Thesis of doctoral (PhD) dissertation

THE ROLE OF ANTHOCYANINS AS NATURAL HUMAN SALIVA AMYLASE INHIBITORS ISOLATED FROM HUNGARIAN SOUR CHERRY VARIETIES IN THE PREVENTION OF CARIES

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1. INTRODUCTION AND AIMS OF THE RESEARCH

The World Health Organisation (WHO) showed the first global map about dmf number of cariological status of 12-year old children in 1969. These results certified that in the industrial countries the prevalence of the caries is at a high level, while in the developing countries its presence is low. Some years ago, WHO developed a mouth disease monitoring system, espeially in connection with caries of children. According to the reports, in Europe 20-90% of 6-year old children have a tooh with a hole in it, and about 100% of adults have experienced this disease. Caries is a multifactorial, civilization folkdisease which causes local destroying of the hard tissue of teeth. It is caused by acidic by-products of bacterial fermentation of carbohydrates from food (Marsh et al., 1999; Fejerskov et al., 2003). In the last decade, interest in the medicine from herbs significantly increased. It is well-documented, that herbs and natural compounds have important antibacterial effects against different microorganisms including cariogen and periodontal pathogens. The natural phytochemicals are effective alternatives of antibiotics and can prevent and treat caries and other mouth infections (Yamanaka et al., 2004.; Hevesi et al., 2012.; Zhou et al., 2016.; Balci Yuce et al., 2016). Several studies deal with biologically active components of sour cherry and we know the exact structure of anthocyanins (Silva et al., 2002) and melatonin which certifies their role in eliminating free radicals (Martín et al., 2000.; Galano et al., 2013.; Reiter et al., 2014). From the structure we can conclude that these anthocyanins are farmacologically very active molecules. A lot of studies write about the effects of anthocyanins on enzymes (Wang et al., 1999; Jayaprakasam et al., 2005; Akkarachiyasit et al., 2010). So our aim was to investigate the effect of the anthocyanins in the Hungarian sour cherry varieties on the activity of human saliva amylase and what effects they have on the number of *Streptococcus mutans* cells in the oral cavity and to identify the role of anthocyanins in the prevention of caries.

2. MATERIALS AND METHODS

2.1 MEASURE METHODS DURING *IN VITRO* INVESTIGATION

2.1.1 Sample preparation for measuring dry content

Sour cherry was grinded, then frozen at -20°C for 8 hours. We lyophilized it with CHRIST ALPHA 1-4 LSC equipment at -20°C, 1,030 mbar pressure, for 16 hours. We repeated lyophilization three times. The lyophilized samples were milled in mortar, then from the measured mass we calculated the dry content of each varieties.

2.1.2 Extraction methods and UHPLC analysis

We chose bathing from extraction methods, which we continuously swirled. Its advantage is that from the same starting materials we get the extract with the same active agent content.

Anthocyanin extraction and UHPLC analysis

All the samples were homogenised with Braun Multiprimer (300 Watt) equipment. The extraction miwture is methanol: water: acetic acid 25:24:1. It was swirled with a magnetic stirrer (MSH 300, BIOSAN) for 60 minutes at room temperature. The extract was filtered and evaporated at 40°C with BÜCHI ROTAVAPOR R-210 (Switzerland). The samples were redissolved in formic acid solution (3%). Then sour cherry extract was fractionated, pre-conditioned with Supelclean ENVI-18 SPE column in order to separate anthocyanins more easily (Kim és mts., 2005). After purification the fraction rich in anthocyanin was evaporated.

The samples were investigated with CromasterUltraRs ultra high pressure liquid chromatography, detector with diode-line, automatic sample-taker and Agillent OpenLAB software, where stationary phase was Phenomenex Kinetex 2,6 μ, XB.C18, 100A, 100 x 4.6 mm. The moving phase was methanol on pump, 3% formic acid on B pump (Mozetic et al., 2002). We did the detection at λ=535 nm and applied gradient elution (0 min: 15:85, 25 min: 30:70, 30 min: 40:60, 40 min: 50:50). The identification of the components was done by HPLC standards: Cyanidin-3-glucoside chloride (AppliChem), Malvidin-3-glucoside chloride (AppliChem), Malvidin-3,5-diglucoside chloride (AppliChem), Cyanidin-3-ruthinoside chloride (Fluka).

We did three paralel measurements for each sample. We gave the anthocyanin concentration in mg/100g fresh mass.

