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Potravinarstvo Slovak Journal of Food Sciences
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Slovak Journal of Food Sciences

Potravinarstvo Slovak Journal of Food Sciences
vol. 11, 2017, no. 1, p. 480-488
doi: https://dx.doi.org/10.5219/786
Received: 19 March 2017. Accepted: 17 May 2017.
Available online: 14 July 2017 at www.potravinarstvo.com

ISSN 1337-0960 (online)

APPROACHES TO THE DETERMINATION OF ANTIOXIDANT ACTIVITY OF EXTRACTS FROM BEE BREAD AND SAFFLOWER LEAVES AND FLOWERS

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ABSTRACT

The main objective of this study was to develop approaches for the determination of total antioxidant activity of natural products (bee bread and safflower extracts) using DPPH radical scavenging assay. Considering that analytical procedures and results related to this assay and reported by many authors are significally differed between each other and depend on many factors (the nature of tested extracts, the nature of solvents for extraction, a reaction time of DPPH with a sample, DPPH solvents and concentration, ratio between DPPH and an extract, etc.), the methodology of the evaluation of antioxidant capacity of different origin extracts by DPPH radical scavenging assay was developed. Ascorbic acid (AA) was used as standard antioxidant and the correlation between the percentage of DPPH scavenging and AA concentration was determined at two different initial absorbances of DPPH solution. Average concentration of AA which inhibited 50% of DPPH radicals (IC_{50}) was equal to 156.0 – 171.26 $\mu g.mL^{-1}$. The reaction kinetics of DPPH inhibition by bee bread and safflower extracts was described by the curves of the dependence of the total antioxidant activity on time with squared correlation coefficients (R^2) in the range of 0.89 – 0.98. The reaction times for these extracts were from 40 to 70 min at the correct ratio of volumes between the tested extracts and a DPPH solution. These studies demonstrated that the extracts obtained from bee bread of 2016 year of pollen collection had significantly higher the total antioxidant activity compared with the extracts of bee bread of pollen collection of 2015 considering the ratio of bee bread and the solvent in the extracts and volume of the extract for the procedure. This fact is explained not only botanical origin bun also the time of the storage of bee bread before the preparation of extracts. There was not found significant differences in the total antioxidant activity of extracts from flowers of safflower sown in fall and in spring. Antioxidant activity of the extracts from leaves of spring sown safflower was higher compared with the total antioxidant activity of the extracts from fall sown plants. Ascorbic acid equivalents of the tested extracts could be ranged as follows: bee bread of 2016 pollen collection >bee bread of 2015 pollen collection >leaves of safflower spring sown >flowers of safflower spring sown >flowers of safflower fall sown >leaves of safflower fall sown.

Keywords: bee bread; safflower; antioxidant activity; DPPH; phenolic compounds

INTRODUCTION

Herbal preparations and food of plant origin are considered to be the most promising sources of antioxidants. In the initial stages of the development of herbal preparations or food herbal products, especially from nonpharmacopeia plant material, the determination of the sum of such analytical markers as phenolic compounds, provitamins (carotenoids) and vitamins (vitamin C, vitamin E (tocopherols)), microelements with changeable valence (manganese, iron, selenium etc.), amino acids and enzymes, having antioxidant properties, unsaturated acids could generally define biological value including nutritional properties of these products. Se, Cu and Zn are considered antioxidant nutrients **(Salem et al., 2011; Yu et al., 2013; Markievicz-Żukovska et al., 2013; Ivanisova et al., 2015; Rzepecka-Stojko et al., 2015; Zuluaga et al., 2015)**. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly being used in the food, pharmacetical and cosmetics industry for their antioxidative properties and health benefits **(Salem et al., 2011; Baba and Malik, 2015)**.

According to the nature all antioxidants are divided into two large groups: hydrophilic and hydrophobic. The hydrophilic antioxidants (vitamin C, phenolic acids and others) are reacted with oxidants in the cytoplasm of the cell, inside cell organelles, and in exracellular fluid, while the lipophilic antioxidants (carotenoids, tocopherols, flavonoids, etc.) protect cell membranes from lipid peroxidation. Bee pollen collected from willow, pear and

apple trees, and from dandelion has the highest content of ascorbic acid **(Rzepecka-Stojko et al., 2015)**. Antioxidant properties are closely related to chemical structure of phenolic compounds, especially with the number of hydroxyl groups on the aromatic ring and conjugated double bonds **(Rzepecka-Stojko et al., 2015; Zuluaga et al., 2015)**. The high phenolic and flavonoid content is responsible for the bioactivity of crude extracts. Flavonoids are highly effective scavengers of most oxidizing molecules, including singlet oxygen, suppress reactive oxygen formation, chelate trace elements involved in free-radical production, scavenge reactive species and up-regulate and protect antioxidant defenses **(Baba and Malik, 2015)**. The presence of a double bond between C2 and C3 in the C-ring in a flavonoid structure influences antioxidative properties. A carbonyl group at the C4 position enables the compounds to scavenge hydroxyl radicals. This group is presented in flavons, flavonols, flavanons, and isoflavons. The ability to scavenge hydroxyl radical increases with the number of hydroxyl groups present in the B-ring, especially at the positions C3′ and C4′. The presence of hydroxyl groups at the C5 and C7 positions in the A-ring, C3′ and C4′ in the B-ring, as well as C3 in the C-ring enhances the inhibition of lipid peroxidation **(Rzepecka-Stojko et al., 2015)**.

Among natural sources of antioxidants are bee pollen, bee bread, leaves and flowers of safflower **(Salem et al., 2011; Yu et al., 2013; Ivanišova et al., 2015; Kuşoğlu and Kahraman, 2015; Rzepecka-Stojko et al., 2015; Bogdanov, 2016; čeksteryte et al., 2016)**.

According to different authors, content of phenolic compounds amounts to 3.0 – 5.0% (including phenolic acids 0.19% and flavonoids 0.25 – 1.4%) **(Rzepecka-Stojko et al., 2015)**, 1.05 – 1.68% **(Markievicz-Żukovska et al., 2013)** in bee pollen and 0.25 – 1.37%, in particular flavonoids 0.19 – 0.45%, **(Zuluaga et al., 2015)**, 2.12 ±0.008% **(Čeksteryte et al., 2016)**, 1.35 – 2.54% **(Ivanisova et al., 2015)** in bee bread.

