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**APPLICATION OF COMMERCIAL WHEY PROTEINS FOR EXTRACTION OF
BIOACTIVE COMPOUNDS FROM ELDERBERRY**

M. MALYSA-PASKO, M. LUKASIEWICZ, P. JAKUBOWSKI, M. KULIG

University of Agriculture in Krakow, Department of Food Technology,
ul. Balicka 122, 30-149 Krakow, Poland
e-mail: malysamagdalen@gmail.com

ABSTRACT:

Recently, interest in food of plant origin and its pro-health properties have increased. Plant products due to its significant antioxidant activity should be a constant part of the human diet. Elderberry is a plant with high potential as a source of functional component obtained from flowers, fruits, bark and roots. Wild elderberry flowers are used as a antipyretic, diuretic and sealing blood vessels. On the other hand elderberry fruits are known as the source of many substances that have detoxifying, antiviral and strengthening effects on human body. Outside of therapeutic significance both fruits and flowers are used increasingly in the food industry.

Micelle-mediated separation is similar to traditional liquid-liquid extraction. In this method the organic solvent is replace with an aqueous surfactant solution. Micelles are colloidal type cluster that are spontaneously formed when the concentration of surfactant molecules increase above called critical micellar concentration (cmc).

The aim of this work was to extract bioactive components from elderberry (*Sambucus nigra*) fruits and flowers by means of various extraction solutions. The extraction solution were WPC (whey protein concentrate) and WPI (whey protein isolate) solution. Comparative extracts were also made using ethanol solutions and distilled water as control. Obtained extracts were analyzed by determination of: total reduction capacity using Folin-Ciocalteu reagent, flavonoid content, antioxidant activity using DPPH reagent. In WPC and WPI protein extracts, the extraction of bioactive components is more efficient than in the case of water extraction. This could be seen at the lowest concentration of both WPI and WPC.

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Keywords

Protein, elderberry, micelle mediated extraction, WPI, WPC

Introduction

Plants of the Musk family (Adoxaceae) are derived from the order of plants called dipterocarpus (Dipsacales). Adoxaceae includes five types of plants, among others: elderberry (*Sambucus L.*) [Bell and Donoghue 2005]. The elderberry is a deciduous shrub or a small tree growing to a height of 3 to 7 meters. It is widespread throughout Europe and Poland [Moszowicz 1982; Grau et al. 1996]. *Sambucus nigra* is short-lived and rarely lives for more than 35 years [Enescu et al. 2016]. It can be found in bushes (especially riversides), moist deciduous forests, mixed forests, hedges, courtyards, fields, pastures and dry soils, but above all in sunny locations [Miraj 2016]. It grows on fertile and humus soils [Grau et al. 1996]. Bark, light gray in the young plant, turns into thick, rough and gray with an unpleasant odor. The leaves are arranged in opposite pairs, odd-shaped, 10 to 30 cm long, with serrated leaves of 5 to 10 cm length. Inflorescences grow in the form of canopies with five major twigs. The flowers have 5 - 6 mm of diameter, with five petals. They are white, collected in a five-seater crown with a sphere up to 25 cm in diameter. Flowers have a very characteristic smell. [Moszowicz 1979; Grau et al. 1996; Miraj 2016]. The fruit is a shiny dark purple blackberry, 3-5 mm in diameter, produced in falling clusters. They are an important food for many birds [Miraj 2016]. Young fruits are green and proper dark purple color is reached when they mature. Each fruit contains one to three brown, ovoid seeds [Mumcuoglu et al. 2010]. It blooms from May to June, in some places even until July, while the fruits ripen from August to the end of September [Grau et al. 1996; Moszowicz 1979; Moszowicz 1982; Senica et al. 2016].