Melatonin extraction and HPLC analysis

All the samples were homogenized with Braun Multiprimer (300 Watt) equipment. The extraction mixture consist of 600 ml chloroform and 300 µl 0,8 M Na_2HPO_4. It was swirled with a magnetic stirrer (MSH 300, BIOSAN) for 40 minutes at room temperature in dark because the molecule is sensitive for the light (Reiter, et al., 2005, modified by Remenyik, but not published 2012). Rude filtration was applied, then the two phases were separated in a separation cone. 2 spoons of dried magnesium-sulphate were added to the organic phase. Organic phase was evaporated with Büchi Rotavapor R-210/215 (Switzerland) vacuum evaporator in 40°C water bath, the extract was evaporated dry. The samples were analysed with Hitachi LaChrom high pressure liquid chromatography, diode-line detector L-7455, automatic sample-taker L-7250, interface L-7000, pumpa L-7100 and HPLC Manager software, where the stationary phase was Chromolith RP-18e 100 x 4,6 mm. The moving phase was 18% acetonitrile on pump A, Sorensen buffer on pump B (Burkhardt, és mts., 2001). Detection was done at λ=275 nm and isocratic elution was applied. Melatonin was identified with HPLC-pure Melatonin standard (Alfa Aesar). We did three paralel measurements for each sample. We gave the melatonin concentration in ng/100g fresh mass.

2.1.3 MALDI-TOF MS

To investigate the sour cherry extract from 'VN1' variety among 'Bosnyák' landraces andeach component of the HPLC fraction we recorded mass spectrum with MALDI-TOF Mass Spectrometer (Bruker Biflex III) in a positive ion way. The matrix was 2,5-dihidroxi-benzoe acid (DHB) or 2,3,6-trihidroxi-acetophenon (THAP). We calibrated the equipment for measuring m/z values of $[M+Na]^+$ ions of anthocyanins and melatonin m/z értékeire (449,26g/mol; 232,29 g/mol) with outer calibration.

2.1.4 Determination of anthocyanins with pH differencial method

2 g of homogenised samples was extracted in 10 ml 2% (V/V) HCl-Ethanol solution. We made pH=1,0 buffer, which consists of 67 ml HCl-solution and 25 ml KCl-solution. KCl solution: 1,49 g KCl is dissolved in 100 ml distilled water. HCl solution: 1,7 ml

cocentrated HCl is diluted in 100 ml distilled water. pH=4,5 buffer: 1,64 g Na-acetate is dissolved in 100 ml distilled water, then concentrated HCl is added until pH=4,5. The measure is simultaneously done at λ=530 and 700 nm (Amersham Biosciences Ultraspec 2100 pro type spectrophotometer). During the measure 400 µl sample is added into 3600 µl buffer (Lee et al., 2005). Three paralel measures are done. Anthocyanin concentration is given in mg cianidin-3-glucoside equivalent /100 g value for fresh and dry mass. Calculation with the following formula:

$$\Delta A = (A_{530nm}\ pH1,0 - A_{700nm}\ pH1,0) - (A_{530nm}\ pH4,5 - A_{700nm}\ pH4,5)$$

$$\%\left(\frac{m}{m}\right) = \frac{\Delta A}{\varepsilon L}\ MD\frac{V}{m}\ 100$$

A: Absorbance
ε: 26900
L: length of cuvette (1 cm)
M: Molar weight (449,2 g/mol)
D: Dilutions
V: sample volume (ml)
m: measured sample mass (mg)

2.1.5 Measure of antioxidant capacity

Antioxidant capacity of each sample was determined by photochemiluminescent (PCL) method with Photochem (Analytik Jena, Germany) equipment. The cirve was evaluated with PCLsoft (Analytik Jena AG, Jena, Germany) software.

2.1.6 The determination of the participants in clinical examination

If we want to examine the occurence of caries, we need quantitative measure numbers. This is caries index. Caries forms a hole after a certain period of time which causes irreversible change on teeth. If this state is durable, an untreated lesion forms (fillings, crowned and lost teeth). Teeth suffer from caries can be determined with DMF- measure number, where D is decayed, F is filled and M is missing tooth (DMF-T) or surface (DMF-S).

Every volunteer took part in a detailed history recording. We got information about the caries index of the participants, which means the number of decayed, lost and filled tetth for permanent and temporary teeth (DMF-T, dmf-t). The number of decayed surfaces was determined (DS). Besides caries, other oral infection and systematic disease did not occur.

The participants had to be suitable for the following citeria: a written agreement (patient prospectus and declaration) and suitable age. The criteria of exclusion are smoking and regular drug-taking, allergic reaction for sour cherry and/or the components of the chewing gum. Every participant filled a questionnare about the health state, eating and dental care habits of the patient.