Polyphenols are components of safflower and bee pollen that determine their antioxidative activity as they scavenge free radicals and chelate metal ions. Flavonoids are the largest group of phenolic compounds, which have antioxidant, antimicrobial, antitumor, antiinflamatory activities, regulate free-radical reactions in the body. The pollen flavonoids quercetin, rutin and chrysin demonstrated a chemopreventive activity by increasing apoptosis. Flavonoids in plants constitute the antioxidant system, in which various antioxidants restore each other and exhibit a synergistic effect **(Silva et al., 2014; Barene et al., 2015; Rzepecka-Stojko et al., 2015; Zuluaga et al., 2015; Bogdanov, 2016)**. The content of polyphenols in bee bread extracts can strongly vary depending on the botanic origin of the raw material, the time and location of collection of bee bread, drying and/or storage conditions and extraction conditions as well (nature of a solvent, its concentration, ratio raw material-solvent, a method of extraction, etc.) **(Markievicz-Żukovska et al., 2013; Rzepecka-Stojko et al., 2015; Zuluaga et al., 2015; Bogdanov, 2016; čeksteryte et al., 2016)**. Among phenol compounds of bee bread were identified p-coumaric acid and trace amounts of ferulic and caffeic acids, and flavonoids kaempferol, apigenin, chrysin, isorhamnetin, naringenin and quercetin **(Markievicz-Żukovska et al., 2013; Čeksteryte et al., 2016)**. According to **Čeksteryte et al. (2016)** flavonoids in the form of glycosides were not found in bee bread.

Bee bread is a fermented mixture of plant pollen, honey, and bee saliva that worker bees use as food for the larvae, and for young bees to produce royal jelly. Pollen collected by bees is mixed with a small amount of honey and saliva and packed into the cells of the honeycomb where it undergoes a chemical change (lactic fermentation) **(Brovarskij et al., 2010; Brindza et al., 2013; Markievicz-Żukovska et al., 2013; Eswaran and Bhargava, 2014; Zuluaga et al., 2015)**. As a rule, biochemical composition of pollen changes in the process of fermentation: concentration of proteins reduces because of degradation to amino acids, contents of lactic acid and carbohydrates increase, and fats decrease. As concentration of lactic acid increases, pH of suspension of bee bread is lower (3.8 – 4.3) compared with pollen (approximately 6.3) **(Barene et al., 2015; Zuluaga et al., 2015)**. Bioactive properties and content of compounds in bee bread are associated mainly with its botanical origin. Bee bread contains a wide variety of compounds. More than 200 compounds were identified in extracts of bee bread samples. Among them are phenolic antioxidants (phenolic acids and flavonoids), unsaturated fatty acids (α-linolenic, linoleic, oleic and 11, 14, 17-eicosatrienoic acids), carbohydrates, free amino acids, C21 – C35 alkanes, unsaturated alcohols, carbohydrate acids, vitamins (B1, B2, B6, pantothenic acid, folic acid), carotenoids, microelements, lipids (1.65 – 5.50%), proteins (19.1 – 27.3%) **(Markievicz-Żukovska et al., 2013; Eswaran and Bhargava, 2014; Barene et al., 2015; Hudz et al., 2015; Ivanišova et al., 2015; Rzepecka-Stojko et al., 2015; Zuluaga et al., 2015; Bogdanov, 2016; čeksteryte et al., 2016; Gálik et al., 2016; Hudz et al., 2016)**. Such a fact as a precipitation in our investigations at the change of a solvent for dilution of extracts of bee bread does indicate the diversity of biologically active substances in it **(Hudz et al., 2016)**. Therefore, it is necessary to pay attention on the choice of reagents during development of analytical procedures of the determination of antioxidant activity and contents of flavonoids of bee bread extracts with the purpose of avoiding precipitation during a reaction in these analytical procedures.

Safflower (*Carthamus tinctorius* L.) plants are valuable agronomic species for their multipurpose usage: the presence of red and yellow pigments as food colorants **(Jadhav and Joshi, 2015)**; as the source of phenolic substances as natural antioxidants **(Yu et al., 2013; Kuşoğlu and Kahraman, 2015)** and vegetable oil as source of alphatocopherol **(Zubkov et al., 2014; Matthaus et al., 2015)**. Flowers and seeds are extensively used in traditional herbal medicine in Asian countries (China, Korea, and Japan) for treating various diseases such as gynecological, cardiovascular and cerebrovascular ones **(Yu et al., 2013; Gautam et al., 2014; Zhou et al., 2014)**. Pharmacological effects of safflower are due to its ability to accumulate the biologically active secondary metabolites, especially phenolic compounds, in different parts of plant **(Lee et al., 2002; Salem et al., 2011; Zhang et al., 2016)**. Using the secondary metabolites from flowers and seeds of safflower some new pharmaceutical

preparations were obtained **(Kharisova, 2014)**. More than 200 compounds were isolated from of different parts of C. tinctorius L. **(Asgarpanah and Kazemivash, 2013; Zhou et al., 2014)** and identified as flavonols and flavanones **(Zhao et al., 2005)**, chalcone flavonoids **(Salem et al., 2011; Yue et al., 2013)** and their glycosides **(Zhou et al., 2006)**. The accumulation of secondary metabolites in safflower depends on eco-geographical area and various technological approaches of plant cultivation **(Treutter, 2010)**, and in particular on the time of sowing. It is well-known that safflower seeds can be sown in the fall or in the spring because of its low temperatures tolerance. **Petrie et al. (2010)** reported that fall seeding resulted in earlier flowering and maturity and increased yield compared to spring seeding. However, the comparative studies of biologically active secondary metabolites accumulation in safflower of different time of sowing and their antioxidant activity were not carried out enough yet.

The DPPH scavenging radicals assay procedures reported by many authors are significally differed by their performing. Among such differencies are time and kinetics of the reaction between DPPH and bioactive constituents of tested extracts, solvents and concentration of DPPH, used wavelength in the procedure **(Yu et al., 2013; Carmona-Jiménez et al., 2014; Fadda et al., 2014; Nicklisch and Waite, 2014)**. As **Fadda et al. (2014)** have reported the reaction of DPPH with lemon juice is completed in 3 min; 87 and 98 min are necessary for green tea infusion and pomegranate juice, respectively; 283 min are needed for rosemary essential oil. **Yu et al. (2013)** incubated reaction mixtures of safflower extracts with DPPH during 4 min and measured their absorbance at wavelength of 490 nm. While **Salem et al. (2011)** and **Kuşoğlu and Kahraman (2015)** employed the analytical procedure which established an incubation period of 30 min the absorbance read at 517 nm. However, they used the different ratio of an extract to volume of DPPH **(Salem et al., 2011; Kuşoğlu and Kahraman, 2015)**. Different modifications and optimizations of DPPH assay are performed with the purpose of their adaptation to tested samples or are the invention of researchers. Therefore, it is not easy to compare results of different scientists.

