 80 and 100 V/V% by ethanol, respectively. The centrifuge tubes were mixed with Vortex and repeated every day. After the extraction periods (1 hour, 1 day, 1 week and 1 month) were mixed again and centrifuged at 1600 g,

10 min, and then the supernatant was filtered in Filtrak 388 paper (Sartorius Stedim Biotech S.A., Gottingen, Germany). Tinctures were kept in a dark place at room temperature till measurement.

Digestion of the tinctures

The details of the sample preparation were described elsewhere (Soós et al. 2017). Briefly, 2–2 ml tinctures were weighted into quartz tubes. Samples were dried in an oven at 45 °C until constant mass. Samples were digested by microwave digestion. Then 2 ml HNO_3 were added into dried samples and kept overnight. Next day 0.6 ml H_2O_2 was added and the tubes were closed with Teflon tapes. Closed tubes were placed into Teflon vessels and digested by the followings: heating up to 180 °C in 15 min, kept at 180 °C in 20 min. Digested solutions were washed into plastic tubes and filled up to 10 ml.

Elemental analysis

The elemental analysis was made by Thermo Scientific iCAP 6300 inductively coupled plasma optical emission spectrometry (ICP-OES) and Thermo Scientific X-Series II inductively coupled plasma mass spectrometry (ICP-MS) (Thermo Scientific, Bremen, Germany). The main parameters were the following:

ICP-OES: RF power 1350 W, plasma gas flow rate 12.0 l min^{-1}, sample gas flow rate 1.00 l min^{-1}, auxiliary gas flow rate 1.00 l min^{-1}, peristaltic pump speed 50 rpm, plasma view axial, integration time 10 sec. Concentric nebulizer was used with cyclonic spray chamber. Analyzed elements and their wavelengths (nm) were the following: Ca (315.887), P (213.618).

ICP-MS: RF power 1400 W, plasma gas flow rate 14.0 l min^{-1}, sample gas flow rate 0.86 l min^{-1}, auxiliary gas flow rate 0.88 l min^{-1}, pole bias -16.0, hexapole bias -10.0, dwell time 100 ms, sweep 9, main run 3, peristaltic pump flow rate 20 rpm. CCT gas was the mixture of H_2-He in 7:93 ratios, the CCT gas flow rate was 6.00 ml min^{-1}. Concentric nebulizer was used with Peltier cooled (2 °C), spray chamber. Copper was analyzed in ^{65}Cu isotope.

Detailed information about the performance indicators are described elsewhere (Soós et al. 2017).

Dry matter analysis

2–2 ml tincture was weighted into quartz tubes. Samples were dried in an oven at 45 °C until constant mass. Dry matter content (m/V%) was calculated by the followings: (mass of the dried sample and the tube – mass of the tube)*100/pipetted volume.

Polyphenol analysis

Polyphenol analysis was made by Folin-Ciocalteu method (Singleton et al. 1999) with minor modification. Samples were diluted with 80 V/V% ethanol by the followings: 10 times dilution from 0 V/V%, 50 times dilution from 50 V/V% and 250 times dilution from 80 and 100 V/V% tinctures, respectively. Samples were diluted from filtered solutions, so they were not filtered again. From the diluted samples 0.5 ml were pipetted into tubes and 2.5 ml 0.2 N Folin-Ciocalteau reagent (Scharlab SL, Sentmenat, Spain), after 5 min 2 ml 75 g l^{-1} Na_2CO_3 (Scharlab SL, Sentmenat, Spain) were added to the tubes and mixed well. Samples were kept at room temperature in 2 hours. Calibration standards were made from gallic acid standard (Scharlab SL, Sentmenat, Spain) as the same way, between 0 and 200 mg l^{-1}. Absorbance was measured by Nicolet Evolution 300 (Thermo Electron Corporation, Madison, WI USA) spectrophotometer in 10 mm cuvettes at 760 nm. Results were expressed in gallic acid equivalent (mg GAE l^{-1}).

Flavonoid analysis

The flavonoid content was analyzed with the method of Zhishen et al. (1999) with minor modification. From the aqueous tinctures 10 times dilution, from the other tinctures 50 times dilution was made by 80 V/V% ethanol. Samples were diluted from filtered solutions, so they were not filtered again. From the diluted samples 1–1 ml were added into plastic tubes and 4 ml 80 V/V% ethanol were added to it. After it 0.3 ml 5% $NaNO_2$ (VWR International, Leuven, Belgium), 5 min later 0.3 ml 10% $AlCl_3$ (VWR International, Debrecen, Hungary), 1 minute later 2 ml 1 M NaOH (Sigma Aldrich, St. Louis, USA) were added into the samples. All the solutions were completed up to 10 ml with 80 V/V% ethanol. The absorbance was measured by Nicolet Evolution 300 (Thermo Electron Corporation, Madison, WI USA) spectrophotometer in 10 mm cuvettes at 510 nm compared to catechin solutions (TCI Development Co, Shanghai, China). Results were expressed in catechin equivalent (mg CE l^{-1}).