2.2 APPLIED METHODS DURING THE CLINICAL EXAMINATION

2.2.1 Production of sour cherry extract

For our examination and the production of the chewing gum 'VN1' variety selected from „Csengődi csokros" was used. Fruit samples were taken to the laboratory, washed, pitted and homogenized with a Braun Multiquick hand blender. The totally homogenized samples were extracted with twice volume of sour ethanol (0,1% HCI) with a magnetic stirrer for 1 hour. Then the samples were filtered and the solvent was evaporated at 40°C in a vacuum rotation evaporator (Heidolph, Germany). Previously microbiological examination was done, which certified that the developed extract can be used for human research. The results of microbiological examination are shown in 1. appendix.

2.2.2 Production of chewing gum with or without sour cherry extract

The chewing gum was produced with a melting method by Dr. Váradi Judit and her collegues, DE GYTK Pharmacology Department. Geminis T BHA rubber basic (CAFOSA), xilit, citric acid, glicerin, sacharin (Sigma), peppermint volatile oil, Kirsch aroma (Akras) were used for the product. Xilit was dissolved in pure water at 80°C, then rubber basic was added and melted in water bath. The flavour -masking substances, flavorings (citric acid, glicerin, sacharin) and 0,1 g 'VN1' sour cherry extract with 11,87 mg/100mg anthocyanin concentration were dissolved in pure water. In control chewing gum the sour cherry extract was not added into the water phase. The water phase was emulsified in the melting rubber basic phase at 60°C. Finally, the peppermint volatile oil was added at 40°C. The mixture was divided into 2,5 g pieces and ball like products were made. After the 12-hour conditioning time, the chewing gum balls were stored at 8-15°C in a plastic jar until utilization.

2.2.3 The examination of the chewing gum

During our study, we determined the percentage of the sour cherry released from the chewing gum in connection with the time. A mortar was used because there was not an equipment with a bubble chamber. Chewing was modelled with the pistilus of the mortar, which was always done by the same person because of 1 pressure effect/second frequency. The releasing medium which symbolized the saliva had pH=6,00 according to VIII. Hungarian Medicine Book. During our study, 20,0 ml releasing medium was added into the sample. In 1, 2, 3, 4, 5, 7, 9, 11, 13, 15 minutes 0,5 ml sample was taken, which was supplemented back after each sample-taking. The test-tubes were labelled and the absorbance of the samples was measured at λ= 530 nm with spectrophotometer. The study was done with chewing gum without sour cherry extract and these results were used as control. We get the absorbance of the sour cherry extract from the difference between the absorbance of the chewing gum with sour cherry extract and that of the control (chewing gum without sour cherry extract). From these results the percentage of the sour cherry released from the chewing gum in connection with the time was determined.

We certified that 45% of anthocyanin content of the chewing gum released during the 15-minute examination. The anthocyanin concentration releasing at a certain period of time was the most intensive in the first 2-3 minutes which is ideal for the prevention of caries.

2.2.4 The measure of α-amilase activity of saliva samples

For the kinetical measure 50 mM pH = 6,0 MES buffer was used, which contained 5 mM calcium acetate, 51,5 mM NaCl and 152 mM NaN_3 (Paolucci et al., 2002). To set pH NaOH and HCl were used. The buffer was stored in the fridge until utilization. Short substrate was applied (chromogen 2-chlor-4-nitrophenil-4-O-α-D-galactopiranozil-maltozid ($GalG_2CNP$, SORACHIM SA, Lausanne, Switzerland). The kinetical measures were done with Jasco V550 type two-ray spectrophotometer, which can be thermostated. The final volume of the mixture was 500 µl which contained 200 µl 5 mM 2-chlor-4-nitrophenil-4-O-α-D-galactopiranozil-maltozid ($GalG_2CNP$, SORACHIM SA, Lausanne, Switzerland) substrate and 285 µl MES buffer, which was incubated at 37°C for 5 minutes. Saliva samples (10 µl) were diluted in the rate 1:200. The reaction was started by adding 15 µl diluted saliva and the initial reaction speed was measured. The absorbance-change, which was created by chromophore group, was measured for 120

seconds at 400 nm opposite the blind buffer (Gyémánt et al., 2003). Time course measurement function of Jasco Spectra Manager software was applied for determining the gradient of the beginning part of the curve. Jasco Spectra Manager software Time course measurement. Every experiment was repeated three or four times. The alpha-amylase activity of the saliva (sAA) (1 unit = μmol/min) was calculated with the beginning speed (ΔA/min) value.

