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ANTIOXIDANT PROPERTIES OF FLAVONOIDS AND HONEYS STUDIED BY OPTICAL SPECTROSCOPY METHODS

BADANIE WŁAŚCIWOŚCI ANTYOKSYDACYJNYCH FLAWONOIDÓW ORAZ MIODÓW METODAMI SPEKTROSKOPII OPTYCZNEJ

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S u m m a r y

Flavonoids are considered very efficient radical scavengers and found in almost every plant. Antioxidant activities of flavonoids and a few popular kinds of bee honeys were investigated using absorbance spectroscopy. Spectral methods are powerful tools for the study of biologically active compounds and allow measurements of low concentrated substances under physiological conditions. Quercetin, naringenin, catechin, 3-hydroxyflavone were studied during the reaction with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·). The results suggest that quercetin and catechin are better antioxidants than vitamin C. Among the analyzed honeys buckwheat honey exhibited the strongest antioxidant properties. Kinetic studies of the radical-scavenging activity were also performed. One can observe that popular vitamin C reduces free radicals instantly after adding to the solution, while flavonoids cause rather an exponential decay in concentration of the free radicals in the solutions. Our results show that adding of $CuCl_2$ to the solution of DPPH and quercetin did not influence the level of antioxidant capacity of quercetin.

S t r e s z c z e n i e

Flawonoidy uważane są za bardzo skuteczne wymiatacze wolnych rodników i występują niemal w każdej roślinie. Właściwości antyoksydacyjne flawonoidów oraz kilku najbardziej popularnych rodzajów miodów pszczelich zbadano metodami spektroskopii absorpcyjnej. Metody optyczne są przydatnym narzędziem do badania związków biologicznie czynnych i umożliwiają pomiary w niskich stężeniach badanych substancji oraz w warunkach fizjologicznych. Kwercetynę, naringeninę, katechinę, 3-hydroksyflawon badano w reakcji z rodnikiem 2,2-diphenyl-1-picrylhydrazyl (DPPH•). Wyniki pokazują, że kwercetyna oraz katechina są lepszymi przeciwutleniaczami niż witamina C. Spośród analizowanych miodów miód gryczany wykazuje najsilniejsze właściwości antyoksydacyjne. Przeprowadzono także badania kinetyki wymiatania wolnych rodników. Zaobserwowano, że popularna witamina C wymiata wolne rodniki niemal natychmiast po dodaniu do roztworu DPPH, zaś flawonoidy raczej wywołują wykładniczy zanik stężenia wolnych rodników. Nasze wyniki wskazują także, że dodanie $CuCl_2$ do roztworu DPPH i kwercetyny nie wpływa znacząco na zdolności przeciwutleniające kwercetyny.

Key words: flavonoids, bee honey, DPPH
Słowa kluczowe: flawonoidy, miód pszczeli, DPPH

INTRODUCTION

Free radicals are particles or ions that have an unpaired electron. In the human body, as a result of metabolic processes, reactive oxygen species (ROS) arise.

The ROS include superoxide anions ($O^{\cdot}{}_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) [1]. Free radicals attack cell membranes, proteins,

unsaturated fatty acids and lipids causing oxidative stress. This leads to the destruction of cells and atherosclerosis [2]. Free radicals interact with DNA leading to tumour formation [3].

Antioxidants are substances that may protect other molecules against the effects of free radicals. The ROS level in the body is controlled by antioxidative enzymes [4]. Situation may change after 40 years of age when defense mechanisms are less effective [5]. Besides, as a result of environmental stress (environmental pollution, UV radiation, magnetic field, the use of chemicals in food production, cigarette smoke, etc.), ROS level can increase dramatically. Therefore, it is important to enhance the antioxidant mechanism. It seems that one of the most promising antioxidant substances are flavonoids. They are found mainly in fruit and vegetables [6, 7]. Antioxidant properties of flavonoids are probably related to their polyphenolic structure [7]. Vitamin C is one of the most powerful and well-known antioxidants as an electron donor. The supplements containing Vitamin C are extremely popular although it is found in fresh fruit and vegetables. The structures of studied flavonoids and ascorbic acid are presented in Fig.1. One of the sources of natural flavonoids is honey bee. In literature one can find a lot of information about the medicinal properties of honey [8].