Total antioxidant activity of extracts of bee bread and leaves and flowers of safflower may serve an important marker on the initial stages of the DPPH assay development, including stage of their extracts obtaining (choice of an optimal solvent for extraction, duration of extraction, the type of extraction, the time of collection of bee bread etc.). Therefore, the main objective of this study to develop approaches for determination of the total antioxidant activity of extracts from bee bread as well as leaves and flowers of safflower sown in fall and spring by means of DPPH and evaluate the differences in antioxidant activities of the tested extracts.

MATERIAL AND METHODOLOGY

Biological material

Bee bread was obtained from Mykolajev and Poltava regions of Ukraine. Samples were collected in the summer (July) of 2015 and May – June of 2016. Three samples of bee bread were collected in Mykolajev region and one sample in Poltava region. Leaves and flowers of safflower were collected in July 2016 in Chisinau area of the Republic of Moldova. Leaves and flowers were collected from at least 10 safflower plants of fall as well as spring sowing. The extracts were prepared from medium sample of identical plant material in 5 replicates.

Chemicals

The chemicals such as 1,1-Diphenyl-2-picrylhydrazyl (DPPH), methanol, ethanol, ascorbic acid were analytical grade and were purchased from Reachem (Slovakia), Sigma Aldrich (USA) and Ukraine (ascorbic acid).

Ethanol extraction

Some bee bread samples in the amount 2.5 g were extracted with 50 mL of 50% ethanol for 9 days, or in the amount 10.0 g were extracted with 100 mL of 50% ethanol for 21 days. The process of the preparation of bee bead extracts is described in Table 1. Supernatants were decanted and filtered through filter paper and stored in fridge (2 – 8 °C).

1, 7-11 batches of extracts were prepared from bee bread samples collected in Mykolajev region and 3 batch was prepared from bee bread collected in Poltava region. 7, 8, 9 and 10, 11, respectively, were prepared from different granules of the same example.

The extraction from leaves and flower of safflower was performed by maceration procedure using 70% aqueous ethanol. Ratio between the fresh plant material and ethanol solution was 1 **:** 10 for leaves and 1**:** 20 for flowers. After 7 days of extraction, the extracts were filtered through paper filter and stored at 4 – 6 °C.

Potentiometric determination of pH

Values of pH of bee bread samples was determined by pH meter WTW 3110 (Germany) previously calibrated with buffers pH = 4.01 and pH 6.86. 2% suspensions of three samples of bee bread were used for determination of their value of pH.

DPPH free radical scavenging assay

Total antioxidant activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$) (DPPH) according to modified analytical procedure **(Baba and Malik, 2015**).The extracts or their appropriate dilutions of bee bread (0.05 mL) were mixed with 1.95 mL of DPPH solution (0.0025 g DPPH in 100 mL 50% methanol) and incubated in the darkness for 60 – 70 min. Every 10 min the absorbance of the reaction mixtures was determined using the UV/Vis spectrophotometer (Genesys 20, USA) at 515 nm. The blank of a sample consisted of 0.05 mL of the corresponding extract or its appropriate dilution and 1.95 ml of 50% methanol. The blank of DPPH solution consisted of 0.05 mL of 50% ethanol and 1.95 mL of 50% methanol.

This analytical procedure was tested on leaves and flowers of safflower in the same way with one exception: initial extracts (0.05 mL) were mixed with 1.95 mL of DPPH solution. For positive control 0.05 ml solutions with diferent concentration of ascorbic acid were mixed with 1.95 mL of DPPH solution (at two different absorbances of DPPH solution).

Table 1 Preparation of the extracts of bee bread.

Number of batch of extract	Date of collection of bee pollen	Ratio of bee bread to 50% ethanol	Dates of extraction	Time of maceration
1	15/07/2015	10 g : 100 mL	19/04/2016 – 10/05/2016	21 day
3	03/07/2015	10 g : 100 mL	19/04/2016 – 10/05/2016	21 day
7	10/06/2016	2.5 g : 50 mL	28/09/2016 – 07/10/2016	9 days
8	10/06/2016	2.5 g : 50 mL	28/09/2016 – 07/10/2016	9 days
9	10/06/2016	2.5 g : 50 mL	28/09/2016 – 07/10/2016	9 days
10	23/05/2016	2.5 g : 50 mL	28/09/2016 – 07/10/2016	9 days
11	23/05/2016	2.5 g : 50 mL	28/09/2016 – 07/10/2016	9 days

All the tests were performed in triplicate. The radical scavenging activity of a tested extract or its dilution was calculated as follows:

$$\%inhibition = \frac{[A(DPPH) - A(extract)]x100}{A(DPPH)}$$

where: A *(DPPH)* – the absorbance of a solution DPPH; A *(extract)* – the absorbance of a sample or its dilution. Every 10 min absorbances of the solution DPPH and the tested sample were measured.

Ascorbic acid (AA) was used as standard antioxidant, its calibration curves were determined in the range of concentrations from 0.04 to 0.4 $mg.mL^{-1}$ at the different initial absorbances (Figure 1). The squared coefficients of correlation of the linear equation for these curves were 0.9371 and 0.9755 that permitted to express the obtained results of the total antioxidant activity of the tested extracts in equivalents of ascorbic acid. Concentration of AA which inhibited 50% of DPPH radicals (IC_{50}) was equal to 163.5 ±7.5 $\mu g.mL^{-1}$.

RESULTS AND DISCUSSION

Values of pH of the three suspensions were in the range of 4.09 – 4.17 (4.19, 4.17, 4.09). Our results were in conformity to **Barene et al. (2015)** data 4.01 – 4.23 (in general not less than 3.7), **Ivanisova et al. (2015)** data (3.8 – 4.3) and the data of **Ukrainian standard 7074:2009 (2010)** 3.5 – 5.0. It seems that the limits of Ukrainian standard are too wide. It is suppose that bee bread with pH higher than 4.3 can be of poor quality because of low content of lactic acid.

One of the methods for assessing the total antioxidant activity is determination of the absorption of the solution of DPPH, dissolved in methanol or etanol of certain concentration in water, without addition of a sample (a herbal product) and with the addition of this sample at specific intervals of time.