2.2.5 Microbiological measures

To identify and determine the number of the alive β-haemolitic *Streptococcus mutans* cells, saliva samples were cultured with 5% defibrinated sheep blood (Biocenter) at cocentrated triptase-soya-based agar (Sigma Aldrich). On this medium the β-haemolitic *S. mutans* cells have a characteristic morphology (Wolff and Liljemark, 1978). The total lysis of erythrocytes shows pellucid, yellow, hollow circles around the lonely *S. mutans* colonies, which make the morphological identification of these cells easy. To get an individual bacterium colony 10^4 times dilution was done in a second-class laminar-flow boksz to avoid the infection from the surrounding. Agar plates were incubated at 37°C in aerob condition containing 5-10% CO_2 for 4 days. Then morphological identification was done and we calculated the number of β-haemolitic *S. mutans* cells and multiplied it with 10^4. In the case of pure agar plates we repeated the culture in 10^3; 10^2; 10^1 dilution.

2.2.6 Statistical analysis

To evaluate the results we calculated the mean and standard deviation of the values. The data was analysed with SPSS statistical software, 17th version (SPSS Inc, Chicago, IL). One-direction variance analysis (ANOVA) was applied. The significance level was set on $p<0,05$ value.

To evaluate our results descriptive statistic was applied for summarising the results of saliva amylase activity and the number of *Streptococcus mutans* cells. The mean and the standard deviation values from non-stimulated saliva were calculated in each time. The normal distribution of the data was examined with Kolmogorov-Smirnov test.

T-probes of the two independent samples were used to compare the difference between the categories and the levels of saliva amylase activity in the case of each parameter.

Pearson correlation coefficient (r) and linear regression method were applied to analyse the correlation between the amylase activity in the rest saliva and the dental status (DMF-T).

In the three age groups the oral status (DMF-T) was examined with Welch Anova instead of ANOVA. When ANOVA was significant, a post-hoc test was applied for multiple compare. If we assume equal variances, Bonferroni test was applied, if the variances are not equal, Games-Howell test was applied. The evaluation of time effects between the categories (control and treated) was done with one-variable ANOVA, multiple measures to compare the average differences of the saliva amylase activity. The two-variable ANOVA was done with multiple measures to evaluate the time effects among the categories (control and treated), the three age groups and the interaction effect for comparing the average differences of *Streptococcus mutans* levels.

The differences were determined according to Bonferroni correction. Two-variable tests with 5% significance level were applied. Every statistical calculation was done with the 23.0 statistical pack (SPSS, Chicago, IL, USA).

3. RESULTS

3.1 Results of analysing sour cherry varieties:

Dry-content of each sour cherry varieties was investigated and we found that ‘VN1’ variety has the highest dry-content (19,32%) and ‘Cigánymeggy 59’ has the lowest dry-content (14,12%). Then the total anthocyanin concentration of these sour cherry varieties was determined by isolation with pH differential method and solid phase extraction (SPE).

According to our results, ‘Cigánymeggy 59’ has significantly higher anthocyanin concentration ($p<0,05$).

Anthocyanin profiles of the five sour cherry varieties were determined by UHPLC, a suitable chromatographic method was developed for qualitative and quantitative analysis of the components. In case of all studied varieties, the concentration of cianidin-3-rutinoside was the highest.

‘VN1’ variety has significantly high cianidin-3-rutinoside concentration (147 mg/100g). ‘Cigánymeggy 59’ and ‘VN1’ varieties have significant cianidin-3-glucoside concentration (58,3 mg/100g, 58,8 mg/100g) opposite the other varieties. This variety has low cianidin-3-glucozil-rutinoside/ malvidin-3,5-O diglucoside. ’Debreceni bőtermő’ variety among ’Kántorjánosi’ landraces has the highest cianidin-3-rutinoside concentration (64,3 mg/100g), the cianidin-3-glucoside concentration is (25,1 mg/100g) and this variety also contains 16,3 mg/100g cianidin-3-glucosil-rutinoside/ malvidin-3,5-O diglucoside. According to the one-variable analysis ‘VN1’ among ‘Bosnyák’ landraces have significantly higher anthocyanin concentration than that of ‘Cigánymeggy 59’ and ‘Kántorjánosi’ landraces measured by UHPLC ($p<0,05$).

The major components of sour cherry were determined by MALDI-TOF method. During the identification three major components were identified: cianidin-3-glucosil-rutinoside 757,5 Da, cianidin-3-glucoside 449,1 Da, cianidin-3- rutinoside 595,6 Da. Several smaller nathocyanin components were determined in sour cherry extracts with HPLC.

Then we investigated the antioxidant capacity of each anthocyanin standard. According to the one-variable ANOVA the mean values of water-soluble antioxidant capacity of cianidin-3-O monoglucoside, cianidin-3-O-rutinoside and malvidin-3,5-

diglucozóside were significant ($p<0,05$). The values of fat-soluble antioxidant capacity of the studied anthocyanin standards did not show significant difference ($p<0,05$).
We studied the fat- and water-soluble anioxidant capacity of the five Hungarian sour cherry varieties with photochemiluminescent method. 'VN1' and 'Cigánymeggy 59' varieties have significantly high water- (ACW) and lipid- soluble (ACL) antioxidant capacity ($p<0,05$).