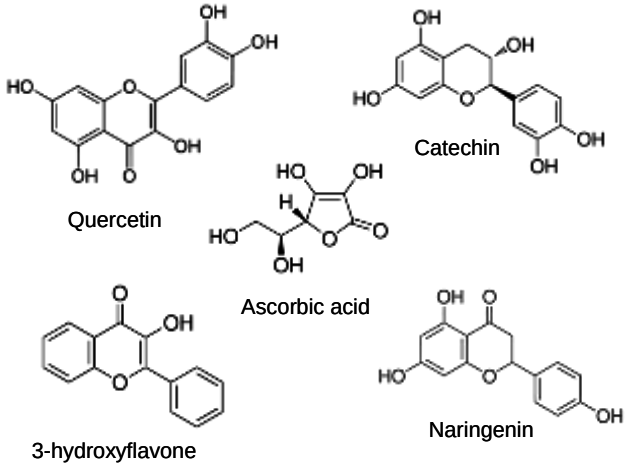

Fig. 1. *Structures of antioxidants in this study*
Rys. 1. *Struktura badanych antyoksydantów*

In the present study the antioxidant properties of selected flavonoids, ascorbic acid and honeys are investigated using absorption spectroscopy. A good marker of antioxidant capacities of studied substances is free DPPH radical (2,2-diphenyl-1-picrylhydrazyl) [9]. The antioxidants as a source of hydrogen and electron convert DPPH into hydrazine. Antioxidant properties of flavonoids can be estimated on the basis of determination DPPH concentration after adding antioxidant. DPPH shows an absorbance maximum at 517 nm which disappears upon reduction by an antiradical compound.

MATERIALS AND METHODS

Quercetin, naringenin, catechin, 3-hydroxyflavone, ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) were purchased from Sigma Aldrich (St. Louis, USA). A solution of DPPH was prepared by dissolving 5mg DPPH in 2ml of methanol and the solution was stored in the dark at 4°C. 5mM stock solution of flavonoids and ascorbic acid were prepared in 96% ethanol (POCh S.A) and kept at 4°C protected from light. The suitable amount of the above substances was rapid added to DPPH solution (0.1mM) and the absorbance was recorded. The value of used concentration of flavonoids (1-700μM) and ascorbic acid (1-16.6μM) depended on the capability to scavenge the DPPH radical. The 2010 year honey samples were delivered by experienced beekeepers. The stock solutions of all honeys were prepared by dissolving 2 mg of each honey in distilled water. Copper (II) chloride was purchased from POCh S.A and dissolved in distilled water to prepare 5mM stock solution. To examine the impact of copper (II) on antioxidant properties of quercetin, solution of quercetin (1-15μM) and the same amount of $CuCl_2$ were added to a solution of DPPH (0.1mM) in methanol.

To show the antioxidant properties of the studied substances one can use their ability to free radicals destroying. The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) solution after receive an electron and hydrogen from antioxidant changes color from purple to yellow [9]. The absorbance was measured at a wavelength of 517 nm and observed in time. Absorbance spectra of DPPH were recorder 10 minutes after adding quercetin into the DPPH solution. The capability to scavenge the DPPH radical as a function of antioxidants concentration was determined after 2 hours. Kinetic studies of the radical-scavenging activity of quercetin and other components were being performed for 90 min.

The capability to scavenge the DPPH radical (A%) was calculated using following equation:

$$A\% = \frac{A_0 - A_A}{A_0} 100\%,$$

where A_A means absorbance of the studied sample and A_0 - absorbance of the control sample. The parameter IC_{50} which means a concentration of antioxidant substance causing 50% decrease in the concentration of free radicals was also determined. The study was performed in a methanol medium. IC_{50} parameters were calculated from linear fitting of the capability to scavenge the DPPH radical as a function of antioxidants concentration. The number of points was equal to 5. Correlation coefficients and standard error of slopes and intercepts were calculated with the ORIGIN 7.0. Then standard error of IC_{50} was determined. The absorbance of the mixture was being recorded at 517 nm against a second cuvette with a blank solution of DPPH. Absorption measurements were performed using spectrophotometer UV-VIS Jasco 550. The sample temperature was 20°C.