All parts of the wild elderberry plant (leaves, berries, inflorescences, roots, shoots, bark) have a long history of use in herbal or culinary applications, widespread on almost every continent of the world [Petrut et al. 2017]. Dark purple elderberry berries can be eaten when fully matured, but are mildly poisonous in the unripe state. All green parts of the plant are poisonous because they contain cyanogenic glycoside - sambunigrin. In Scandinavia and Germany *Sambucus nigra* (eg German Fliederbeersuppe) is a traditional meal [Miraj 2016]. Elderberries were and are also used for jams, jellies and wines along Europe. Dried ripe or fresh berries are recommended for the treatment of constipation, as a diuretic and as a diuretic, for upper respiratory tract infections and for the alleviation of pain [Duymuş et al. 2014]. In Austria, the fruit and flowers of *Sambucus nigra* were used in traditional medicine: fruits were made of infusions, jellies, juices or syrups, while flowers were used as brew or syrup. These medications are used to treat respiratory, oral, gastrointestinal and skin disorders and viral infections, fever, colds and flu [Miraj 2016]. Both flowers and fruits have antimicrobial,

antiviral, immunomodulatory, antioxidant, anti-inflammatory, diaphoretic and expectorant effects [Krajewska 2014]. One possible mechanism of action of lilac extracts in the treatment of influenza is that flavonoids stimulate the immune system by increasing the production of cytokines by monocytes. It has also been shown that they inhibit the influenza virus hemagglutinin and thus prevent the adherence of the virus to cellular receptors [Wadstein et al. 2004]. The fruits include choline, carotenoids, tannins, B vitamins, vitamin C [Kuznicka and Dziak 1987], sambunigrin, anthocyanins - chrysanthemum or cyanidin 3-glucoside, which accounts for 65,7% of all cyanidin derivatives contained in 32,4% cyanidin 3-sambucoside, and other lesser ones: cyanidine 3,5-diglycoside, cyanidin-3-sambucoside-5-glucoside, cyanidin 3-rutazide, pelargonidine 3-glucoside, and pelargonidine 3-lipidazide [Krajewska 2014, organic acids: citric, tartaric, nicotinic and p-aminobenzoic acids and others [Broda and Moszowicz 1979]. Flowers contain compounds such as choline, organic acids, tannins, phenolic compounds (coffee, ferulic, chlorogenic), mineral salts [Kuznicka and Dziak 1987], essential oil (0,3%), cyanogenic glycoside - sambunigrin, saponins triglycerides (α - and β -amyrin, ursolic acid and oleate) and sterols, mucus, flavonoids - rutin, derivatives of kemferol and quercetin; [Broda and Moszowicz 1979; Krajewska 2014]. The bond that must be paid attention is sambunigrin. It is a cyanogenic glycoside causing poisoning. Occurs in all parts of the green plant, in fresh flowers and unripe fruit. This compound is thermolabile and after heat treatment there is no risk of poisoning [Krajewska 2014]. Flower heads are commonly used to make infusions, giving a very popular refreshing drink in Northern Europe and the Balkans [Miraj 2016]. You can also bake with cakes at the cakes [Kuznicka and Dziak 1987].

The aim of the study was to extract bioactive ingredients from selected musk plants by means of various extraction solutions. The plant used for the study was wild black with no *Sambucus nigra*.

Experimental

Materials and equipment

Aqueous solution of WPC (SFD, Poland): 0,01% (0,0113 g/ 100cm³), 0,025% (0,0253 g/100 cm³), 0,05% (0,0506 g / 100 cm³), 0,1% (0,1029 g/ 100 cm³); aqueous solution of WPI (SFD, Poland): 0,01% (0,0105 g/ 100 cm³), 0,025% (0,0256 g/ 100 cm³), 0,05% (0,0502 g/100 cm³), 0,1% (0,1000 g/100 cm³).

For the preparation of extracts used in two materials derived from the wild bushes Elderberry - flowers and fruit. The flowers were harvested at the end of June 2016, while the fruits were at the beginning of September 2016. After harvesting, both types of raw materials were sorted and left frozen until analysis.

The research material was extracts made of black elderberry. Extraction solutions were 40%, 60%, 80% and 96% ethanol solutions and WPC (whey protein concentrate) and WPI (whey protein isolate) solutions at concentrations of 0.01%, 0.025%, 0.05%, 0.1%. Comparative extracts were also obtained using distilled water - control. Each fruit was rubbed in a porcelain mortar and transferred quantitatively by means of an extraction solution to the conical flasks which were subjected to 2 hour extraction at room temperature on a Wigan ES24H magnetic stirrer. After this time, the extracts were centrifuged in a MPW-350R laboratory centrifuge for 15 minutes and 12,000 rpm, filtered and filled to a specified volume.