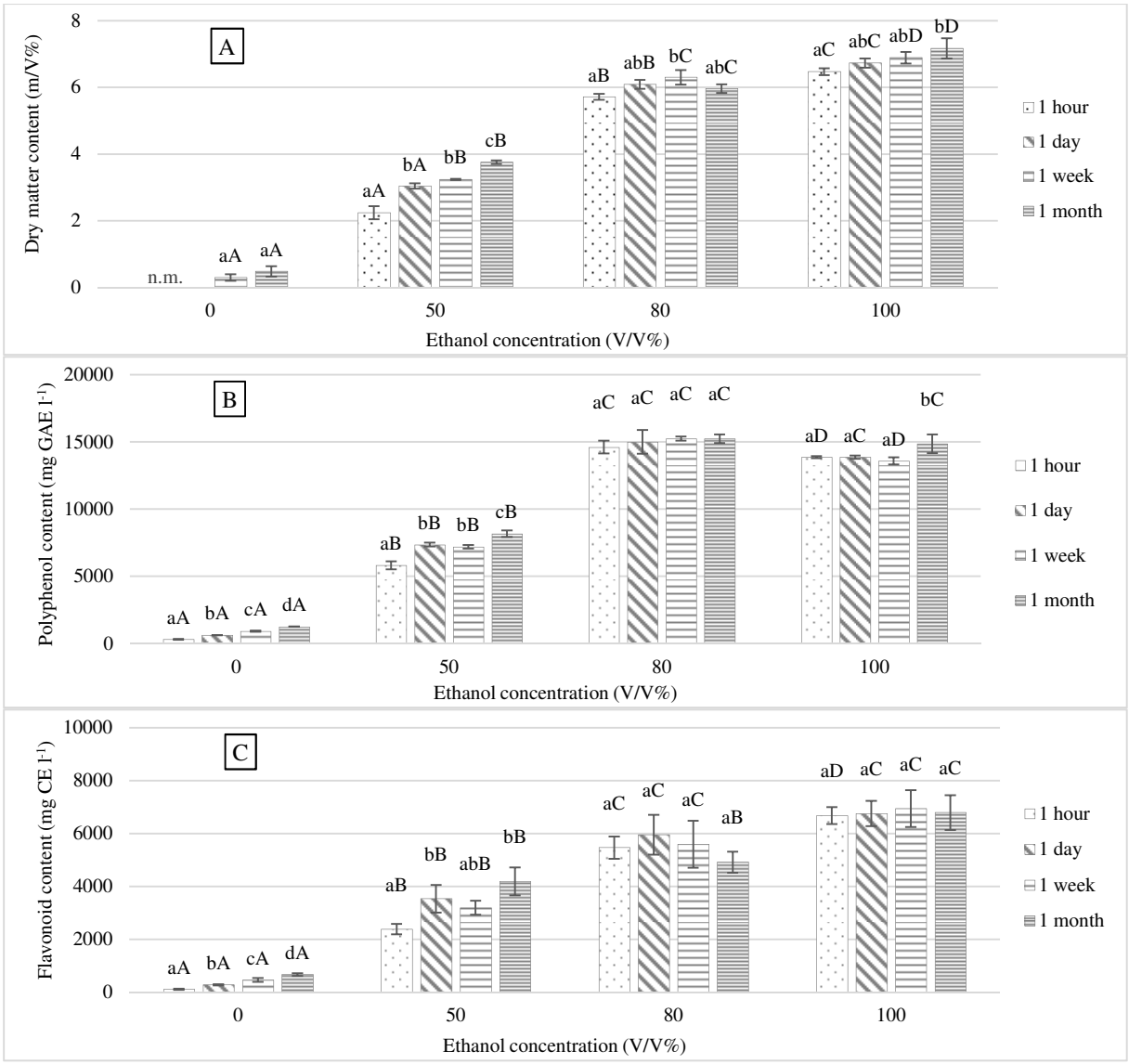
RESULTS AND DISCUSSION

Dry matter, polyphenol and flavonoid content in tinctures

The dry matter, polyphenol and flavonoid contents of the propolis tinctures are shown in *Figure 1*. It is observed, that the tendency of the results are similar. The lowest concentrations of all the parameters were found in aqueous extracts. The dry matter content was below 1 m/V%, moreover the content was below the measurable limit (0.1 m/V%) after 1 hour and 1 day. However an increasing tendency was found depending on the time of the extraction. The highest concentration was 0.49 m/V% after 1 month. The polyphenol and flavonoid contents were also the lowest in aqueous solutions, however a significant increasing tendency was found depending on the time in all the two