RESULTS AND DISCUSSION

The Figure 2 shows the absorbance spectra of DPPH• solutions – free of quercetin and containing quercetin with different concentration. Absorbance measurements were made 10 minutes after adding quercetin to the solution.

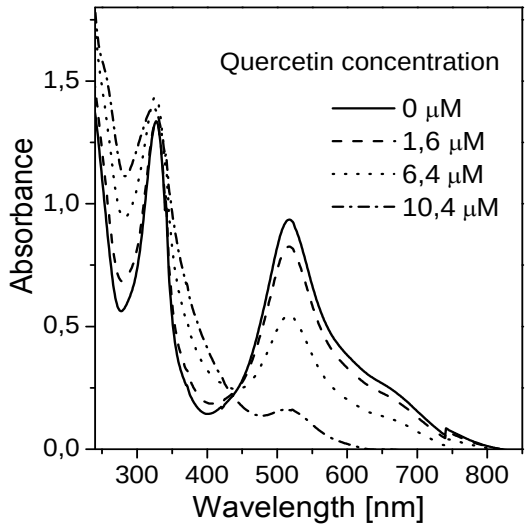

Fig. 2. *Absorption spectra of DPPH in presence of quercetin with various concentrations*

Ryc. 2. *Widma absorpcji DPPH w obecności kwercetyny o różnych stężeniach*

As it shows Figure 2. DPPH radical has two characteristic absorption bands: first with the maximum at 325 nm and second with maximum at 517 nm. After adding quercetin the second band decreases. The quercetin donates hydrogen or electron to the DPPH radical. In this way DPPH becomes non-reactive molecule. To monitor changes in radical concentrations the changes in intensity of 517 nm band during 90 minutes were measured. Then the percentage of DPPH scavenging is calculated and plotted versus time for 5 different concentration of quercetin. Obtained results are presented in Figure 3.

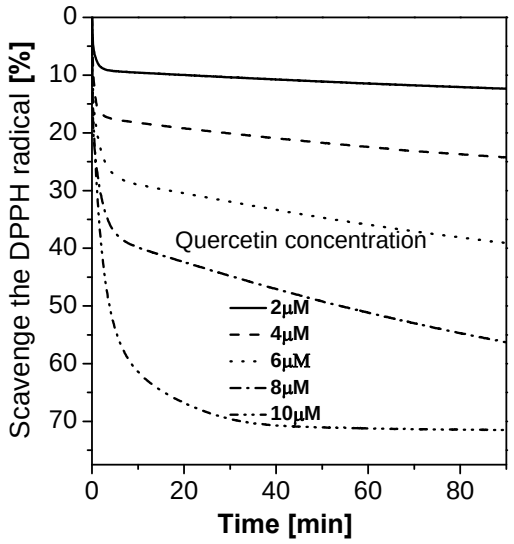

Fig. 3. *The capability to scavenge the DPPH radical by quercetin at various concentrations as a function of time*

Ryc. 3. *Zdolność wymiatania rodnika DPPH przez kwercetynę o różnym stężeniu w funkcji czasu*

There are two constants of the decay rate of absorbance. At the beginning rapid exponential decay of radical concentration is observed, later changes are rather slow and proportional to the time. First step is related with abstraction of two hydrogen atoms from quercetin until the conversion of quercetin into quinine. Second step leads to degradation of quercetin [9]. Initial rate of the decrease depends on quercetin concentration. With increasing quercetin concentration the initial fast phase of absorbance decay is getting longer.

Figures 4 A and B show concentration dependence of the DPPH reduction for selected antioxidant substances. Absorbance measurements were performed 2 hours after adding a substance to the solution. One can see from the figures that 3-hydroxyflavon and naringenin are weak antioxidants. The strongest capacity for free radicals scavenging has catechin.

