Thesis of doctoral (PhD) dissertation

THE ANTIOXIDANT COMPOUNDS IN SOUR CHERRY, AND THE ROLE OF ANTIOXIDANTS IN THE PREVENTION OF INFLAMMABLE PROCESSES IN THE BODY

Andrea Kun-Nemes

Supervisor:

Judit Gálné Dr. Remenyik

UNIVERSITY OF DEBRECEN

Kerpelyi Kálmán Doctoral School

Debrecen

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INTRODUCTION AND AIMS OF THE RESEARCH

Consumers are increasingly refraining from artificial additives. In terms of toxicology, synthetic antioxidants are not advantageous for pharmacological use, so more and more research groups are focusing on the study of phytonutrients. The analysis of plant extracts and the identification and quantification of the active ingredients in them, as well as the development of gentle extraction, high-efficiency isolation were started. At the same time, the investigation of the physiological effects of these bioactive components has become the focus of scientific interest.

At the beginning, these studies were carried out mainly among berries due to their outstanding antioxidant capacity, but the berries are no too ideal for the industry in terms of production (yield, harvest) and shelf life.

In contrast, the peel, flesh and kernels of cherries also contain a number of antioxidant compounds with biological activity. Our country has a huge assortment of varieties, the conditions for its cultivation are ideal and in Hungary the fruit grown of sour cherry is the largest amount after the apple, so we have a large amount at our disposal. Although a number of publications have been published on the study of antioxidants (anthocyanins), no systematic analysis has been performed in the case of Hungarian sour cherry cultivars, so our studies were aimed at examining the antioxidants of the most common hungarian sour cherry cultivars and their physiological effects.

Therefore, during my doctoral research, I set the following aims: to analyze the tocopherol content of oil obtained from the seeds of Hungarian sour cherries, to assess and compare the antioxidant capacity of major sour cherries grown in Hungary, to compare the methods, that are used to measure the antioxidant capacity of cherry varieties, the extraction and identification of extractable and non-extractable compounds in sour cherries, mapping of their usability, investigation of the effect of anthocyanin-rich sour cherry extract in diet-induced obese mice.

MATERIALS AND METHODS

Preparation and storage of cherries

The cherries were freshly deseeded, cut, pre-frozen (-80 °C, 2 hours), then lyophilized and stored frozen (-20 ° C) until extraction.

Experimental animals and their housing conditions

After arrival, the animals were habituated to the new environment for a week. Mice were housed in an animal room with 22–24 _C and 50–70% relative humidity. The lighting was set to 12 h light and 12 h dark period (lights o_ at 7 am). The animals received standard mouse chow (S8106-S011 SM R/M-Z+H; ssni_ Spezialdiäten GmbH, Soest, Germany) and tap water ad libitum during the acclimatization period.

A week later, the animals were randomly divided into three groups. The control group (n = 11) eceived standard mouse chow and tap water ad libitum. The second group (n = 12) was switched to a high fat diet (RM AFE 45% FAT SY (P), Special Diets Services, United Kingdom) and tap water supplemented with 5% sucrose (MEÉ). The third group (n = 12) received a high fat diet and sucrose as the second group did, but also received anthocyanin-rich tart cherry extract in a daily dosage (D) of 60 mg/kg dissolved in their drinking water (MEÉA). The experimental protocol lasted for six weeks. The weight of the animals was registered twice a week, the water consumption was registered on a daily basis, and the anthocyanin solution was prepared accordingly to maintain the daily dosage protocol. The sucrose solution was freshly prepared every day, and the anthocyanin solution was prepared on a daily basis according to the daily water intake (WId) and the body weight (BW) of the mice following the formula below:

$$\mathrm{c\ (mg/mL)} = \frac{\mathrm{D\ (mg/kg)} * \mathrm{BW(kg)}}{\mathrm{WI_d\ (mL)}}$$

Ethics

All experiments involving mice were conducted in accordance with the European Community guiding principles for the care and use of experimental animals and the University of Debrecen Ethics Committee for Animal Research (ethical code number: 15/2013/DEMÁB, the date of approval of ethical submission: 15 April 2013).

Thirty five male C57BL/6J mice (obtained fromInnovo Ltd. (Isaszeg, Hungary), the local distributor of The Jackson Laboratory) were used throughout the study.

Extraction of the oil content of cherry seeds

After removal of the flesh, the seeds were dried at room temperature, the weight of the seeds was weighed per variety, and the seeds were broken to separate the bark from the endosperm, which was homogenized in a mortar. The samples were stirred with 45 ml of n-hexane on a magnetic stirrer (1 hour). After extraction, the samples were filtered through filter paper and the resulting permeate was evaporated in a vacuum evaporator. As a final step in the method, the remaining apolar fraction was centrifuged (5 min, 10000 rpm). The oils were stored refrigerated (-20 °C) until instrumental measurements.

Preparation of lyophilized samples for determination of antioxidant capacity

To test for antioxidant activity, lyophilized samples were prepared at 25 mg / cm^3 in eppendorf tubes. The following solvents were used for the tests: FRAP - distilled water, DPPH - methanol, TEAC - methanol, ACW - distilled water, ACL - methanol. Separation of the solid and liquid phases was facilitated by laboratory centrifugation (5 min, 10000 rpm) and the supernatants thus obtained were used for the assays.

Extraction of anthocyanins

The method of Homoki et al. (2016) was used to extract the anthocyanin content of the samples.

Preparation of cherry extracts

For our studies, we used two extraction methods (solvent combinations 1 and 2) to extract the extractable antioxidants from sour cherries. The residue, that remained after the extractions was hydrolyzed to get the antioxidant compounds retained in bound form.