aforementioned parameters. The concentration of the polyphenols after 1 month was 1260 mg GAE l^{-1}, and the flavonoid content was 679 mg CE l^{-1}, respectively. The second lowest concentrations of these parameters were found in the 50 V/V% solutions. Although we found an increasing tendency, but it was not significant in all the cases. In the case of polyphenols and dry matter the difference was not significant between 1 hour and 1 day, moreover in case of flavonoid was an increasing after 1 day and a small decreasing after 1 week. After 1 month an increasing was observed again, although it was not significant compared to the extract after 1 day. The dry matter contents were between 2.25 and 3.75 m/V%, the polyphenol contents were between 5820 and 8160 mg GAE l^{-1} and the flavonoid content was between 2390 and 4200 mg CE l^{-1} in 50 V/V% tinctures, respectively.

Figure 1: **Dry matter (A), polyphenol (B) and flavonoid (C) content of the propolis tinctures depending on the ethanol content of the extraction solvent and the extraction time**



Note 1: different small letters mean the significant differences ($p<0.05$) between different extraction solvents. Capitals mean significant differences ($p<0.05$) between different ethanol content of the extraction solvent. Note 2: n.m.: not measurable.

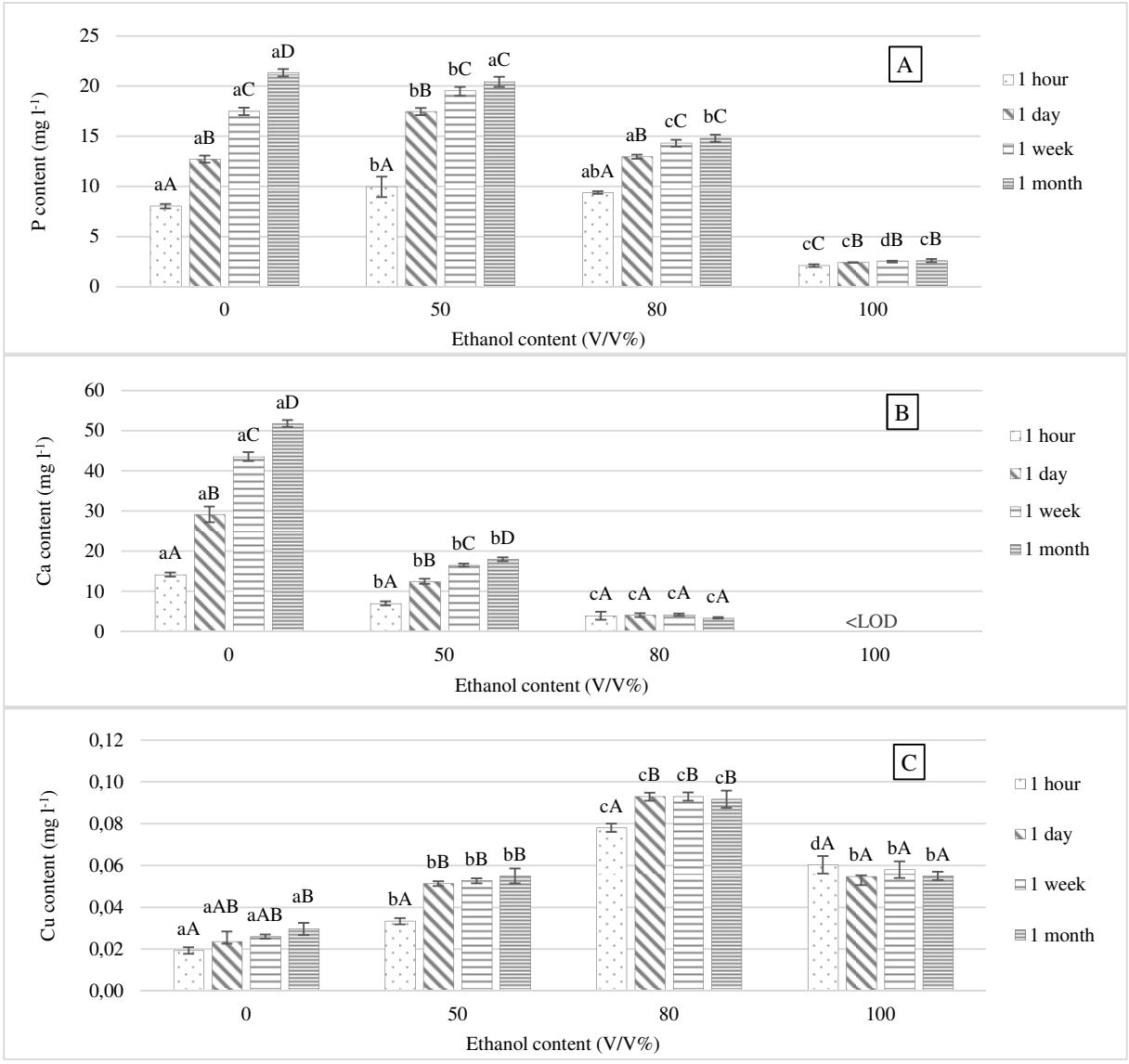
The second highest results were found in 80 V/V% extracts in case of dry mass and flavonoid content, whereas the second highest results were found in 100 V/V% extracts in case of polyphenols. Although results were not significantly different in all cases, or if they were significant, difference was small. There was no significant difference in case of flavonoids by the extraction time in 80 and 100 V/V% extracts and in polyphenol content in 80 V/V% tinctures. Low, but significant increase was found in polyphenol and dry matter content in 100 V/V% tinctures, as well as in dry matter in 80 V/V% extracts, except after 1 month