3.2 Effect of anthocyanins and sour cherry extract on the activity of human saliva amylase (in vitro):

Inhibitory activity of sour cherry extract and some anthocyanins was studied in the presence of human saliva amylase on $GalG_2$-CNP substrate (EC 3.2.1.1). We firstly proved that sour cherry extracts and some anthocyanins can inhibit in µM concentration, we determined the type of inhibitory, which inhibits in a purely competitive way.
IC_{50} was determined for characterising sour cherry extracts and pure anthocyanin structures. So malvidin-3,5-diglucoside has the highest inhibitory activity. Then cianidin-3-glucoside, cianidin-3-rutinoside follow and finally malvidin-3-glucoside has the lowest inhibitory activity. IC_{50} values of each sour cherry extract were determined. We found that 'Cigánymeggy 59' and 'Debreceni bőtermő' are better inhibitors than the other sour cherry extracts. Good inhibitory feature of 'Cigánymeggy 59' correlates with its high anthocyanin content, while 'Debreceni bőtermő' has better inhibitory activity than we calculated from its anthocyanin content which refers to the presence of other inhibitory compounds.
Bonding of our studied anthocyanin structures to the active center of HSA was modelled by Dr. Jérémie Mortier and Dr. Gerhard Wolber at Freie University, Pharmaceutical Institute, Pharmacautical and Medicinal Chemistry Department, Berlin.
As we know, the occupancy of -1, -2, -3 subplaces of the human saliva amylase enzyme play a central role in the catalytic activity. Our results show that the studied anthocyanins bond into the -1, -2, -3 subplaces of alpha-amylase enzyme providing inhibition activity. Opposite the cianidin-glucosides, malvidin-3,5-diglucoside can stable bond into the active center of the enzyme. The better the bonding of an inhibitor molecule is in the active place of the enzyme, the more difficult the substrate can supersede it during the competition. This can explain that malvidin-3,5-diglucoside has better inhibitory activity

than the other standards and 'Cigánymeggy 59' is better inhibitor of the human saliva amylase enzyme than 'VN1' sour cherry variety.

3.3 Investigation of the effect of chewing gum with sour cherry extract on the activity of saliva amylase

At first, in clinical investigation the rest saliva amylase activity was determined in control and treated groups. The statistic analysis of the results was made according to age and sex. We found that the rest saliva amylase activity of the three age groups did not show significant difference. Furthermore, the rest saliva amylase activity of the three age groups did not show significant difference between women and men.

The effect of chewing gum with sour cherry extract was investigated during 30 minutes and the results were shown by Figure 1 and compared with the control group.
In control and treated groups amylase activity of the first taken saliva samples was low. This can be explained, that the conformation of the alpha-amylase enzyme characterizes tercier structure which cannot allow the substrate to bond. In the second sample taking (after chewing) it can be seen that saliva amylase activity significantly increased in both groups because of intensive chewing. The stimulus salive was formed by chewing. Ion composition of the stimulus saliva significantly differ from the rest saliva. In the stimulus saliva the concentration of Na^+, K^+, Ca^{2+}, HCO^-_3, és a Cl^- increases. As we know, alpha-amylase is an allosteric enzyme, which means that one allosteric activator must bond to the enzyme in order to its quaternary structure formation. The allosteric activator of the enzyme is Cl^- ion. Stimulus saliva formed because of the intensive chewing which provided the right conditions to the enzyme for conformation change and the quaternary structure of the saliva amylase formed. In the second sample taking time, saliva amylase activity significantly increased in both groups. Repeated measuring with one-variable ANOVA showed significant time effect ($p<0,05$).

In the third smaple taking we can see that in the 10. minute after chewing the amylase activity significantly decreased in the treated group (chewing gum with sour cherry extract) in connection with the control group. This means that anthocyanins of the sour cherry extract bonded into the active center of the enzyme. In the 4. and 5. sample taking, the enzyme activity of the saliva decreased further in the treated group, it was near the activity of the rest saliva. During chewing chewing gum which contains sour cherry extract rich in anthocyanin, anthocyanins released inhibited the demolition of the starch in the oral cavity in the exaperimental time (30 minutes). We verified that sour cherry

extract rich in anthocyanins has a significant role in the prevention of caries and the preservation of oral health.