Methods

Content of reducing compounds

Extracts were transferred quantitatively from 0,2 cm³ of the solution and diluted with 9,8 cm³ of distilled water. The solutions thus obtained were taken 5 cm³ and mixed with 0,25 cm³ of Folina-Ciocalteu reagent and 0.5 cm³ of a 7% Na₂CO₃ solution was added and the mixture was stirred. Samples were incubated for 30 minutes in a dark place and after this time the absorbance of samples was tested. Measurement of absorbance was done with Spectro UV - VIS Dual Beam UVS - 2800, Labomed inc., at wavelength $\lambda = 760$ nm. The calibration curve was made from a solution of gallic acid in the concentration range of 0,01-0,1 mg / ml and the equation describing it was: $y = 7.4608x + 0.0004$ with $R^2 = 0,9957$. The study was performed in duplicate [Duda-Chodak et al. 2011].

Content of flavonoids compounds

The extract was diluted with 1 cm³ and stirred with 1 cm³ of distilled water. The solution thus obtained was taken 1 cm³ into a 10 cm³ volumetric flask, 5 cm³ of redistilled water and 0,3 cm³ of 5% (w / w) aqueous sodium nitrate solution were added. The resulting solution was stirred and allowed to stand for 5 minutes, 0,6 cm³ 10% (w / w) aqueous hexahydrated aluminum chloride solution and remixed. After 5 minutes 2 cm³ of a 1 M aqueous NaOH solution was added and the water was redistilled to the mark. The absorbance of the samples so prepared was measured at wavelength $\lambda = 510$ nm against the zero test. Calibration curve was made from quercetin solution in the concentration range 0, 1-0,7 mg / ml and the equation describing it was: $y = 0.5415x + 0.024$ at $R^2 = 0,9959$. The study was performed in duplicate [Cieszynska et al. 2011].

Ability to scavenge free radicals

Extracts were extracted quantitatively from 0,2 cm³ of the solution and diluted with 9,8 cm³ of methanol. From these diluted solutions, 0,25 cm³ was taken, mixed with 2,25 cm³ DPPH solution and incubated for 1 hour in a dark place. After this time, the absorbance of the samples at wavelength $\lambda = 517$ nm was tested. A blank test was done in which, instead of the extracted test, 0,25 cm³ of distilled water was used. The results were calculated on the basis of the absorbance difference between the zero and the specimens. The calculated antioxidant activity is shown for dilutions 1:49. The study was performed in duplicate [Kowalski 2013].

Results and discussion

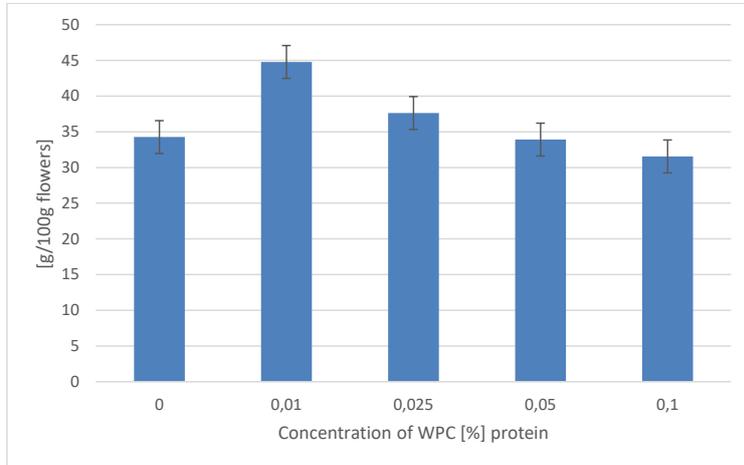
Extraction in protein solutions is a micellar extraction in which surfactants (surfactants) were WPC and WPI proteins. Each surfactant consists of a hydrophilic and hydrophobic part, and in the water solvent the hydrophobic parts accumulate to separate the hydrophilic part from the water. In such concentrations during the extraction, insoluble or poorly soluble compounds may be dissolved in water itself [Kiszkiel and Hryniewicka 2011].