Extraction with the mixture of ethanol and water (Solvent combination 1)

200 g of sample was extracted with 150 mL ethanol (96%) and 150 mL distilled water for 2 h (1/A). The samples were centrifuged (Eppendorf Cetrifuge 5810R)) for 15 min at 4000 rpm and the supernatant was recovered. 150 mL ethanol (96%) was added to the residue, and the mixture was mixed for 2 h (1/B). After centrifugation (15 min, 4000 rpm), the supernatant was recovered. For the first time, supernatants from 1/A and 1/B were evaporated together (1/AS + 1/BS), but in the second case separately (1/AS and 1/BS). Evaporation was performed at 40 ^{o}C, 10 mbar. The residue (1/R) was lyophilised (ScanVac CoolSafe 55-4 Pro lyophilizer), homogenized (Gorenje SMK 150 B coffee grinder), and stored in freezer (-20 ^{o}C) before use.

Extraction of extractable antioxidants according to Saura-Calixtoa and Goñi [28] (Solvent combination 2)

During the dissolution, the solvents in solvent combination 1 were replaced by the first extraction in 300 mL of acidic methanol / water (50:50; pH 2) according to the method of Saura-Calixto nad Goñi (2006) and the second extractant was 300 mL of acetone / water (70:30, v / v), but all other parameters were unchanged.

Extraction of hydrolysable tannins

10–10 mg dried sour cherry residue powder from the two extractions were subjected to hydrolysis with 2 mL methanol and 200 μL sulphuric acid for 20 h at 85 ºC. Samples are then centrifuged (2500 g, 10 min) and supernatants recovered. The residues was washed with 2–2 mL distilled water two times [HARTZFELD et al., 2002]. The supernatant from 1/R residue is 1/RH, and the extract from 2/R residue is 2/RH.

Extraction of condensed tannins

10–10 mg dried sour cherry residue powder from the two extractions were treated with 3 mL HCl/butanol (5:95) and 100 μL FeCl3 (2 wt %) at 100 ºC for 3 h. After centrifugation (2500 g, 10 min), the supernatant was recovered. The residues were washed with 2–2 mL HCl/butanol (5:95) two times. The supernatant from 1/R residue is 1/RC, and the extract from 2/R residue is 2/RC [PORTER et al.,1985; REED et al., 1982].

Alkaline hydrolysis

0.1 g dried sour cherry residue powder from the two extractions were treated with 5 mL NaOH (4 mM) at 25 ºC for 1 h. After centrifugation (4000 rpm, 10 min), the supernatant was recovered. The supernatant from 1/R residue is 1/RA, and the hydrolysate from 2/R residue is 2/RA [ANOKWURU et al., 2018].

Enzymatic hydrolysis

We also attempted to extract the bound antioxidants from the dried residue, that remaining from the extraction with the two solvent combinations by enzymatic hydrolysis using three different enzymes. In case of protease and α-amylase, we followed the method of Anokwuru et al. (2018) while for pectinase we used the method of Guo (2017).

Preparation of blood samples

A total of 500 μL of whole, fresh blood was centrifuged for 10 min at 3000 rpm from every animal. Erythrocyte sediment (max 500 μL) was re-suspended three times in 1.5 mL normal saline solution and centrifuged again for 5 min at 3000 rpm each time before the supernatant was discarded. Lysis of the erythrocytes was achieved by adding 1 mL of cool water to the sediment and storing the sample at 4 _C in the dark for 15 min. Then, the sample was centrifuged for 5 min at 4 ^{o}C at 3000 rpm. The upper phase was frozen and stored at -70 ^{o}C for subsequent determinations.

Determination of antioxidant capacity by spectrophotometric methods

The extractable and non-extractable antioxidant capacity of cherries was determined by the following methods:

- The ferric reducing antioxidant power (FRAP) assay: FRAP was performed as previously described by Benzie and Strain (1999). It is based on the reduction of the Fe^{3+}-TPTZ complex to the ferrous form at low pH. As a result of the reaction, the color of the initially golden-yellow complex changes to blue. This reduction is monitored by measuring the absorption change at 593 nm.

- Method for determining antioxidant capacity based on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging: DPPH is a purple stable radical with characteristic activity at 517 nm. In the reaction that takes place during the analysis, the antioxidant compounds of the test sample provide H atoms to the DPPH radical, which reacts with them to lose their color, so that the absorbance decreases in proportion to the antioxidant capacity of the sample [BRAND-WILLIAMS et al., 1995].

- Determination of Trolox Equivalent Antioxidant Capacity (TEAC): the principle of the method was developed by Miller et al. (1993). The ABTS + cation used in the method is an artificially produced radical that reacts with the antioxidants in the sample. During the reaction, the originally dark green radical becomes colorless, so that the color intensity decreases and the degree of color change is proportional to the antioxidant capacity of the sample.

Determination of anthocyanins by pH differential method

One of the special properties of anthocyanins is that their color changes depending on the pH of their environment, so the measurement method uses this phenomenon. Samples were tested at two pH values (pH 1 and 4.5) and the measurement was also performed on two waves (530, 700 nm) [Lee et al., 2005].

Determination of vitamin C

In this measurement method, the reducing property of vitamin C is used, during the reaction an equivalent amount of Fe (II) ions is formed from Fe (III) ions. The latter form a colored complex in the presence of α, α'-dipyridyl reagent, thus the amount of vitamin C can be measured on a spectrophotometer [KANDRA, 2006].

Determination of total phenol content (TPC)

This reducing ability-based method was originally developed by Singleton et al. (1999). During the measurement, the originally yellow reagent turns blue due to the reducing action of antioxidant compounds, which can be monitored spectrometrically at 765 nm.

Determination of procyanidin content

Prior and colleagues (2010) developed this colorimetric method based on the reaction of 4-dimethylaminocinnamaldehyde (DMAC) and flavan-3-ols. The absorbance of the reaction products can be measured at 640 nm and compared to a standard which is A2-procyanidin.

Determination of antioxidant capacity by chemiluminometric method (PLC)

Antioxidant capacity was measured on a Photochem® (Analytik Jena AG, Germany) using the appropriate ready-to-use measuring kits: ACW kit: water-soluble components [POPOV and LEWIN, 1994], ACL kit: fat-soluble components [POPOV and LEWIN, 1996] to determine its antioxidant capacity, and ACW kit: to determine the concentration of superoxide dismutase (SOD) [POPOV and LEWIN, 1999].