a decrease was found. Dry masses were between 5.72 and 7.17 m/V%, polyphenols were between 13 600 and 15 200 mg GAE l^{-1} and flavonoid contents were between 4920 and 6940 mg CE l^{-1} in 80 and 100 V/V% tinctures.

The elemental content in tinctures

The P, Ca and Cu contents of the propolis tinctures were shown in *Figure 2*. The concentration of the P was the highest in 0 V/V% extract after 1 month with 21.3 mg l^{-1}, however there was no significant difference compared to 50 V/V% tincture in the same time. Significant increasing by the extraction time were found in 0, 50 and 80 V/V% tinctures, respectively. There was no tendency in the extract of 100 V/V% depending on time, just after 1 week was significantly higher concentration than before, but it was decreased after 1 month. The lowest concentrations were in 100 V/V% tinctures between 2.12 and 2.61 mg l^{-1}, respectively.

Figure 2: **P (A), Ca (B) and Cu (C) content of the propolis tinctures depending on the ethanol content of the extraction solvent and the extraction time**



Note 1: different small letters mean the significant differences ($p<0.05$) between different extraction solvents. Capitals mean significant differences ($p<0.05$) between different ethanol content of the extraction solvent. Note 2: <LOD: under the limit of detection.

The Ca dissolved in the highest quantity in every extraction times in the aqueous extracts. There was also an increasing tendency depending on the extraction time in 0 and 50 V/V% extracts. The highest concentration was 51.8 mg l^{-1} in the aqueous extracts. However a significant difference was occurred compared

to 50 V/V% solution, reduced up to 18.0 mg l^{-1} concentration.

Cu was not dissolved in the highest quantity as well as the other elements in 0 V/V% solutions. The increasing ethanol content increased the Cu content up to 80 V/V%, then the concentrations in the 100 V/V% tinctures were reduced to 50 V/V% extracts, except in the extract after 1 hour. As it was shown, there was lower difference depending on the time, compared to the Ca or the P.

The connection between the flavonoids and the elements

Previous articles highlights that flavonoids can make complexes with metals, such as with Fe^{2+} and Cu^{+} ions, respectively (Havsteen 2002). The physiological effect of the flavonoid-metal complex may like the original flavonoid, usually has great stability, but the toxicity of the metal is significantly decrease. The complex making of the flavonons and flavonols are often studied with Cu(II), Fe(III), Zn(II), Ni(II), moreover other ions (Selvaraj et al. 2014).