The reduction of a DPPH absorbance with compounds with antioxidant properties (compounds containing hydrogen) changes purple color of a reaction mixture into slightly purple or even pale or yellowish depending on the content of antioxidants in a sample and their nature. The reduce of staining intensity is controlled by change in the absorbance of the reaction mixture at 515 nm over time. According to the literature, DPPH has an absorption maximum at 514 – 517 nm. Fixing a reduce of a color intensity of a reaction mixture is carried out by various authors at various time intervals: from 4 – 15 minutes to 1 hours **(Meda et al., 2005; Yu et al., 2013; Eswaran and Bhargava, 2014; Ivanišova et al., 2015; Čeksteryte et al., 2016)**.

Assay of the total antioxidant activity foresees the selection of appropriate conditions for every extract. As evidenced by the literature data, modifications of this analytical procedure involve a solvent selection for DPPH, concentration of DPPH in solution, ratio of the volume of an extract to a DPPH solution, time of interaction of an extract with DPPH, the wavelength at which absorbance of the reaction mixture is measured, the pH of the reaction medium, the composition of a blank, methods of calculating an antioxidant activity, presence or absence of a positive control, the nature of the antioxidant in the positive control **(Meda et al., 2005; Ivanišova et al., 2015; Čeksteryte et al., 2016)**.

Fifty percent ethanol was chosen as a solvent for the extraction from point of view of the extraction of both hydrophilic and hydrophobic compounds from bee bread simultaneously. In addition to this, some literature data confirm that aqueous and 50% ethanolic extracts have higher antiradical scavenging activity compared to 90% ethanol **(Eswaran and Bhargava, 2014)**.The studies have been started with the selection of the methanol concentration for dissolution of DPPH. The studies conducted on two native extracts of bee bread demonstrated the dependence of radical scavenging activity on methanol concentration in solvent for DPPH.

As below provided results show (Figure 2), the total antioxidant activity of bee bread extract of batch 1 in the presence of 100% methanol is lower. In addition to it, the obvious visible precipitation was observed for batch 1 using 100% methanol as a solvent of DPPH. It is supposed that 100% methanol as a solvent of DPPH precipitates more hydrophilic substances with antioxidant activity. This assumption is agreed with literature dates that one of the major drawbacks of measuring antioxidant activity by means of DPPH is missing a large contribution of antioxidant activity from proteins and other hydrophilic antioxidants because they are precipitated by methanol **(Nicklisch and Waite, 2014)**. Among more hydrophilic antioxidants could be flavonoids in the form of glycosides with 3 monosaccharide remnants or proteins. These flavonoids are soluble in water but not soluble in strong alcohols **(Anonym, 2009)**.

The next stage of the elaboration of the analytical procedure of total antioxidant activity measuring by means of DPPH was to select the volume of a sample for the determination of the reliable total antioxidant activity of extracts.

Figure 3 demonstrates that if the concentration of antioxidants is too high, then DPPH is reduced very fast.

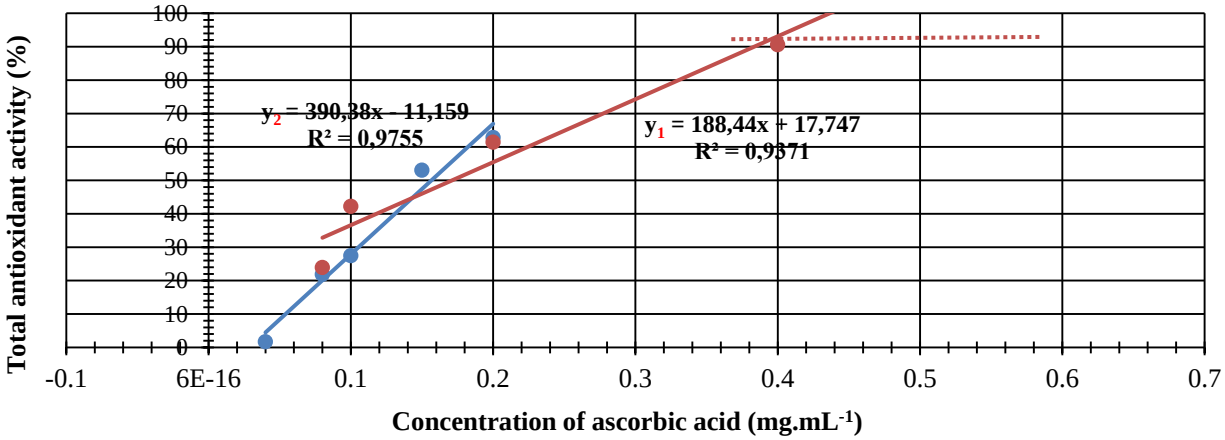

Figure 1 Dependence of the total antioxidant activity on concentration of ascorbic acid: 1 – curve at the initial absorbance of DPPH 0.603 (R^2 = 0.9371); 2 – curve at the initial absorbance of DPPH 0.520 (R^2 = 0.9755).

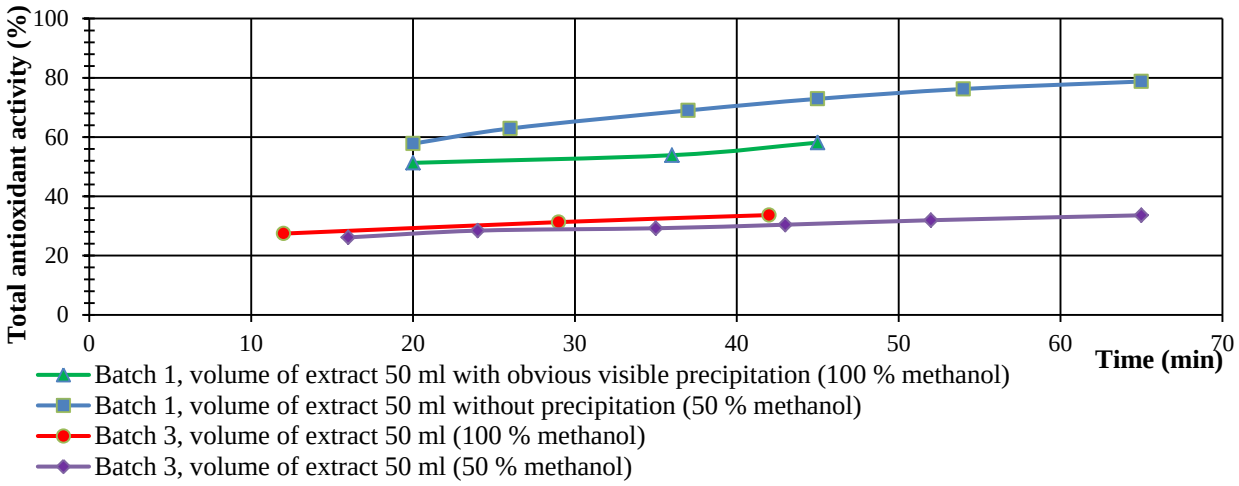

Figure 2 The dependence of the antioxidant activity of two extracts of bee bread on the concentration of methanol in DPPH solution.