Chromatographic methods

The following methods were developed and used for the quantitative and qualitative determination of each compound in cherries:

- UHPLC analysis of the sour cherry extract: Measurements were carried out using CromasterUltraRs UHPLC, equipped with diode array detector, automatic sampler and Agillent OpenLAB software. The sample components were separated on a Phenomenex Kinetex column (2.6 μm, XB.C18, 100A, 100 - 4.6 mm). UHPLC running conditions consisted of the following linear gradient steps 0 min solvent A 15%, 0–25 min solvent A to 30%, 25–30 min solvent A to 40%, 30–40 min solvent A to 50%. Solvent A: MeOH; Solvent B: 3% HCOOH (Formic acid) in water. Flow rate was 0.7 μL/min and oven temperature was kept at 25 ^{o}C. The

anthocyanin content was analyzed quantitatively by comparison with the corresponding authentic standards. UV–vis detection was used at 535 nm wavelength for anthocyanins and 340 nm for flavonoid and phenolic compounds. The appropriate amounts of sour cherry extracts were measured and dissolved in solvent A. Injection volume was 10 μL.

• UHPLC-MS analysis of sour cherry extract: The UHPLC system (Dionex Ultimate 3000RS) was coupled to a Thermo Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, USA) equipped with an electrospray ionization source (ESI). The HPLC separation was achieved on a Themo Accucore C18 column (100 mm × 2.1 mm × 2.6 μm). Sampler and oven temperature were maintained at 25 ^{o}C, flow rate was 200 μL /min.

Eluent A was water containing 0.1% formic acid and eluent B was methanol containing 0.1% formic acid. The following gradient elution program was used: 0 min, 95% A; 0–3 min, 95% A; 3–43 min, →0% A; 43–61 min, 0% A; 61–62 min, →95% A; 62–70 min, 95% A. 2 μL of the samples were injected in every run. The Q Exactive hybrid quadrupole-orbitrap mass spectrometer was operated with the following parameters: capillary temperature 320 ^{o}C, spray voltage 4.0 kV in positive and 3.8 kV in negative ionization mode. The resolution was set to 35000. The mass range scanned was 150–1500 m/z.

The maximum injection time was 100 ms. The resolution was set to 17,500 in the cases of MS2 scans. The collision energy was 35 NCE. Sheath gas and aux gas flow rates were 32 and 7 arb, respectively. Xcalibur 4.0 (Thermo Fisher Scientific Inc., Waltham, USA) software was used to collect and analyze data.

• Quantification of tocopherols by HPLC: Samples were analyzed by a Merck-Hitachi LaChrom HPLC consisting of the following systems: diode array detector L-7455, autosampler L-7250, interface L-7000, pump L-7100. "Manager" software was used to operate the chromatography system and to evaluate the results. A phenomenex luna NH2 5 μm, 250 mm × 4.6 mm (283104-16) column was used as the stationary phase. Separation was performed by isocratic elution (n-hexane: dioxane = 80:20) at a flow rate of 1 mL / min and detection was performed at 295 nm.

Measurement methods used for the analysis of blood samples

• Oral Glucose Tolerance Test (OGTT): Glucose tolerance was determined by means of an oral glucose tolerance test (OGTT) in week 6 of the experimental period. After an overnight fast, the baseline blood glucose levels were measured (0 min), then the mice were given 2 g/kg glucose orally. Blood glucose levels were determined by means of a glucometer (Accu-Chek,

Roche Diagnostics, Budaörs, Hungary) at 15, 30, 60, 90, and 120 min. Blood samples were collected by making a small incision in the lateral tail vein.

- Determination of plasma adiponectin and resistin concentrations: An enzyme-linked immunosorbent assay kit (MRP300 and MRSN00, R&D Systems, Minneapolis, USA) developed to measure adiponectin and resistin concentrations in mice was used according to the manufacturer's instructions.

- Quantification of cytokines: Plasma levels of cytokines / chemokines were determined by means of the MILLIPLEX MAP Mouse Metabolic Hormone Magnetic Bead Panel (MMHMAG-44K, EMD Millipore Corp., Billerica, MA, USA) according to the manufacturer’s instructions.

RESULTS

Cherry seed oil

The aim of the work was to investigate the oil yield of the Hungarian sour cherry cultivars and to assess their tocopherol content in order to create an alternative mode of use of sour cherry seeds. Accordingly, at first the weight ratios of the gut (endosperm) of different Hungarian cultivars to the bone shell were determined, after that the oil yields of the endosperms, and the amount of tocopherol isomers in the seed oil were analyzed.

Our values show that, the kernel accounts for an average of 20% of the whole seed, but for some varieties, such as cherries called 'Éva', this value can be significantly higher (25%). We found that the oil yield of the endosperm was between 10 and 15% and that three tocopherol isomers (alpha, gamma, delta) were present in measurable amounts in the extracted oil, as the beta isomer was not detected, but they were very rich in gamma-tocopherol, as its average concentration 1.19 mg γ-tocopherol / g endosperm.

Table 1. The amount of gamma-tocopherol in the whole seed.

Notations: * significantly different from 'E', 'Kántorjánosi' and 'Petri' cultivars ($p < 0.05$).

Meggyfajták	**Az γ-tokoferol koncentrációja a magban (mg/100 g)**
M	28,0±2,7*
A	29,3±2,9*
E	19,8±1,2
Éva	25,1±2,3*
Kántorjánosi	20,9±1,2
Petri	20,7±1,5

Summarizing the results (Table 1), we can say that, the production of seed oil is a good alternative for the processing of sour cherry seeds, because there are some varieties, where the oil extraction could be a particularly economical, efficient and ideal source of industrial gamma-tocopherol due to high active ingredient content. To facilitate this, further studies may be warranted to examine the varieties of sour cherries (the amount of sour cherries produced, seed oil and γ-tocopherol), that processed in large quantities in the industry.