WPC protein extracts

Extracts made of black elderberry flowers

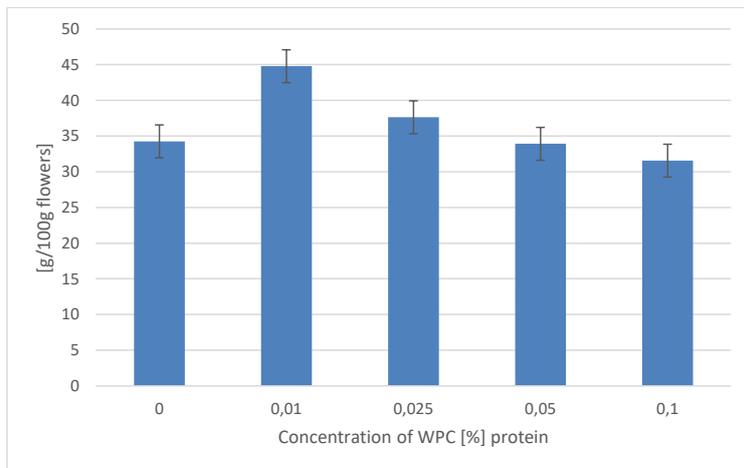
Mean total polyphenol content

Fig. 1. Average content of total polyphenols in elderberry flowers depending on the concentration of WPC protein solution used.



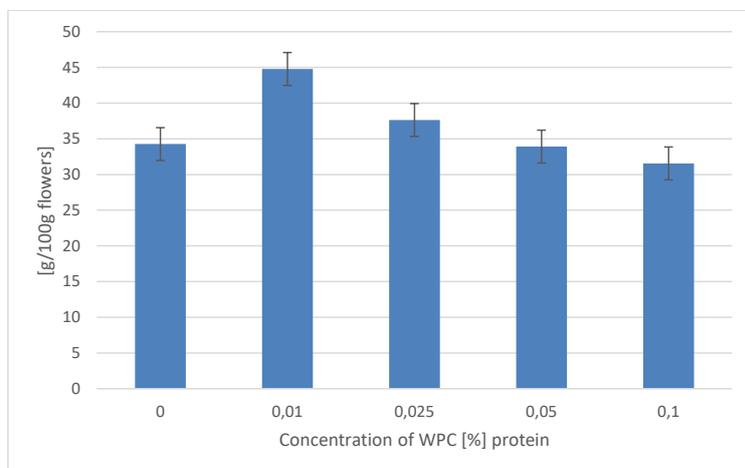
Average content of flavonoids

Fig. 2. Average content of flavonoids in elderberry flowers depending on the concentration of WPC protein solution used.



Average antioxidant activity

Fig 3. Average antioxidant activity of black elderberry extracts according to the concentration of WPC protein solution used.



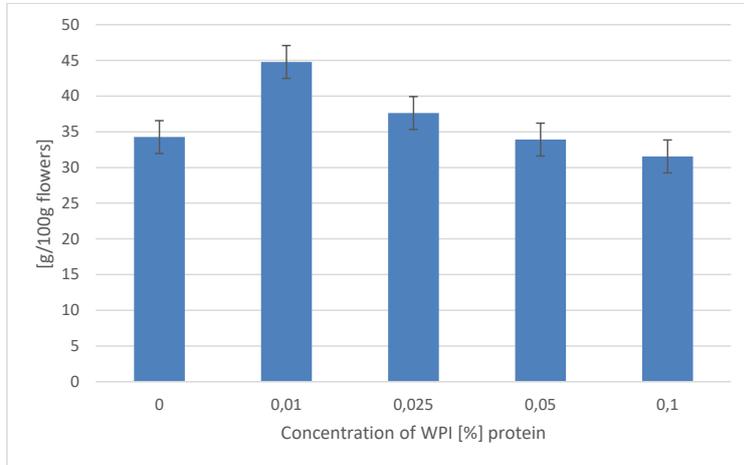
In flower's extracts, the extraction was more efficient in a 0,01% WPC protein solution. The total polyphenol content was higher than that of the control and WPC extracts at concentrations of 0,025%; 0,05% and 0,1%. These, however, did not differ between them. The average content of flavonoids was highest in the extract from the 0,01% protein solution compared to the aqueous extract and the rest of the tested solutions and amounted to an average of 0,92g / 100g flowers. Meanwhile, the others ranged from 0,65 to 0,69g / 100g of flowers. In the case of antioxidant activity also the highest values were reached by extracting flowers in a WPC protein solution of 0,01% concentration. Using the remaining concentrations, the results were statistically insignificant compared to the control extract.