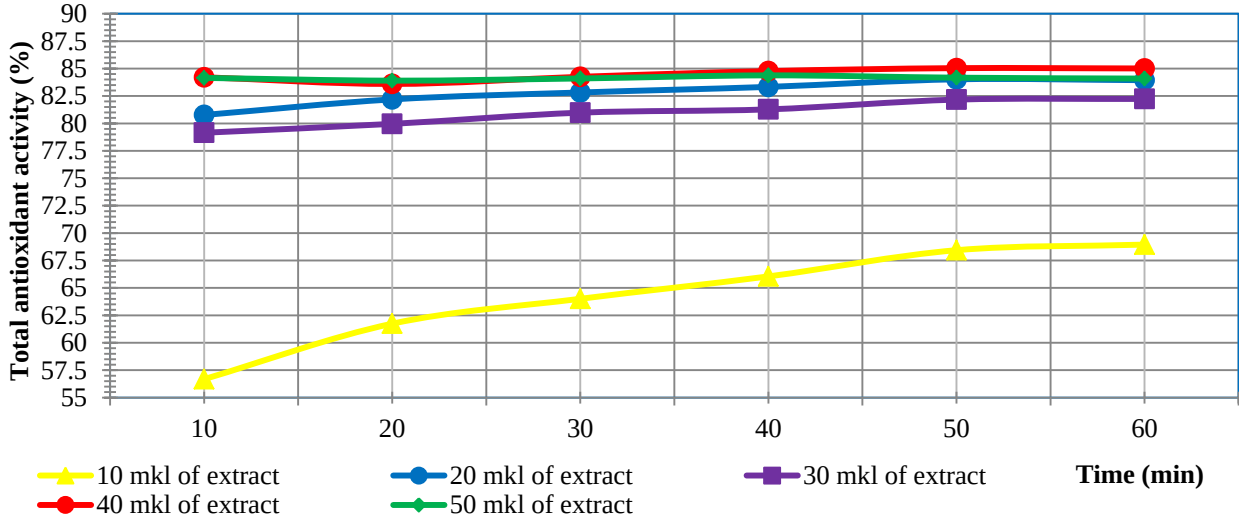

Figure 3 The dependence of the total antioxidant activity on the volume of the native bee bread extract (the initial absorption of DPPH was approximately 0.500).

In that case, the kinetic curves of antioxidant activity changes on time are more parallel to axis X (time) and what is more the total antioxidant activity is equal 80 – 85% and does not depend on the volume of the extract (20 – 50 mkl). These studies were conducted for batch 7. The same situation was observed for positive control: at too high ascorbic acid concentration (0.4 – 0.6 mg.mL^{-1}) the part of kinetic curve of antioxidant activity change on AA concentation is parallel to axis of concentration.

Another stage of our study was to check antioxidant activity of bee bread extracts using 0.0025% DPPH

solution with date of its storage for 24 hours. These studies showed that absorption of a DPPH solution was reduced with the time of its storage and the total antioxidant activity of extracts dilutions increased, respectively (Figure 4). In these studies the following regularity was observed: the less the absorption of a DPPH solution the more the total antioxidant activity of a sample was.

Figure 4 shows that antioxidant activity of the extract dilution of batch 8 is significantly higher at the less initial absorption of DPPH 0.471 (80.51%) compared with 64.25 – 68.64% at the initial absorption of DPPH 0.587 – 0.592. There is the same situation with batch 9. On the base of obtained results, it is concluded that it is possible to compare results of measuring the total antioxidant activity only at the same values of the initial absorption of a DPPH solution. Consequently, the total antioxidant activity of batch 8 is higher than that of batch 7 if we take into consideration the initial absorpion of DPPH 0.466 and 0.471, respectively. Similarly, the total antioxidant activity of batch 9 is higher than that of batch 8 if we take into consideration the initial absorpion of DPPH 0.576 and 0.587 or 0.592, respectively.

One more stage of the development of analytical procedure of measuring the total antioxidant activity was approbation of the developed procedure for assay of the total antioxidant activity of safflower leaves and flowers extracts. The results are presented in Figure 5.

The reaction kinetics of DPPH inhibition by safflower extracts is good described by the curves of the dependence on time with squared correlation coefficient in the range of $R^2 = 0.89 – 0.98$. The reaction times for these extracts were in the range from 40 to 60 min. As figure 4 indicates that total antioxidant activity of the extract from leaves of spring sown safflower is much higher compared with flowers spring sown safflower. It is explained by the ratio between the fresh plant material and ethanol: 1: 10 for leaves and 1: 20 for flowers. Content of AA equivalents in the extract from leaves of spring sown safflower was higher, but no significantly, than that in the extract from leaves of fall sown plants. Content of AA equivalents in the extract from flowers of spring sown safflower was similar with that in the extract from flowers of fall sown plants. Considering that the antioxidant activity is predetermined by accumulation of secondary metabolites on plants, the obtained data correlated with the content of total polyphenols and flavonoid glycosides, in safflower plants of spring and fall sown **(Ivanova et al., 2016)**. The total phenolic content in flower extracts collected from plant sown in fall and in spring was similar (0.25 ± 0.02 mg.mL^{-1}). Leaves of spring sown safflower contained the total polyphenols as well as the flavonoid glycosides more by 20 – 30% than leaves of fall sown plants.

Despite fact that the ratio between fresh plant materials and ethanol is 1: 10 for leaves compared to flowers 1:20, the total antioxidant activity of the extract from leaves of spring sown safflower is only higher 1.67 times compared with flowers spring sown safflower.The same situation is observed with the extracts from leaves and flowers of fall sown safflower (71.46 : 42.41 = 1.68) (Table 2). Ascorbic acid equivalent of antioxidant activities of tested extracts could be ranged following: leaves of safflower spring sown ≥flowers of safflower spring sown ≥flowers of safflower fall sown >leaves of safflower fall sown. The present study revealed that the extract from leaves of safflower sown in spring possessed the greatest antioxidant activity in ascorbic acid equivalent.

In these studies we also observed the following regularity: the less the absorption of negative control was the higher the total antioxidant activity of extract from leaves or flowers of fall sown safflower was (Table 2). For comparison, the total antioxidant activity of extract from flowers of fall sown safflower was 34.54% at the initial absorbance of DPPH 0.587 and, respectively, 42.41% at the initial absorbance of DPPH 0.466.