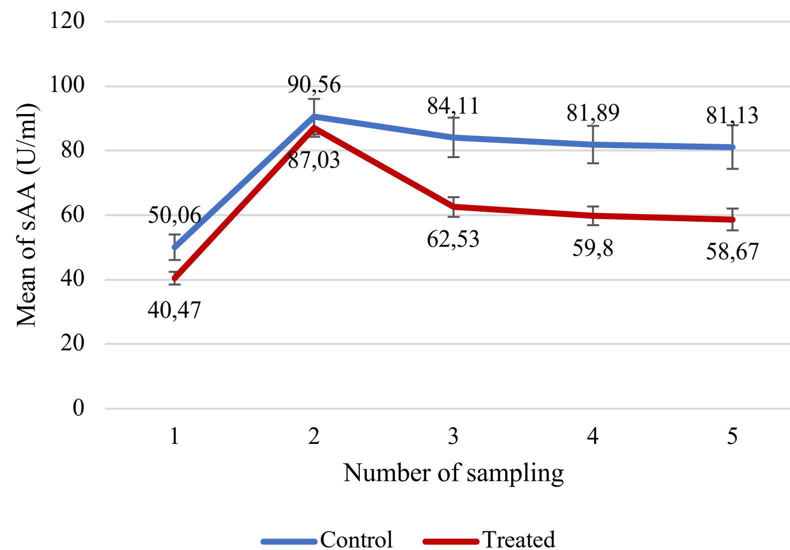

Figure 1: The level of saliva amylase activity in control (n = 34) and treated (n = 36) group before and after stimulation. Data was given in average ± standard error (SE) ($p<0,05$).

We statistically evaluated the answers of the questionnare and found that the frequency of gum blooding and the use of dental floss increased the rest saliva amylase activity which can explain the effect of inflammation. Surprisingly, the regular use of chewing gum decreased the level of the rest saliva amylase activity, but not significantly.

The oral condition was studied in the three treated groups. DMF-T values were higher by the elderly patients. Saliva amylase activity of adult patients was higher than those volunteers who have more decayed teeth, we did not experienced this effect by children. The correlation was significant by adults, and we found positive linear correlation in both adult age groups. Our results show that the higher saliva amylase activity resulted in higher DMF-T level in the groups between 18-20 years and over 30 years.

3.4 Investigation of the effect of chewing gum with sour cherry extract on the change of the number of *Streptococcus mutans*

There was not significantly difference among the number of *S. mutans* colonies of the rest saliva in the three age groups and these values were not influenced by the sex. We evaluated the number of *S. mutans* colonies in the control and treated groups of the three age groups and it significantly differed among the age groups after chewing. We studied the effect of the chewing gum with sour cherry extract on the number of *Streptococcus mutans* during 30 minutes. The results were compared to the control group (Figure 2).

In control and treated groups there was similar beginning *S. mutans* level before chewing in the rest saliva samples. This is caused by bacteria bonding to the biofilm in a sessile way. So the *S. mutans* level of the rest saliva samples is very low in both the control and the treated groups.

In the samples right after chewing the number of *S. mutans* increased in both control and treated groups. The effect is significant in every treated age group opposite the control.

The unit of the biofilm splits up for the effect of intensive chewing and large amount of *S mutans* gets into the oral cavity. In the third sample taking saliva the level of *S. mutans* significantly decreased in every treated age group opposite the control groups. So anthocyanins of sour cherry extract inhibit bonding of *S. mutans* to the biofilm. Because of the intensive chewing large amount of saliva is producted which helps with emptying the microorganisms unable to adhesion from the oral cavity because of the continuous swallow opposite the control groups. In the 4. and 5. sample taking we can see the number of *S. mutans* colonies of those who use the chewing gum with sour cherry extract is near the level of *S. mutans* in the rast saliva in every age group opposite the control group. We can conclude that releasing anthocyanins during chewing chewing gum rich in sour cherry in the studied time (30 minutes) inhibit adhesion of *S. mutans* bacteria to the biofilm. Because of the continuous chewing, the large amount of saliva empties the microorganisms from the oral cavity.

These results show that sour cherry extract rich in anthocyanin plays a significant role in prevention of caries and preserving the health of oral cavity.