Comparison of antioxidant content of cherry cultivars with different measurement methods

In the case of 12 Hungarian sour cherry cultivars, the capacity of all antioxidants was measured by five methods (FRAP, DPPH, TEAC, ACL, ACW). In addition, the vitamin C content of the samples was measured and the anthocyanin compounds were quantified in order to have a reference value for the sour cherry cultivars, against which we can determine the correctness of the obtained antioxidant capacity values and check the efficiency of the methods.

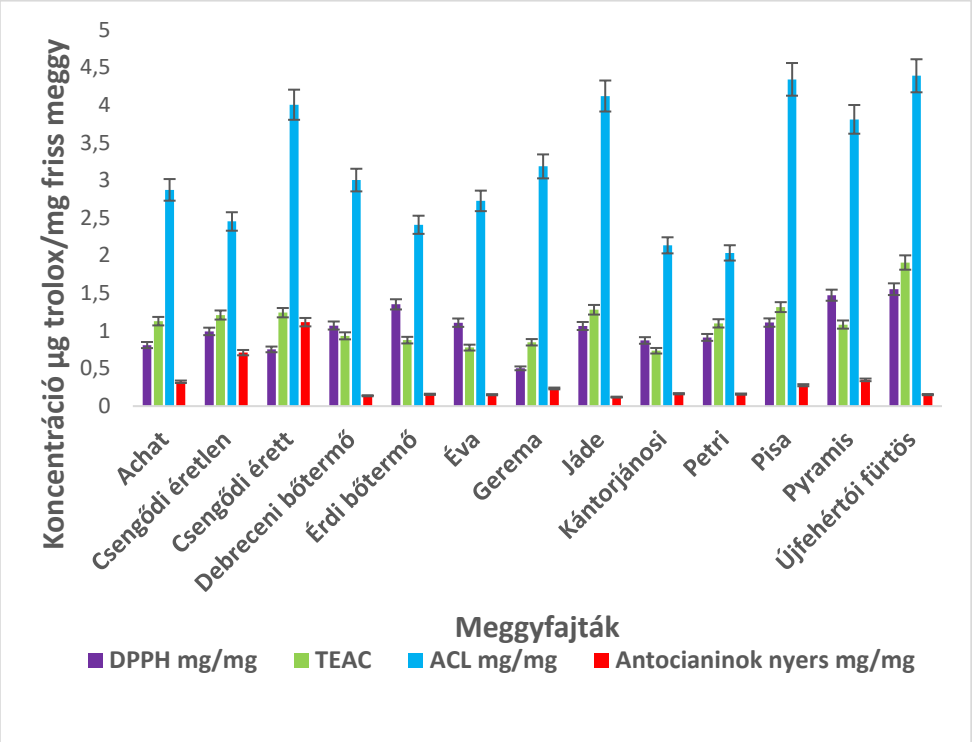

Figure 1. Comparison of fat-soluble antioxidant capacity and anthocyanin content of cherry cultivars measured by DPPH, TEAC and ACL methods.

We found that we were able to measure the amount of anthocyanins and vitamin C, respectively, in the case of lipid- (Figure 1) and water-soluble (Figure 2) methods, but we also found that there are significant differences between not only the varieties but also the measurement methods too.

It can also be said that chemiluminescence-based PCL (ACW, ACL) methods proved to be the most effective in determining the antioxidant capacity of cherries, as we measured the highest values, which were typically multiples of the results obtained by spectrophotometric measurements.

We also found that each variety contained different proportions of lipid- and water-soluble antioxidant compounds, and that in this sense the ‘Újfehértói fürtös’ variety was the most valuable of the examined cherries, so we used this fruit for our further studies and extracts.

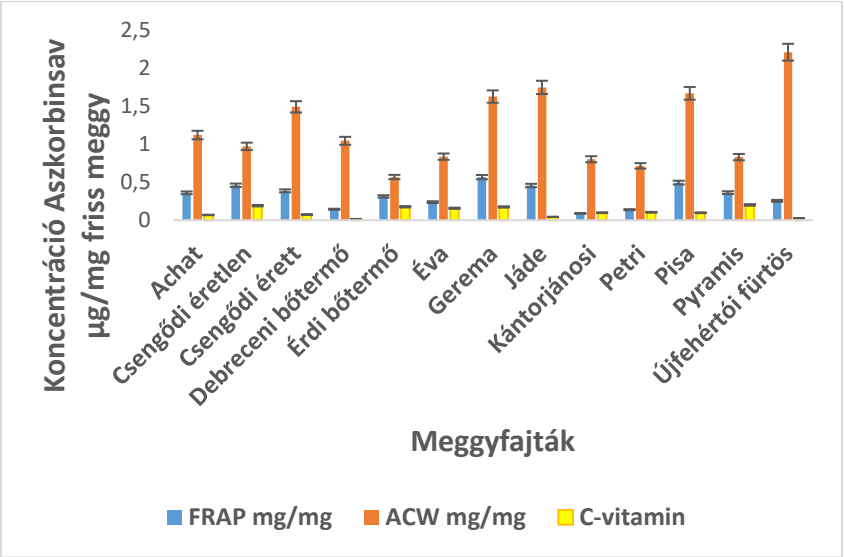

Figure 2. Comparison of water-soluble antioxidant capacity and vitamin C content of cherry cultivars measured by FRAP and ACW methods.

Extraction and identification of antioxidant compounds in sour cherry

For the quantitative and qualitative determination of antioxidants, extractable and non-exractable antioxidant compounds were separated by means of solvents and hydrolysing agents, and then the antioxidant capacity of the extracts was determined by analytical methods (Figure 3).