Table 2 also shows that samples of bee bread extracts of samples 7, 8, 9, 10 and 11 had significantly higher total antioxidant activity compared with batches 1 and 3 considering the ratio of bee bread and the solvent in the extracts and volume of the extract for the procedure. This fact can be explained not only botanical origin bun also the time of the storage of bee bread before the preparation of extracts. Considering the same ratio 1:10 of raw material and the solvent in the tested extracts of bee bread and safflower and the same initial absorbance of DPPH (0.589 – 0.612**),** the results of the total antioxidant activity and AA equivalents are comparable for the extracts from leaves of spring sown safflower (67.14%), flowers of

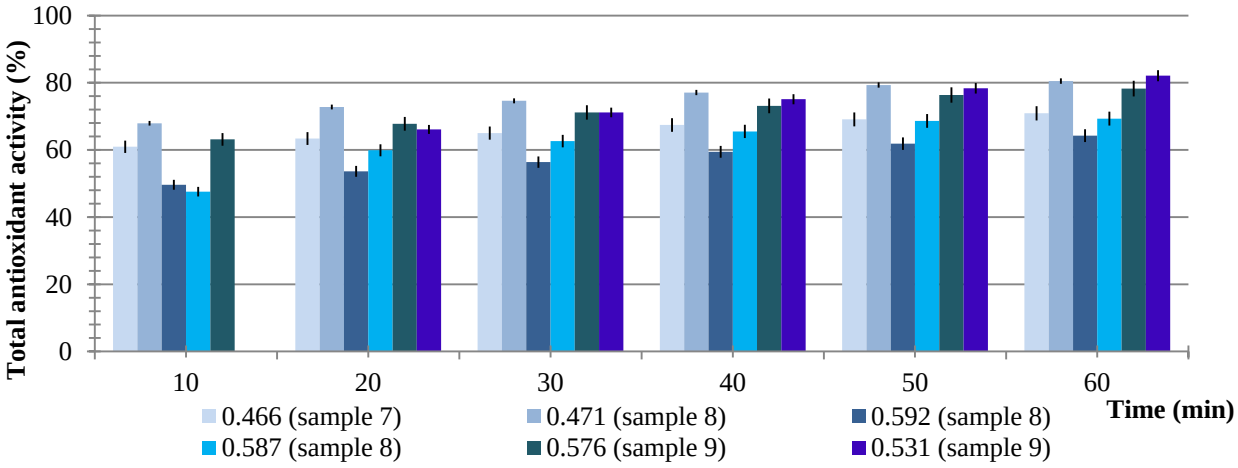

Figure 4 The dependence of the total antioxidant activity of the extracts dilutions on the initial absorption of DPPH solutions.

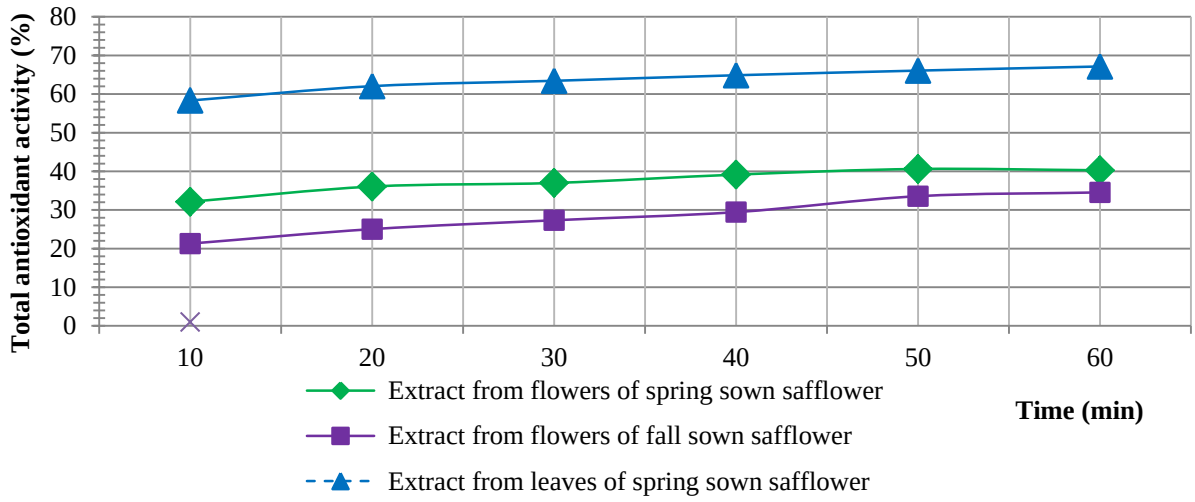

Figure 5 The dependence of the total antioxidant activity of the safflower extracts on the time.

Table 2 The generalized table of the measure of the total antioxidant activity of the extacts of bee bread and safflower.

Number of sample	Volume of extract (mL)	Volume of dilution of an extract 1:5 (mL)	Total antioxidant Activity (%)	The initial absorbance of DPPH	Content of ascorbic acid equivalents, mg in 1 mL a extract	Average content of ascorbic acid equivalents, mg in 1 g of raw material
Bee bread						
1	0.05	–	82.39 78.76	0.589 0.612	0.34^{1} 0.32^{1}	0.033
3	0.05	–	35.88 33.61	0.589 0.612	0.10^{1} 0.08^{1}	0.009
7	–	0.05	70.91	0.466	1.05^{2}	0.210
8	–	0.05	80.51 64.25 68.64	0.471 0.592 0.587	1.17^{2} 1.25^{1} 1.35^{1}	0.250
9	–	0.05	78.26 82.10	0.576 0.531	1.60^{1} 1.20^{1}	0.280
10	–	0.05	45.15	0.638	0.75^{1}	0.150
11	–	0.05	46.89 47.33	0.604 0.638	0.75^{1} 0.80^{1}	0.155
Safflower						
flowers of spring sown safflower	0.05	–	40.27	0.576	0.12^{1}	0.024
flowers of fall sown safflower	0.05	–	34.54 42.41	0.587 0.466	0.09^{1*} 0.13^{2*}	0.022
leaves of spring sown safflower	0.05	–	67.14	0.592	0.26^{1}	0.026
leaves of fall sown safflower	0.05	–	71.46 65.34 63.18	0.471 0.511 0.513	0.21^{2*} 0.20^{2*} 0.19^{2*}	0.020

Note: * – average 0.11 $mg.mL^{-1}$ (0.09 +0.13)/2; ** – average 0.20 $mg.mL^{-1}$ (0.21 +0.20 +0.19)/3.
1 – calculations were conducted using y_1 = 188.44x +17.747; 2 – calculations were conducted using y_2 = 390.38x +11.159.

spring sown safflower (40.27%), flowers of fall sown safflower (34.54 – 42.41%), and bee bread of batch 1 (82.39 – 78.76%) and 3 (33.61 – 35.88%).