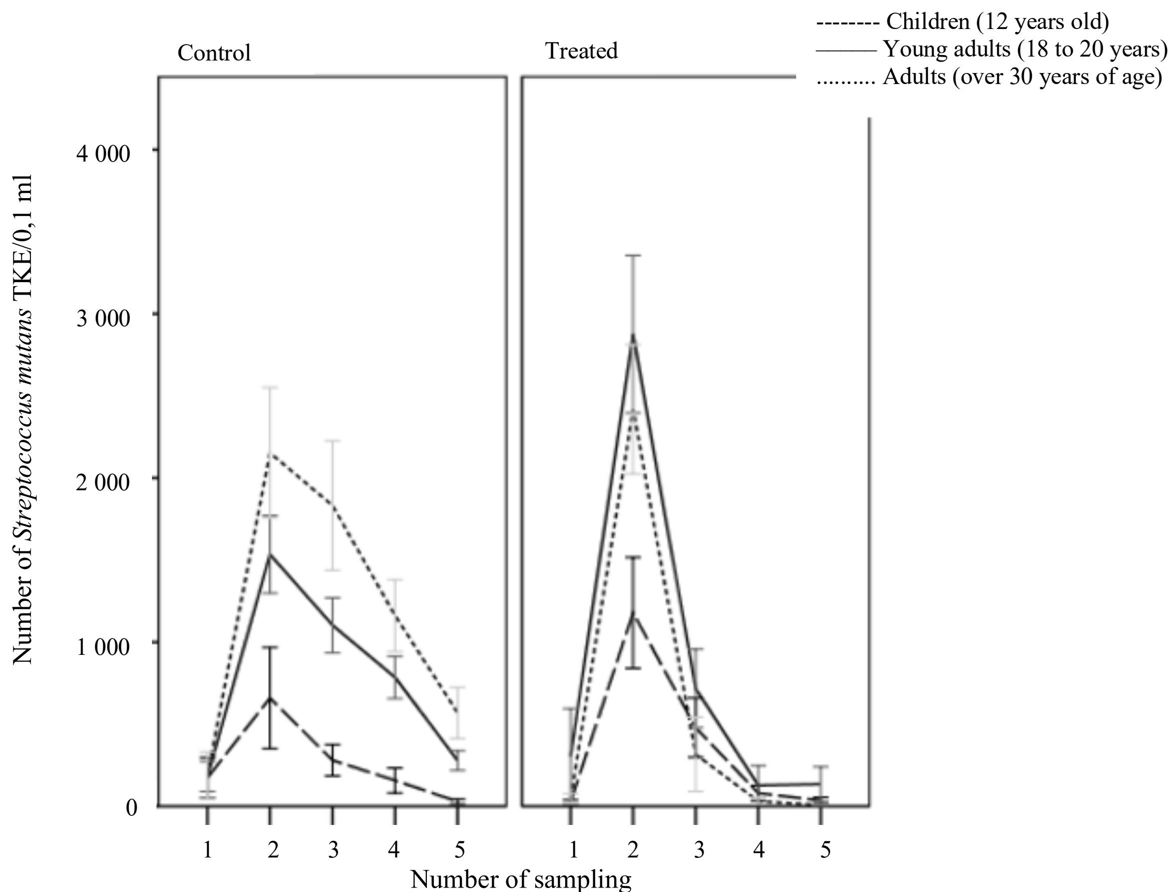

Figure 2: The change of the number of *S. mutans* in the three age groups, control (n = 34) and treated (n = 36) groups. Data was given in average ± standard error (SE) (p<0,05).

Statistic evaluation of the answers to the questionnare showed significant difference in three cases between control and treated groups.

Significant difference in the beginning level of *S. mutans* was between allergic and non-allergic patients. The frequency of the former mentioned dry mouth and bleeding gum decreased the number of *S. mutans* cells while dental floss and chewing gum increased it.

One-variable ANOVA test showed significant difference in three cases (dental floss, tooth status, dry mouth) between control and treated groups.

In the treated group who chew chewing gum with sour cherry extract in the 20th minutes of chewing the number of *S. mutans* significantly decreased regardless of using dental floss. Because of chewing and pumping the stable biofilm changes, which was strengthened by the sour cherry extract in the chewing gum, so the number of *S. mutans* colonies significantly decreased.

In case of tooth status, in the treated group (chewing chewing gum with sour cherry extract) patients with caries (DS>0) and patients without caries (DS=0) had more *S. mutans* cells in their saliva after chewing. The samples from the saliva in the 10th and 20th minutes after chewing had very low bacteria number.

The differences in the treated group (DS>0 és DS=0) completely vanished. This was caused by sour cherry extract. *In vitro* experiments showed that sour cherry has bactericid effect which influences the health of oral cavity (Hevesi et al., 2012). The difference was significantly higher in the stimulated saliva. In case of dry mouth, the number of *S. mutans* cells was high in both control and treated groups opposite the patients who do not suffer from dry mouth. Because of the decreased saliva production the metabolites provide good broth to bacteria. In treated groups from the 20th minutes the differences between the categories completely vanished.

3.5 Melatonin results:

We studied the concentration of another biologically active compound, melatonin in the mentioned sour cherry varieties. Measuring the concentration of this molecule is important because former studies wrote that melatonin could decrease tha viability of the biofilm and block biofilm formation (Zhou és mts. 2016).