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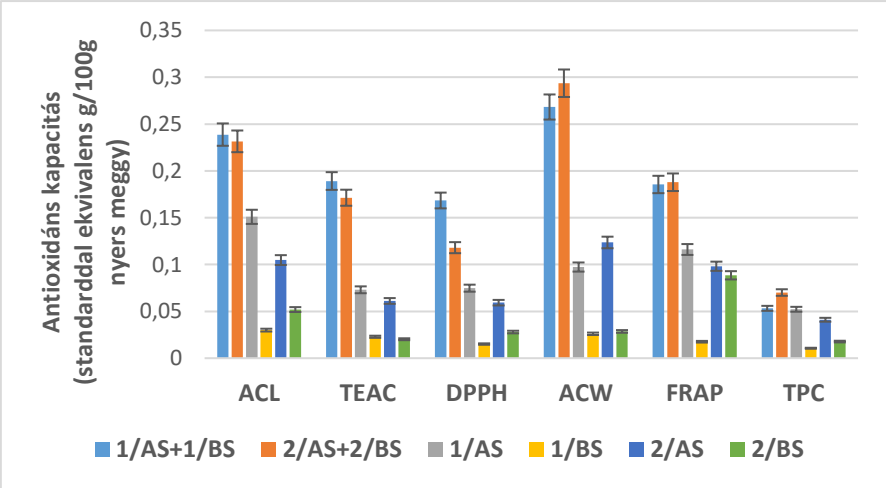

Figure 3: Comparison of antioxidant capacity and total polyphenol content of sour cherry extracts

Standard used for different measurement methods: ACL: trolox; TEAC: trolox; DPPH: trolox; ACW: ascorbic acid; FRAP: ascorbic acid; TPC: gallic acid. Abbreviations: 1 / AS: aqueous ethanolic extract; 1 / BS: ethanolic extract after aqueous ethanol dissolution; 1 / AS + 1 / BS: extract 1 / AS and 1 / BS together; 2 / AS: extract prepared with a mixture of acidic methanol and water; 2 / BS: aqueous acetone extract after extraction with a mixture of acidic methanol and water; 2 / AS + 2 / BS: extract 2 / AS and 2 / BS together.

Solvent antioxidants were extracted from the cherries with two multi-step solvent combinations, and then the extracts were compared by six different measurement methods (FRAP, DPPH, TEAC, ACL, ACW, TPC).

Based on the obtained results, we found that the antioxidant capacity and the total polyphenol content of the extracts obtained by the two extraction methods did not differ significantly, but we could also see that the mixtures used in each step of the solvent combinations alone were not suitable for complete extraction of bioactive compounds. its antioxidant capacity is significantly lower than the values obtained for solvent combinations.

In the case of cherries, the differences already observed in the efficiency of each measurement method were also shown in this study, and, as in previous experience, measurements based on chemiluminescence proved to be the most appropriate in this comparison.

In addition to the determination of antioxidant capacity, the extracted compounds were identified for both solvent combinations using UHPLC-MS and standards. We found that although there was a difference in the number of compounds recovered by the two extraction methods, the groups of compounds extracted (anthocyanins, procyanidins, flavonoids, and other polyphenols) were the same. This finding is very important to us because aqueous-alcoholic extraction (solvent combination 1) is more advantageous for the processing industry and based on the measured values this extraction method is suitable for the etxraction of the most important compounds of sour cherry (anthocyanins, procyanidins, phenolic components and flavonoids).

From the extract obtained with combination 1, we were also able to identify a compound which had not previously been detected in sour cherries. This component is cinchonain, whose anti-cancer properties have been confirmed by several studies.

To identify the main antioxidant compounds in sour cherries and to determine their concentration, an UHPLC chromatographic system were developed, which was later used in the quality control of the anthocyanin-rich sour cherry extract used for our in vivo experiments. The main anthocyanin compounds identified in the 'Újfehértói fürtös' variety are: cyanidin-3-O-glucosyl rutinoside, (2 mg / 100 g cherry), cyanidin-3-O-rutinoside, (183 mg / 100 g cherry), cyanidin-3-O - monoglucoside (4.29 mg / 100 g cherry).

In addition to anthocyanins, large amounts of quercetin, quercetin-3 rutinoside and apigenin (flavonoids) were also found in the samples, which are precursor compounds in the biosynthesis of anthocyanins. But it also contains large amounts of other phenolic compounds, such as chlorogenic and caffeic acid, which are characterized by high antioxidant activity.

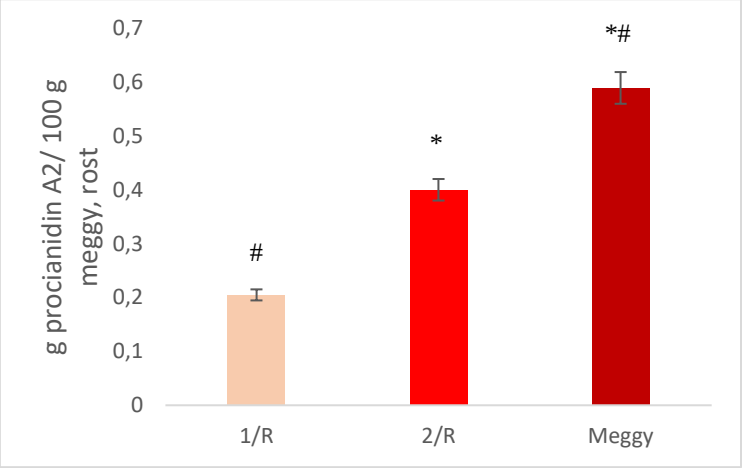

Figure 4. Total procyanidin content of cherries and cherries remaining after extraction.

Units: cherry sample, g procyanidin A2 / 100 g fresh cherries; fiber remaining after dissolution: g procyanidin A2 / 100 g dry residue. Abbreviations: 1/R: residue remaining from sour cherries dissolved by solvent combination 1; 2/R: residue remaining from the sour cherry dissolved by solvent combination 2. Notations: * significantly different from 1/R ($p < 0.05$); # is significantly different from 2/R ($p < 0.05$).

Analysis of the total procyanidin (PAC) content of cherry and residue (Figure 4) showed that although significantly more procyanin could be extract with solvent combination 1 than with method 2, the amount extract was significantly lower than the total procyanidins found in cherry, thus, a significant amount of procyanidin remained in bound form in the samples.

Additional antioxidant compounds were recovered by hydrolysis of the residue remaining after the extraction procedures, although enzymatic hydrolysis did not result in significant antioxidant activity.