The results of AA equivalents in 1 mL of an extract for all the safflower extracts occupy the intermediate place between ones for the two batches of bee bread 1 and 3. Table 2 also demonstrates that the extracts prepared from granules of the same example of bee bread have insignificant distinguished results. The slight difference could be explained by pollen collecion from different flowers in the same time and analysis deviation as well.

CONCLUSION

In conclusion, the results of this investigation confirm that bee bread and safflower are sources rich in compounds with antioxidant properties. The best results were observed for the samples of bee bread with a shorter time of storage. The further studies will be directed on the establishment of correlation of the total antioxidant activity and total phenolic contents and flavonoids.

REFERENCES

Asgarpanah, J., Kazemivash, N. 2013. Phytochemistry, pharmacology and medicinal properties of *Carthamus tinctorius* L. *Chinese Journal of Integrative Medicine*, vol. 19, no. 2, p.153-159. https://doi.org/10.1007/s11655-013-1354-5 PMid:23371463

Baba, S. A., Malik, S. A. 2015. Determination of total phenolic and flavonoids content, antimicrobial and antioxidant activity of a root extract of *Arisaema jacquemontii*. *Journal of Taibah University for Science*, vol. 9, no 4, p. 449-454. https://doi.org/10.1016/j.jtusci.2014.11.001

Barene, I., Daberte, I., Siksna, S. 2015. Investigation of bee bread and development of its dosage forms. *Medicinos teorija it practika*, vol 21, no 1, p. 16-22.

Bogdanov, S. 2016. Pollen: Production, Nutrition and Health: A Review [online] s. a. [cit. 2017-02-01]. Available at: http://www.bee-hexagon.net.

Čeksterytė, V., Kurtinaitienė, B., Venskutonis, P. R., Pukalskas, A., Kazernavičiūtė R., Balžekas, J. 2016. Evaluation of antioxidant activity and flavonoid composition in differently preserved bee products. *Czech Journal of Food Science*, vol. 34, no. 2, p. 133-142. https://doi.org/10.17221/312/2015-CJFS

Brindza, J., Brovarskyi, V. 2013. *Pollen and bee pollen of some plant species*. Kyiv : Korsunskiy vidavnichiy dim Vsesvit, 137 p. ISBN 978-80-552-1073-5.

Brovarskij, V., Brindza, J. 2010. *Včelí obnôžkový peľ*. 1st ed. Kyjev : FOP I. S. Maidachenko, 290 p. ISBN 978-966-8302-31-2.

Eswaran, V. U., Bhargava, H. R. 2014. Chemical analysis and anti-microbial activity of Karnataka bee bread of apis species. *World Applied Sciences Journal*, vol. 32, no 3, p. 379-385.

Carmona-Jiménez, Y., García-Moreno, M. V., Igartuburu, J. M., Garcia Barroso, C. 2014. Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products. *Food Chemistry*, vol. 165, p. 198-204. https://doi.org/10.1016/j.foodchem.2014.05.106 PMid:25038667

Fadda, A., Serra, M., Molinu, M. G., Azara, E., Barberis, A., Sanna, D. 2014. Reaction time and DPPH concentration influence antioxidant activity and kinetic parameters of bioactive molecules and plant extracts in the reaction with the DPPH radical. *Journal of Food Composition and Analysis*, vol. 35, no. 2, p. 112-119. https://doi.org/10.1016/j.jfca.2014.06.006

Gálik, B., Bíro, D., Šimko, M., Juráček, M., Capcarová, M., Kolesárová A., Rolinec, M., Toman, R., Kanka, T. 2016. The effect of dietary bee pollen intake on growth performance and biochemical indicators of rats. Acta Veterinaria Brno, vol. 85, no 1, p. 99-104. https://doi.org/10.2754/avb201685010099

Gautam, S., Bhagyawant, S. S., Srivastava, N. 2014. Detailed study on therapeutic properties, uses and pharmacological applications of safflower (*Carthamus tinctorius* L.). *International Journal of Ayurveda and Pharma Research*, vol. 2, no 3, p. 5-16.

Hudz, N. I., Ivanova, R. A., Šimkova, J. 2016. Study of antioxidant activity as a stage in the development of herbal preparations. In *Modern achievements of pharmaceutical technology and biotechnology: Scientific proceedings*. Kharkow, Ukraine, pp.182-185.

Hudz N., Rehletska A., Filipska A., Vorobets N. 2015. Problems of standartization of bee pollen and bee bread as bioactive components. *Scientific Bulletin of National University of life and Environmental Sciences of Ukraine. Set "Technology of production and processing of livestock products"*, vol. 223, p. 96-103.

Ivanišová, E., Kačániová, M., Frančáková, H., Petrová, J., Hutková, J., Brovarskyi, V., Velychko, S., Adamchuk, L., Schubertová, Z., Musilová. J. 2015. Bee bread – perspective source of bioactive compounds for future. *Potravinarstvo*, vol. 9, no 1, p. 592-598. https://doi.org/10.5219/558

Ivanova, P., Casian, I., Casian, A. 2016. Secondary metabolites accumulation in safflower of fall and spring sowing in the Republic of Moldova. In *Sustainable utilisation of plant genetic resources for agriculture and food*. Piestany, Slovak Republic : National Agricultural and Food Centre, p. 68. ISBN 978-80-89417-69-8.

Kharisova, A. V. 2014. *Pharmacognostical investigation of safflower* (*Carthamus tinctorius* L.) : dissertation theses. Russia : University of Samara. 360 p.

Kuşoğlu, E., Kahraman, S. 2015.Total phenolic content and radical scavenging activity of *Carthamus tinctorius* L. *International Journal of Electronics, Mechanical and Mechatronics Engineering*. vol. 5, no 2, p. 943-947.

Lee, J. Y., Chang, E. J., Kim, H. J., Park, J. H., Choi, S. W. 2002. Antioxidative flavonoids from leaves of Carthamus tinctorius. *Archives of Pharmacal Research*, vol. 25, no 3, p. 313-319.

Matthaus, B., Ozcan, M. M., Al Juhaimi, F. Y. 2015. Fatty acid composition and tocopherol profiles of safflower (*Carthamus tinctorius* L.) seed oils. *Natural Product Research*, vol. 29, no 2, p. 193-196. https://doi.org/10.1080/14786419.2014.971316 PMid:25329876

Markievicz-Żukovska, R., Naliwajko, S. K., Bartosiuk, E., Moskwa, J., Isidorov, V., Soroczyńska, J., Borawska M. 2013. Chemical composition and antioxidant activity of bee bread, and its influence on the glioblastoma cell line (U87MG). *Journal of Apicultural Science*, vol. 57, no 2, p. 147-157.