We developed a chromatographic method to identify melatonin by qualitative and quantitative methods. According to our results we found that Hungarian sour cherry varieties have significantly high melatonin concentration. ‘Cigánymeggy 59’ and ‘Debreceni bőtermő’ have significantly higher melatonin concentration than the other varieties ($p<0,05$).

Sour cherry extracts were further pured with preparative HPLC technique and identified with MALDI-TOF MS analysis on the basis of m/z value: molecule weight of melatonin is 232,29 Da.

4. NEW SCIENTIFIC RESULTS

1. We worked out a method for chinetical identification sour cherry anthocyanins and sour cherry extract *in vitro* on human saliva amylase.
2. *In vitro* we certified that sour cherry extract from the Hungarian sour cherry varieties and the anthocyanin components in μM concentration can purely inhibit the activity of human saliva amylase in a competitive way.

To characterise the inhibitory activity of the pure anthocyanin structures we identified IC_{50} (μM) value: cianidin-3-glucozide: 180 ± 20, cianidin-3-rutinozide: 200 ± 24, malvidin-3-glucoside: 675 ± 73, malvidin-3,5-diglucoside: 80 ± 10. To characterise the inhibitory activity of each sour cherry extracts we identified IC_{50} (μg/ml) value:'Cigánymeggy 59': 330 ± 45, 'VN1': 610 ± 50, 'A': 790 ± 55, 'Debreceni bőtermő': 370 ± 42, 'Kántorjánosi': 892 ± 62.

3. We worked our a method for producing such sour cherry extract rich in anthocyanin in which biologically active molecules preserve their effect.
4. We proved that during the production of the chewing gum with sour cherry extract, the sour cherry extract rich in anthocyanin did not lose its biological activity.
5. In pilot clinical study, we certified that the anthocyanin content of chewing gum with 'VN1' sour cherry extract is 11,87 mg/100mg is effective in the prevention of caries, which was certified with the inhibition of the activity of human saliva amylase and reduction of the number of *Streptococcus mutans* cells.

In the control and untreated groups for the effect of chewing, saliva amylase activity grew by 90,56 and 87,03 U/ml. In the treated group for the effect of anthocyanin, saliva amylase activity decreased by 58,67 U/ml during 30 minutes approaching the rest saliva amylase activity whose value was 50,06 and 40,47 U/ml. Anthocyanins bound to the active centrum of the saliva amylase enzyme inhibiting starch demolition in the oral cavity. In the control and treated groups in every age group for the effect of chewing, a large amount of *Streptococcus mutans* appeared in the saliva samples whose value was 2875-943 TKE/0,1ml. In the treated group during 30 minutes the number of *Streptococcus mutans* decreased by average 35 TKE/0,1ml in the salive in every age group. Anthocyanins inhibited the adhesion of *Streptococcus mutans* bacteria to the biofilm.

5. ADAPTABLE RESULTS IN PRACTICE

With our study we certified that with a suitable extraction technique 'VN1' sour cherry variety rich in anthocyanin can play an important role in the prevention of caries.

Anthocyanins bind to the active site of the salivary amylase enzyme, inhibited starch demolition in the oral cavity. In the treated group for the effect of chewing, the saliva amylase activity decreased by 87,03 U/ml. Fort he effect of anthocyanins in the treated group, the saliva amylase activity decrased by 58,67 U/ml during 30 minutes approaching the rest saliva amylase activity value. The anthocyanins inhibite the adhesion of *Streptococcus mutans* bacteria to the biofilm in the treated group during 30 minutes, a kezdeti 2876-1178 TKE/0,1ml.

The carrier material was the chewing gum made with melting technique which can preserve the biological effect of anthocyanins of sour cherry extract. The chewing gum provides a continuous active agent releasing to biologically active compounds during a certain period. We certified that the chewing gum is suitable for carrying other natural inhibitor compounds. In addition, the chewing gum is a very popular food industrial product, it is widely accepted in the society and applied routinely. Besides regular using oral and dental care instruments, the chewing gum with 0,1 g 'VN1' sour cherry extract, whose anthocyanin content is 11,87 mg/100mg, is a prototype which can play a significant role in the prevention of caries with easy and fast use, because everybody knows such conditions (school, workplace, long-distant travel) when toothbrushing is not possible after eating.

Besides that with other improves it can be solved to make other oral and dental care products from toothpaste, mouth wash or dental floss with sour cherry extract.

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7. PUBLICATIONS

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List of publications related to the dissertation

Hungarian scientific articles in Hungarian journals (2)

1. **Homoki, J.**, Gyémánt, G., Gálné Remenyik, J.: Régi hormon új csodája: magyarországi meggyfajták mint természetes melatonin források. *Agrártud. Közl. 63*, 65-72, 2015. ISSN: 1587-1282.
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