Alkaline and acid hydrolysis have yielded outstanding results, and in these cases the total polyphenol content has also increased, so it is likely that low molecular weight phenolic derivatives from the degradation of bound polyphenols may cause the change. In addition, we found that the effectiveness of the measurement methods resulted in significant differences in this case as well, and that the moderate result of DPPH was greatly influenced by the presence of the strong acid

Overall, the amounts of antioxidants extracted from fresh sour cherry and obtained by hydrolysis of the extraction residues were very similar for the two solvent combinations used, and it can be stated that the acidic (HCl) methanol-acetone solution developed by Saura-Calixto and Goñi (2006) water solvent combination did not significantly extract larger amounts of antioxidant compound. Consequently, the dissolution efficiency is not affected if HCl and methanol are not used in the first step and if the acetone-water combination is replaced by 100% ethanol.

Impact assessment of anthocyanins

The main results of our experiment are illustrated in Figure 5. After reviewing the results and correlations, we found that chronic oral anthocyanin treatment did not protect animals from diet-induced weight gain, and we found that using a high-fat diet and sucrose-supplemented tap water could induce fasting hyperglycemia. chronic anthocyanin treatment could not significantly reduce.

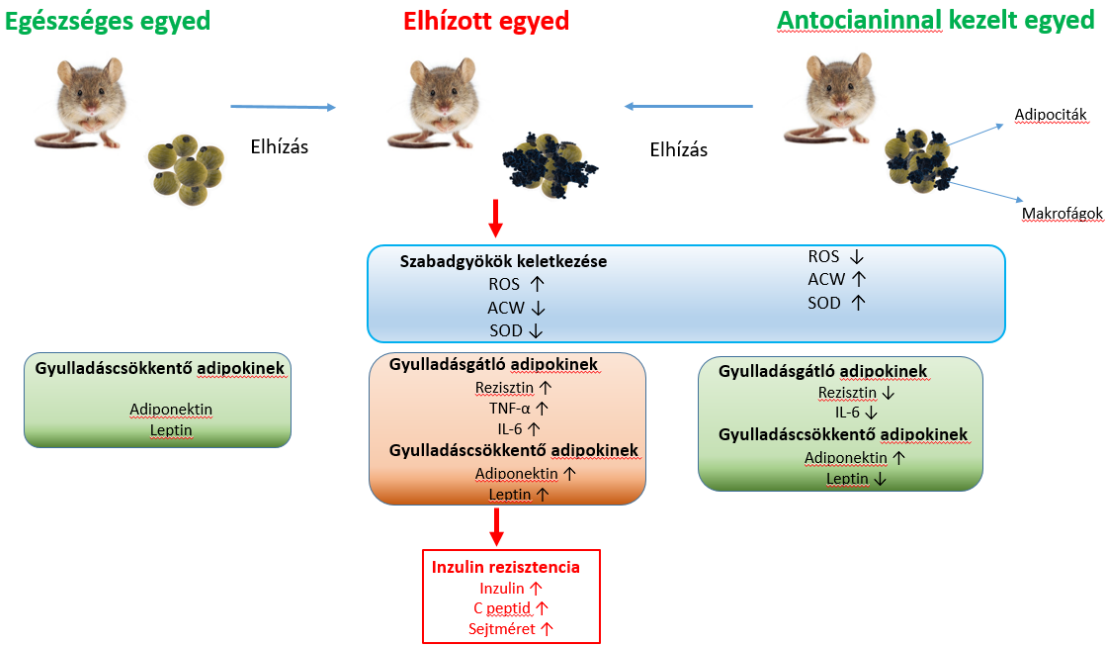

Figure 5. Graphical interpretation of the results of a pet experiment.

We can see that by the end of the experimental period, a high-energy diet resulted in a marked decrease in glucose tolerance in both the MEÉ (high-energy diet) and MEÉA (high-energy diet with anthocyanin-rich sour cherry extract) groups. We also found that the increased insulin response to increased C-peptide secretion associated with oral glucose loading during anthocyanin treatment was not able to significantly offset the elevated changes in blood glucose levels, despite the fact that cyanidin-3-o-β is able to increase cellular insulin sensitivity by inactivating JNK stress kinase or not converting the serine insulin receptor-1 substrate [AGUIRRE et al., 2000].

We found that transient increases in fatty acid levels could also be considered as physiological stimulation of insulin production, as insulin secretion may be temporarily increased to maintain metabolic balance, however, prolonged fatty acid overload impairs β-cell function. In our experiment, we found that a hypercalorie diet increases insulin secretion, and anthocyanin treatment in this case could not offset the effect of a high-energy (high-fat and high-sugar) diet.

Furthermore, we were able to demonstrate that a fat- and sugar-rich diet impairs antioxidant activity (ACW, SOD), but chronic anthocyanin treatment was able to counteract the decrease.

Overall, therefore, in our studies, MEÉ mice showed significant differences compared to healthy controls, but we have to point out that it is difficult to compare the studies in all details because of significant differences in obesity or insulin resistance model (rodent strain, chemical composition, extracts, dosage, duration of treatment) are reported in the literature. Based on this, we can hypothesize that there are differences in the expression patterns of the adipokine gene, which may explain why adiponectin levels did not change in either the obesity model or the response to anthocyanin treatment. It should be emphasized, however, that chronic treatment with sour cherry extract reduced leptin and IL-6 levels, demonstrating that the amount of anthocyanin used has an anti-inflammatory effect, and it can be assumed that higher doses of anthocyanin and / or longer periods of treatment can effectively improve insulin sensitivity. in the diet-induced obesity model, but the presentation of our hypothesis requires further experiments.

Our measurements were able to demonstrate that a diet rich in fat and sugar impairs plasma antioxidant activity, but chronic anthocyanin treatment can offset the decrease in antioxidant capacity in our obesity model and produced a slight yet significant increase compared to healthy controls.