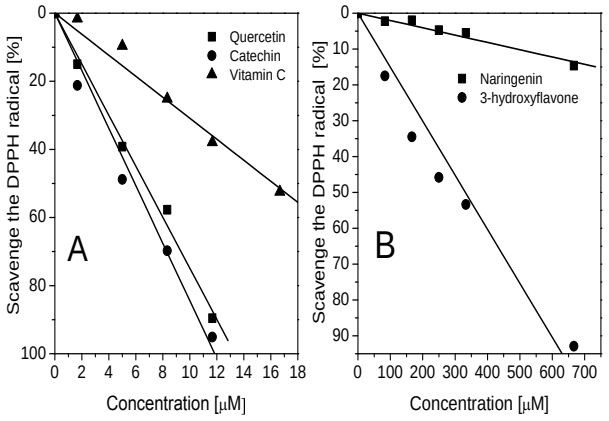

Fig. 4. *The capability to scavenge the DPPH radical as a function of antioxidants concentration*

Ryc. 4. *Zdolność wymiatania rodnika DPPH w funkcji stężenia antyoksydantów*

Parameter IC_{50} that determine the concentration of antioxidant needed to decrease the initial concentration of radicals by half, are summarized in Table 1. From results collected in Table 1 it follows that catechin and quercetin have big capability to scavenge the DPPH radicals. IC_{50} equals 5.8μM and 6.7μM, respectively for catechin and quercetin. Vitamin C has also big capability to scavenge DPPH radicals – its IC_{50} equals 16μM. 3-hydroxyflavone and naringenin exhibit the moderate capability to scavenge DPPH radicals – their IC50 equals 285μM and 2500μM, respectively. Calculated values of IC_{50} suggest that not only the number of hydroxyl group but also their position might be important for antioxidant properties. 3-hydroxyflavon has only one hydroxyl group but is better antioxidant in comparison to naringenin which has 3 groups. One can conclude that the values of IC_{50} parameter determined in this paper are comparable to that one determined by other methods [10-12].

Table. 1. Concentration of antioxidants causing 50% decrease in DPPH concentration (IC_{50})

Tabela. 1. Stężenia antyoksydantów wywołujące 50% spadek stężenia rodnika DPPH

Antioxidant Antyoksydant	Vitamin C Witamina C	Quercetin Kwercetyna	Catechin Katechina	3-hydroxy-flavone 3-hydro-ksyflawon	Naringenin Naringenina
IC_{50} [μM]	16±0.75	6.7±0.17	5.8±0.24	385±19	2500±151

Figure 5 shows the changes of DPPH reduction for 4 selected flavonoids and vitamin C obtained during 90 minutes. In order to illustrate the data on one graph the concentration of 3-hydroxyflavon and naringenin was 10 times higher.

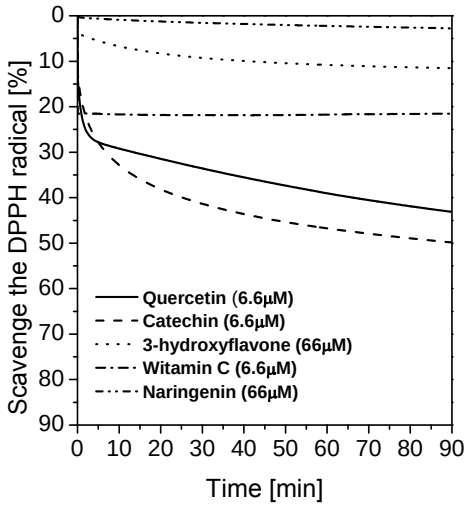

Fig. 5. *The capability to scavenge the DPPH radical by different antioxidant compounds as a function of time*

Ryc. 5. *Zdolność wymiatania rodnika DPPH przez wybrane antyoksydanty w funkcji czasu*

The absorbance at 517 nm decreases over time significantly after addition of antioxidant substances. The mechanism of free radical scavenging by ascorbic acid is different than in case of flavonoids. The hydrogen and electron are transferred from ascorbic acid to the radical form of DPPH almost immediately. Thus it is likely that the administration of synthetic vitamin C can cause the inactivation of free radicals in the gastrointestinal tract only. A better solution seems to be the administration of flavonoids. The antioxidant properties of flavonoids are preserved much longer allowing to their absorption into the bloodstream.