WPI protein extracts

Extracts made of black elderberry flowers

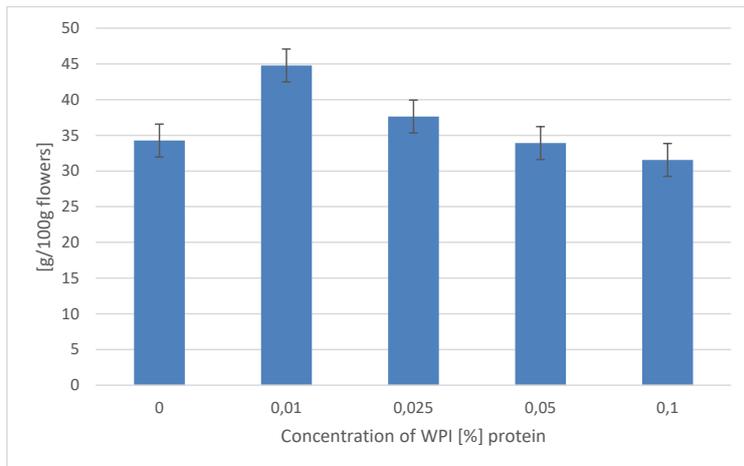
Mean total polyphenol content

Fig 4. Average content of total polyphenols in elderberry flowers depending on the concentration of WPI protein solution used.



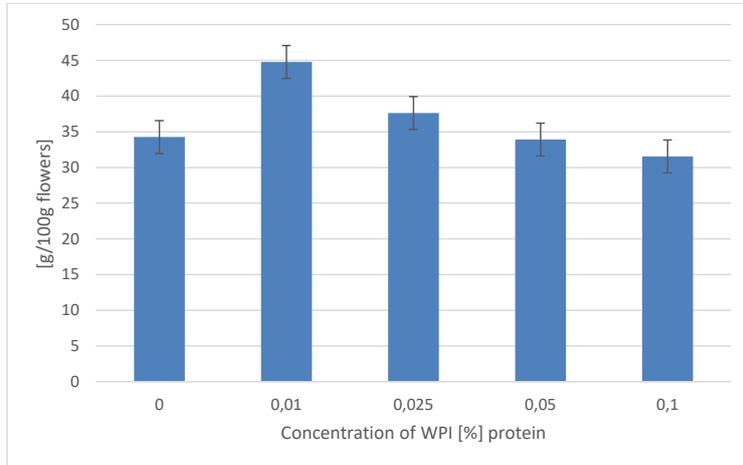
Average content of flavonoids

Fig. 5. Average content of flavonoids in elderberry flowers depending on the concentration of WPI protein solution used.



Average antioxidant activity

Fig. 6. Average antioxidant activity of extracts of elderberry flowers depending on the concentration of WPI protein solution used.

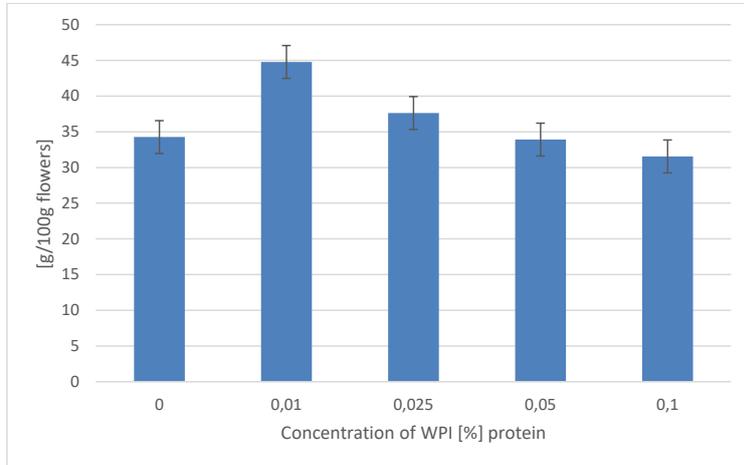


Extraction using different concentrations of WPI protein solution was also found to improve its performance. Total polyphenol content was highest when using 0,01%, 0,025% and 0,05% protein solutions, and the difference between them and the control extract was statistically significant. In contrast, the extract with 0,1% protein solution was statistically no different from the control extract. The highest content of flavonoids was also found in the WPI protein extract of 0,01% but this time it only differed statistically from the extract of flowers in water itself. The rest of the protein solutions used in the extracts did not differ statistically from the flower extract in water itself. The same situation applies to the average antioxidant activity of flower extracts. The highest was determined in a 0,01% protein solution extract and the mean difference was statistically different both from the control extract and the extracts using higher protein concentrations.