NEW SCIENTIFIC RESULTS

1. We have proved that, the ratio of kernel: husk and the oil yield of endosperms are different in Hungarian sour cherry seeds. We found more than 6% differences in the composition of the seeds between some varieties, and almost 7% in the oil yield. The seeds were characterized by significant γ-tocopherol content, that can reach 1.6 mg/g endosperm, while β-tocophorol was not detectable.

2. We proved that the "Újfehértői fürtös" can be characterized by the highest water- and fat-soluble antioxidant capacity (ACW: 2.2 μg/mg; ACL: 4.4 μg/mg) of the Hungarian sour cherry cultivars, and we have developed a new extraction method for the extraction of its valuable compounds using an aqueous-ethanol solvent mixture.

3. In addition to the known anthocyanin compounds, cinchonine was first identified in sour cherries by UHPLC-MS.

4. We have shown in our pet experiments that the use of anthocyanin-rich sour cherry extract can reduce the elevated levels of leptin, IL-6 and resistin caused by a high-fat and high-sugar diet.

In our study, leptin levels decreased by 41%, IL-6 levels by 23%, and resistin levels by 9.5%, thereby demonstrating that the anthocyanin concentration ((60 mg/kg) used had an anti-inflammatory effect in the diet-induced obesity model. Chronic anthocyanin treatment was able to counteract the decrease in antioxidant capacity observed in the obesity model and a significant increase (43 %) compared to the healthy control.

ADAPTABLE RESULTS IN PRACTICE

We hope that our results can be used in practice for the following in the future:

1. We were able to identify some varieties of cherries that have an outstanding seed core and could be a source of γ-tocopherol for the industry, these are the ‘M’, ‘A’ and ‘Éva’ varieties.
2. We have shown that there is a significant difference in antioxidant capacity between the varieties and that among the methods used, PCL (ACW, ACL) methods based on chemiluminescence are the most suitable.
3. We found that the 'Újfehértói fürtös' variety is the richest in water and fat-soluble antioxidant compounds (ACW: 2.2 μg/mg; ACL: 4.4 μg/mg) and has outstandingly high anthocyanin content (0.15 μg/mg).
4. We have developed a method for preparing an anthocyanin-rich extract of cherries, which can be used for both food and pharmaceutical purposes.
5. The experience of our pet experiment may provide a basis for further anthocyanin-based studies in the future in the diet-induced obesity model.

REFERENCES

Aguirre V., Uchida T., Yenush L., Davis R., White M. F. (2000): The c-Jun NH2-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser307. *Journal of Biological Chemistry*, 275: 9047–9054.

Anokwuru C., Sigidi M., Boukandou M., Tshisikhawe P., Traore A., Potgieter N. (2018): Antioxidant Activity and Spectroscopic Characteristics of Extractable and Non-Extractable Phenolics from Terminalia sericea Burch. ex DC. *Molecules*, 23 (6): 1303.

Benzie I. F. and Strain J. J. (1999): Ferric reducing antioxidant power assay; direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods in Enzymology*, 299: 15-27.

Brand-Williams W., Cuvelier M. E., Berset C. (1995): Use of a free radical method to evaluate antioxidant activity. *Journal of Food Science and Technology*, 28: 25–30.

Guo L. (2017): Enzymatic hydrolysis of lotus rhizome starch using alpha-amylase and glucoamylase. *Journal of Food and Nutrition Reserch*, 56: 372–380.

Hartzfeld P. W., Forkner R., Hunter D. M. Hagerman A. E. (2002): Determination of hydrolysable tannins (gallotannins and ellagitannins) after reaction with potassium iodate. *Journal of Agricultural and Food Chemistry*, 50: 1785–1790.

Homoki J. R., Nemes A., Fazekas E., Gyémánt Gy., Balogh P., Gál F., Al-Asri J., Mortier J., Wolber G., Babinszky L., Remenyik R. (2016): Anthocyanin composition, antioxidant efficiency, and α-amylase inhibitor activity of different Hungarian sour cherry varieties (Prunus cerasus L.). *Food Chemistry*, 194: 222–229.

Kandra L. (2006): Biokémiai gyakorlatok.

Lee J., Durst R. W., Wrolstad, R. E. (2005): Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative Study. *Journal of AOAC International*, 88: 1269–1278.

Miller N. J., Rice-Evans C. A., Davies M. J., Gopinathan V., Milner A. (1993): A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clinical Science*, 84, 407–412.

Popov I. N., Lewin G. (1996): Photochemiluminescent detection of antiradical activity; IV: Testing of lipid-soluble antioxidants. *Journal of Biochemical and Biophysical Methods*, 31: 1–8.

Popov I. N., Lewin G. (1999): Antioxidative homeostasis: Characterization by means of chemiluminescent technique. *Methods in Enzymology*, 300: 437–456.

Popov I.N., Lewin G. (1994): Photochemiluminescent detection of antiradical activity. 2. Testing nonenzymic water-soluble antioxidants. *Free Radical Biology and Medicine*, 17, 267–271.

Porter L., Hrstich L., Chan B. (1985): The conversion of procyanidins and prodelphinidins to cyaniding and delphinidin. *Phytochemistry*, 25: 223–230.

Prior R. L., Fan E., Ji H., Howell A., Nio C., Payne M. J., Reed J. (2010): Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders. *Journal of the Science of Food and Agriculture*, 90: 1473–1478.

Reed J., McDowell R. E., Van Soest P. J., Horvath P. R. J. (1982): Condensed tannins: A factor limiting the use of cassava forage. *Journal of the Science of Food and Agriculture*, 33: 213–220.

Saura-Calixto, F., Goñi, I. (2006): Antioxidant capacity of the Spanish Mediterranean diet. *Food Chemistry*, 94: 442–447.

Singleton V. L., Orthofer R., Lamuela-Raventós R. M. (1999): Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in Enzymology*, 299: 152–178.