Strong connection was found between the elements and the flavonoids in the 0 V/V% extracts as shown in *Table 1*. It shows the molar concentrations of the water soluble flavonoids and the element concentrations in aqueous extracts. The molar concentration of the flavonoids were calculated by using 293 g mol^{-1} molar mass (Gates and Lopes 2012). The concentration of the flavonoids were significantly increased in accordance with the element contents. The molar concentration of Ca and P (mmol l^{-1}) were in the same order of magnitude as flavonoids. However the concentration of Cu (µmol l^{-1}) was lower in 3 order of magnitude in any extraction times. The increasing tendency in the aqueous extracts can be explained by the concentration equalization of the elements and the flavonoids between the raw propolis and the extraction solvent depending on the time, which is connected with Fick's law of diffusion (Fonyó and Fábry 2004).

Table 1

The mean concentration of the flavonoid, Ca, P and Cu contents in the water extract (0 V/V%) of propolis

Extraction time	Molar concentration of the components in water extract of propolis			
	Total flavonoid (mmol l^{-1})	Ca (mmol l^{-1})	P (mmol l^{-1})	Cu (µmol l^{-1})
1 hour	0.396	0.354	0.259	0.304
1 day	0.987	0.729	0.410	0.373
1 week	1.610	1.090	0.564	0.409
1 month	2.320	1.300	0.688	0.467

The connection of the flavonoids and the elements were evaluated by Pearson correlation. The Pearson correlation is measuring the amount and the trend of linear connection between two variables. If the correlation coefficient is more than 0.7, there is a strong positive linear correlation between the two parameters (Mitev 2003). The Pearson correlation coefficients depending on the ethanol content of the extraction solvent are summarized in *Table 2*.

The correlation coefficients were greater than 0.98 in the aqueous extracts, moreover is significant in case of P and Cu ($p<0.01$), as well as Ca ($p<0.05$). There is greater than 0.8 correlation coefficient in 50 V/V% tinctures, however is not significant ($p>0.05$). The Ca has a correlation above 0.7, however P has no correlation in 80 V/V% tinctures. On the other hand, P has positive correlation in 100 V/V% tinctures, but correlation cannot be calculated in case of Ca, because it was under the limit of detection.

Table 2

Pearson correlation coefficient between flavonoid and elemental content depending on the ethanol content of the extraction solvent (*p<0.05, **p<0.01)

Ethanol content of the extraction solvent (V/V%)	Pearson correlation coefficient between flavonoid and element content		
	P	Ca	Cu
0	0.996**	0.986*	0.992**
50	0.872	0.848	0.888
80	-0.262	0.876	0.106
100	0.782	n.c.	-0.171

Note: n.c.: not calculable.

The connection depending on the extraction time is also evaluated and summarized in *Table 3.* It is shown that parallel with increasing concentration of flavonoids there was a decreasing concentration of P and Ca. Positive correlation was found only in case of Cu. Although it was not significant but correlation coefficient was above 0.7, which shows a strong positive linear connection, except extraction after 1 month.

Table 3

Pearson correlation coefficient between flavonoid and elemental content depending on the ethanol content of the extraction solvent (*p<0.05, **p<0.01)

Extraction time	Pearson correlation coefficient between flavonoid and element content		
	P	Ca	Cu
1 hour	-0.555	-0.951	0.899
1 day	-0.531	-0.995	0.769
1 week	-0.770	-0.985	0.737
1 month	-0.826	-0.991	0.593

CONCLUSION

The increasing ethanol content increased the dry matter and flavonoid content up to 100 V/V% and the

polyphenol content up to 80 V/V%. Because flavonoids have positive beneficial effects on the human health it is favourable to use high concentration of ethanol for the extraction of propolis. On the other hand the higher ethanol content decreased the content of P and Ca, while increased the Cu content up to 80 V/V%. There was also an increasing tendency depending on the time of the extraction in particular in 0 and 50 V/V% extracts. On the other hand there was no notable increasing in 80 and 100 V/V% tinctures. As a consequence, an extraction up to 1 hour can extract most of the polyphenols and flavonoids from the raw propolis.

There was no significant correlation between the flavonoids and the elements in all the cases depending on the two agents and all the treatments. Because Cu had strong correlation depending on the extraction time and the ethanol content, therefore the Cu was the most probable complex with flavonoids from the measured elements. We compared the molar concentration of the Cu and the flavonoids. The concentration of the flavonoids was 6680 mg l^{-1} in the 100 V/V% tincture after 1 month, therefore the molar concentration was 22.7 mmol l^{-1}, calculated by the average molar mass. The molar concentration of the Cu was 0.0012 mmol l^{-1}, in the same solution. This means that the flavonoid content was more than ten thousand times higher than the copper. So if the copper made complex with flavonoids in the propolis, the concentration was negligible compared to the total flavonoid content.

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