Meda, A., Lamien, C. E., Romito, M., Millogo, J., Nacoulma, O. G. 2005. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry*, vol. 91, no. 3, p. 571-577. https://doi.org/10.1016/j.foodchem.2004.10.006

Nicklisch, S. C. T., Waite, J. H. 2014. Optimized DPPH assay in a detergent-based buffer system for measuring antioxidant activity of proteins. *MethodsX*, vol. 1, p. 233-238. https://doi.org/10.1016/j.mex.2014.10.004

Petrie, S., Machado, D., Johnson, R., Prichett, L., Rhinhart, K., Tuck, B. 2010. Adaptation and yield of spring and fall sown safflower in Northeastern Oregon. Columbia Basin Agricultural Research Center Dryland Research Report 101. Oregon State University, USA. [online] s.a. [cit. 2017-02-19] Available at: http://cbarc.aes.oregonstate.edu/sites/default/files/adaptation_and_yield_of_spring_and_fall_sown_safflower_in_northeastern_oregon.pdf.

Anonym, 2009. Phytochemical analysis of plant raw material containing flavonoids. Chapter: Chemical analysis of medicinal plants. 2009. Methodological Guide in Pharmacognosy. Irkutsk, 67 p.

Rzepecka-Stojko, A., Stojko, J., Kurek-Górecka, A., Gorecki, M., Kabala-Dzik, A., Kubina, R., Mozdzierz, A., Buszman, E. 2015. Polyphenols from bee pollen: structure, absorption, metabolism and biological activity. *Molecules*, vol. 20, no 12, p. 21732-21749. https://doi.org/10.3390/molecules201219800 PMid:26690100

Salem, N., Msaada, K., Hamdaoui, G., Limam, F., Marzouk, B. 2011. Variation in phenolic composition and antioxidant activity during flower development of safflower (*Carthamus tinctorius* L.). Journal of Agricultural and Food Chemistry, vol. 59, no 9, p. 4455-4463. https://doi.org/10.1021/jf1049936 PMid:21434653

Silva, G. R., Natividade, T. B., Camara, C. A., Silva, E. M. S., Assis Ribeiro dos Santos, F., Silva, T. M. S. 2014. Identification of sugar, amino acids and minerals from the pollen of Janaira Stingless Bees (*Melipona subnitida*). *Food and Nutrition Sciences*, vol. 5, no 11, p. 1015-1021. https://doi.org/10.4236/fns.2014.511112

Treutter, D. 2010. Managing phenol contents in crop plants by phytochemical farming and breeding – visions and constraints. *International Journal of Molecular Science*, vol. 11, p. 807-857. https://doi.org/10.3390/ijms11030807 PMid:20479987

Yu, S. Y., Lee, Y. J., Kim, J. D., Kang, S. N., Lee, S. K., Jang, J. Y., Lee, H. K., Lim J. H., Lee, O. H. 2013. Phenolic composition, antioxidant activity and anti-adipogenic effect of hot water extract from safflower (*Carthamus tinctorius* L.) seed. *Nutrients*, vol. 5, no 12, p. 4894-4907. https://doi.org/10.3390/nu5124894 PMid:24288028

Yue, S., Tang, Y., Li, S., Duan, J. A. 2013. Chemical and biological properties of quinochalcone C-glycosides from the florets of Carthamus tinctorius. *Molecules*, vol. 18, no. 12, p.15220-15254. https://doi.org/10.3390/molecules181215220 PMid:24335575

Ukrainian standard 7074:2009. 2010. Kyiv, p. 1-11 p.

Zhao, M., Ito, Y., Tu, P. 2005. Isolation of a novel flavanone 6-glucoside from the flowers of Carthamus tinctorium (Honghua) by high-speed counter-current chromatography. *Journal of Chromatgraphy A*, vol. 1090, no. 1-2, p. 193-196. https://doi.org/10.1016/j.chroma.2005.07.010 PMid:16196150

Zhang, L. L., Tian, K., Tang, Z. H., Chen, X. J., Bian, Z. X., Wang, Y. T., Lu, J. J. 2016. Phytochemistry and Pharmacology of Carthamus tinctorius L. *The Americam Journal of Chinese Mededicine*, vol. 44, no 2, p. 197-226. https://doi.org/10.1142/S0192415X16500130 PMid:27080938

Zhou, X., Tang, L., Xu, Y., Zhou, X., Wang, Z. 2014. Towards a better understanding of medicinal uses of *Carthamus tinctorius* L. in traditional Chinese medicine: a phytochemical and pharmacological review. *Ethnopharmacol*, vol. 151, no 1, p. 27-43. https://doi.org/10.1016/j.jep.2013.10.050 PMid:24212075

Zhou, Y. Z., Ma, H. Y., Chen, H., Qiao, L., Yao, Y., Cao J. Q., Pei Y. H. 2006. New acetylenic glucosides from *Carthamus* tinctorius. *Chemical and Pharmaceutical Bulletin*, vol. 54, no 10, p. 1455-1456. https://doi.org/10.1248/cpb.54.1455

Zubkov, V. V., Milyokhin, A. V., Kurkin, V. A., Kharisova, A. V., Platonov, I. A., Pavlova, L. V. 2014. Prospects of safflower seeds oil in food and pharmaceutical industry. *Bulletin of the Samara Scientific Center*, vol. 16, no 53, p. 1135-1139.

Zuluaga, C. M., Serrato, J. M., Quicazan, M. C. 2015. Chemical, Nutritional and Bioactive Characterization of Colombian Bee-Bread. *Chemical Engineering Transactions*, vol. 43, p. 175-180.

Acknowledgments:

The publication was prepared with the active participation of researchers from international network AGROBIONET, as a part of international program "Agricultural biodiversity to improve nutrition, health and quality of life" within the project ITEBIO-ITMS 26220220115 „Support of technologies innovation for special bio-food products for human healthy nutrition“.

Natalia Hudz (51600967) and Raisa Ivanova (51600341) are thanking to the International Visegrad Scholarship Fund for the realization of research at Institute of Biodiversity Conservation and Biosafety. Experimental activities were realized in laboratories of excellent Center for the conservation and use of agrobiodiversity at the Faculty of Agrobiology and Food Resources, Slovak Agricultural University in Nitra.

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