Extracts made of black elderberry fruit

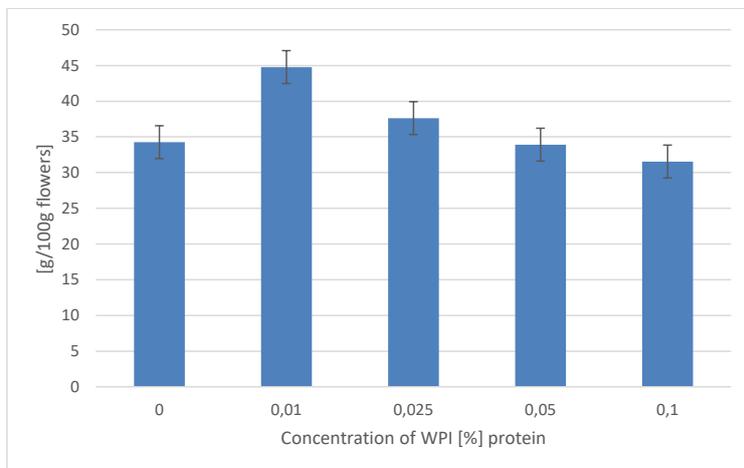
Mean total polyphenol content

Fig. 7. Average content of total polyphenols in elderberry fruit depending on the concentration of WPI protein solution used.



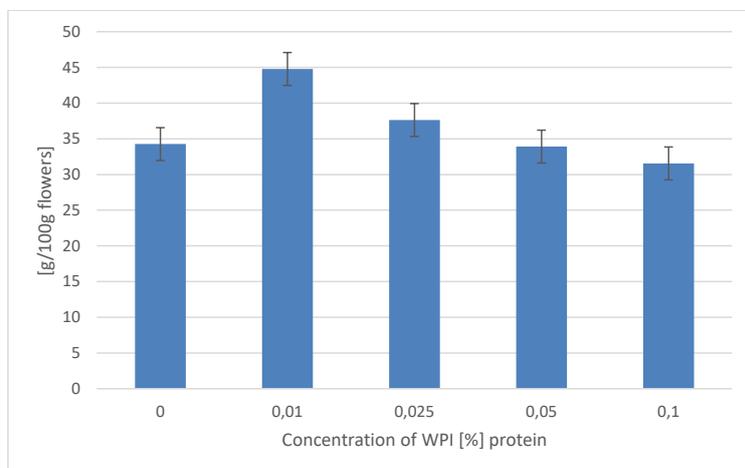
Average content of flavonoids

Fig. 8. Average content of flavonoids in elderberry fruit depending on the concentration of WPI protein solution used.



Average antioxidant activity

Fig. 9. Average antioxidant activity of black elderberry extracts according to the concentration of WPI protein solution used.



In fruit, the degree of extraction of polyphenol compounds did not differ statistically between control extract and protein extracts. The only significant difference was that the polyphenol compounds were better extracted with a 0,01% protein solution than the 0,1% protein.

However, for the average content of extracted flavonoids, a significant difference was observed between the control extract and the protein extract at a protein concentration of 0,01%. The more concentrated extracted flavonoids were compared with both the fruit extract and the higher protein extracts: 0,05% and 0,1%, respectively.

The same relationship was observed in antioxidant activity. Protein extract of 0,01% was the highest activity, and the result was statistically significant compared to aqueous extracts and protein extracts of 0,05% and 0,1%. However, these statistically significant differences did not differ from the control extract.

Conclusions

1. In the WPC and WPI protein extracts, the extraction of bioactive ingredients is more efficient than in the case of water extraction.
2. In WPC solutions, the extraction of both flowers and fruit was best achieved at a concentration of 0,01% protein solution and was more effective than control water extract.
3. In WPI solutions, fruit and flower extraction was best performed at a concentration of 0,01% protein solution and was superior to control water extract.
4. Both flowers and fruits are a rich source of antioxidants.

Literature

Z komentarzem [F.1]: polskie tytuły n angielski
przetłumaczyć w nawiasie

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