The impact of copper (II) on the free radicals reducing properties was also examined. Dependence of the DPPH scavenging on the concentration of quercetin in solution with and without copper is shown in Fig. 6.

The ratio of copper and quercetin concentrations in all samples was the same. Many publications show that quercetin forms an unstable complex with Cu (II) and it leads to pro-oxidant character of quercetin. The mechanism is related to reduce Cu (II) with the formation of Cu (I) and semioxidized quercetin [13]. This way an electron is transferred from quercetin to copper. Our results show that quercetin in the presence of free radicals and Cu (II) cannot be pro-oxidant. It is seen in Figure 6 that the presence of copper does not

significantly reduce the ability of quercetin to free radical DPPH scavenging.

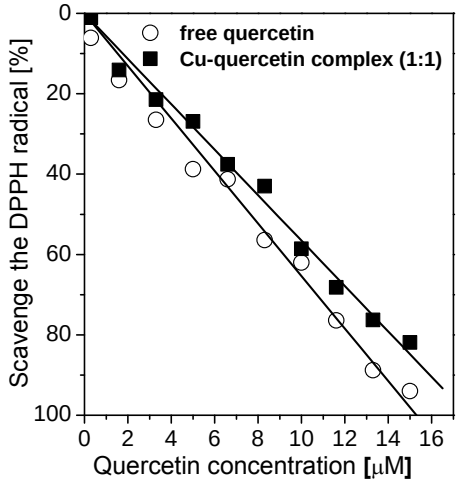

Fig. 6. *The capability to scavenge the DPPH radical as a function of quercetin concentration in the absence and presence of Cu. The Cu/quercetin molar ratios were: 1:1*

Ryc. 6. *Zdolność wymiatania rodnika DPPH w funkcji stężenia kwercetyny w nieobecności i obecności jonów Cu*

The ability to the free radicals scavenging of four popular types of honeys bee was measured using the DPPH method. Honey contains many kinds of flavonoids such as myricetin, tricetin, quercetin, luteolin, quercetin-3-methyl ether, kaempferol, pinocembrin, chrysin, pinobankins, vitexin and many others [14]. The selected honeys were: multi-floral, rapeseed, linden and buckwheat honey. The obtained results are shown in Figure 7.

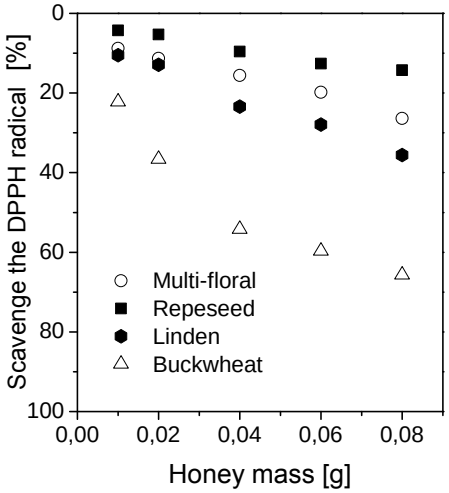

Fig. 7. *The capability to scavenge the DPPH radical as a function of honey mass*

Ryc. 7. *Zdolność wymiatania rodnika DPPH w funkcji masy miodu*

Buckwheat honey has demonstrated the strongest antioxidant capacity. This honey has a little over 2 times more antioxidant properties than rapeseed honey. Linden honey has slightly better antioxidant properties than rapeseed honey and multi-floral honey. Antioxidant properties of honey depend on two factors: the quantity of contained flavonoids and their types. The amount of phenolic compounds in dark honeys is higher than in bright honeys. The buckwheat honey contains flavonoids similar to those reported in honeys from other floral sources but their amount is greater [14].

CONCLUSION

It was confirmed that flavonoids have the ability to capture free radicals. The presented methods of absorption analysis are fast, inexpensive and reproducible. From among the tested substances strongest antioxidant was catechin. It is interesting that ascorbic acid, unlike the flavonoids scavenged DPPH free radicals immediately. The obtained results suggest that administration of copper does not significantly affect the properties of quercetin to reduce free radicals. Among the studied honeys greatest ability to neutralize free radicals have the buckwheat honey.

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