PUBLICATIONS

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Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

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A PhD értekezés alapjául szolgáló közlemények

Magyar nyelvű tudományos közlemények hazai folyóiratban (2)

1. Biró, A., **Kun-Nemes, A.**, Gálné Remenyik, J.: A meggymag mint ipari gamma-tokoferol forrás. *Agrártud. Közl. 63*, 27-33, 2015. ISSN: 1587-1282.
2. **Kun-Nemes, A.**, Stefanovits-Bányai, É., Gálné Remenyik, J.: Új mérési eljárások fejlesztése a növényi antioxidáns státusz meghatározására. *Agrártud. Közl. 63*, 105-112, 2015. ISSN: 1587-1282.

Idegen nyelvű tudományos közlemények hazai folyóiratban (1)

3. **Kun-Nemes, A.**, Homoki, J., Kiss, R., Stündl, L., Gálné Remenyik, J.: Effect of anthocyanin-rich Hungarian tart cherry extract on blood antioxidant status in C57BL/6J mice. *Agrártud. Közl. 150th Anniversary Issue*, 335-341, 2018. ISSN: 1587-1282. DOI: http://dx.doi.org/10.34101/actaagrar/150/1728

Idegen nyelvű tudományos közlemények külföldi folyóiratban (2)

4. **Kun-Nemes, A.**, Homoki, J., Kiss, R., Hegedüs, C., Kovács, D., Peitl, B., Gál, F., Stündl, L., Szilvássy, Z., Gálné Remenyik, J.: Effect of Anthocyanin-Rich Tart Cherry Extract on Inflammatory Mediators and Adipokines Involved in Type 2 Diabetes in a High Fat Diet Induced Obesity Mouse Model. *Nutrients. 11* (9), 1-17, 2019. EISSN: 2072-6643. DOI: http://dx.doi.org/10.3390/nu11091966 IF: 4.546
5. **Kun-Nemes, A.**, Szöllősi, E., Stündl, L., Biró, A., Homoki, J., Szarvas, M. M., Balogh, P., Cziáky, Z., Gálné Remenyik, J.: Determination of Flavonoid and Proanthocyanidin Profile of Hungarian Sour Cherry. *Molecules. 23* (12), 1-20, 2018. ISSN: 1420-3049. DOI: http://dx.doi.org/10.3390/molecules23123278 IF: 3.06

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Magyar nyelvű absztrakt kiadványok (1)

6. **Kun-Nemes, A.**, Szilvássy, Z., Patonay, T., Fári, M., Gálné Remenyik, J.: Antioxidáns kapacitás meghatározása kemiluminometriás módszerrel.
In: XVII. Növénynemesítési Tudományos Napok Összefoglalók. Szerk.: Óvári Judit, Planet Corp. Szolgáltató Kft, Szeged, 66, 2011. ISBN: 97896308.12351

Idegen nyelvű absztrakt kiadványok (1)

7. **Kun-Nemes, A.**, Homoki, J., Kiss, R., Stündl, L., Gálné Remenyik, J.: Effect of anthocyanin-rich tart cherry extract on blood antioxidant status in a high fat diet induced obesity mouse model.
In: 9th Central European Congress on Food, Food Science for Well-being : Abstract book. Ed.: L. Gaceu, M. Mironescu, G. Mohan, Lucia Blaga University of Sibiu Press, Sibiu, 146, 2018. ISBN: 9786061215461

További közlemények

Magyar nyelvű tudományos közlemények hazai folyóiratban (3)

8. **Kun-Nemes, A.**, Fehérné Baranyai, E., Gálné Remenyik, J.: A meggy jelentősége a vaspótlásban.
Agrártud. Közl. 63, 101-104, 2015. ISSN: 1587-1282.

9. Homoki, J., **Kun-Nemes, A.**, Gálné Remenyik, J.: A meggy mint funkcionális élelmiszer.
Agrártud. Közl. 55, 41-47, 2014. ISSN: 1587-1282.

10. Bódi, É., Gálné Remenyik, J., **Kun-Nemes, A.**, Homoki, J., Peles, F., Fekete, I., Kovács, B.: Szelénnel dúsított étkezési csírák antioxidáns aktivitásának meghatározása, valamint mikrobiológiai vizsgálatuk.
Agrártud. Közl. 52, 25-30, 2013. ISSN: 1587-1282.

Idegen nyelvű tudományos közlemények külföldi folyóiratban (2)

11. Homoki, J., **Kun-Nemes, A.**, Fazekas, E., Gyémánt, G., Balogh, P., Gál, F., Al, A. J., Mortier, J., Wolber, G., Babinszky, L., Gálné Remenyik, J.: Anthocyanin composition, antioxidant efficiency, and alpha-amylase inhibitor activity of different Hungarian sour cherry varieties (Prunus cerasus L.).
Food Chem. 194 (1), 222-229, 2016. ISSN: 0308-8146.
DOI: http://dx.doi.org/10.1016/j.foodchem.2015.07.130
IF: 4.529

12. Bódi, É., Gálné Remenyik, J., **Kun-Nemes, A.**, Homoki, J., Peles, F., Fekete, I., Kovács, B.: The determination of antioxidant activity of selenium-enriched wheat and pea sprouts, as well as their microbiological analysis.
Scientific papers. Series A., Agronomy. 56., 196-201, 2013. ISSN: 2285-5785.

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Magyar nyelvű konferencia közlemények (1)

13. Bódi, É., Gálné Remenyik, J., **Kun-Nemes, A.**, Homoki, J., Fekete, I., Kovács, B.: A szelénkezelés és az antioxidáns aktivitás közötti kapcsolat vizsgálata étkezési csírák esetén. In: XIX. Ifjúsági Tudományos Fórum, [s.n.], [Keszthely], 1-6, 2013.

Magyar nyelvű absztrakt kiadványok (1)

14. Homoki, J., **Kun-Nemes, A.**, Hüse, C., Fári, M., Gálné Remenyik, J.: A cyanidin-3-O monoglükozid felhalmozódása a magyarországi meggy fajtákban. In: XVII. Növénynemesítési Tudományos Napok. Összefoglalók: Növénynemesítéssel kultúrnövényeink sokféleségéért. Szerk.: Óvári Judit, Gabonakutató Nonprofit Közhasznú Kft., Szeged, 68, 2011. ISBN: 978963081235

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