Theses of a Doctoral (PhD) Dissertation

INVESTIGATION OF BIOACTIVE COMPOUNDS OF SOUR CHERRY AND PEPPER IN ENDOTHELIAL DYSFUNCTION

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1. Antecedents and Objectives of the Doctoral Dissertation

Both epidemiological, experimental and clinical researches emphasize the close links between nutrition and health. Instinctive dietary changes for some animal species, traditional Chinese medicine dating back to antiquity, the attitude of Ayurveda in India, or the approach attributed to, incorrectly, Hippocrates in the European culture: "*You are what you eat*" then later "*Your medicine should be your food, and your food should be your medicine*", (Feuerbach) can be considered as early, empirical, in a sense intuitive, but still valid nutritional biological results. Diseases caused by vitamin deficiency - scurvy, beriberi - were the first cases in which the above association was confirmed by scientific methods accepted today, and at the same time effective treatment was made available. As a result, it became justified to get to know the nutrient needs of the human body more thoroughly, to carry out extensive research, and then to apply the information derived from them and put it into public health practice.

The research community and the relevant industry actors expected a significant improvement from the fortification of foods with minerals and vitamins. The morbidity of some diseases (rickets, neural tube defects, goiter, early / neonatal cerebral hemorrhage, etc.) has indeed significantly decreased, but in similar statistics for other chronic disorders are still showing a slow increase. Such diseases are, inter alia, II. types of diabetes, atherosclerosis, cardiovascular, gastrointestinal disorders and cancer. The high prevalence in Western societies suggests that this may be due, at least in part, the way of life that has changed in a few decades, from an evolutionary point of view in a matter of moments. Among the civilizational harms we have to the sedentary lifestyle, the sociocultural environment characterized by stress, air pollution, and the harmful habits of the individual, resulted in negative physiological effects, but the transformation of food consumption habits has decisive importance. All of these are capable cause the chronic diseases listed above by upsetting the homeostasis of the human body.

As early as the turn of the millennium, the WHO drew attention to the rising morbidity of diseases of civilization. Their prognosis has proven to be correct, and nutrition-related diseases in civilization place a serious public health burden on the health care systems of the states. Although the number of followers of a health-conscious diet is increasing, it is unfortunately still in its infancy, and it is often influenced by less scientific trends. The harmful consequences of unhealthy nutrition, the partial success of pioneer food modification attempts (e.g. vitamin supplementation), emerging novel health problems

(gluten sensitivity, new viral infections, etc.) call for more sophisticated solutions. In part, they encourage the development of foods that have a preventive effect through their beneficial effects. In part, they inspire the search for active ingredients suitable for the curative treatment of various diseases. In recent decades, a significant proportion of drug development research has turned to pharmacologically active plant-derived compounds (nutraceuticals). Besides the registered medicines already used (aspirin willow, paclitaxel-yew, tamiflu-Japanese star anise, etc.), intensive research is currently underway in order to develop novel products based on pharmacological active components of plant origin.

The regulation of the stable internal environment of each cells, its interaction with its distant environment is realized by the unavoidable participation of endothelial cells covering the inner wall of the blood vessels. Their importance is demonstrated by the fact that endothel dysfunction has a key role of the pathomechanism of above-mentioned chronic diseases mentioned above. It is intensively used in the development of prevention and treatment strategies under laboratory conditions, among other things, in drug research. In our experiments, we studied the effect of biologically active plant molecules in an *in vitro* model of diseases where endothelial dysfunction is a central pathogenetic factor.

In the view of the above, the aim of our research group was to search for, produce and purify plant active ingredients. Subsequently, the effects of compounds of sour cherry and pepper were investigated on the pathophysiological processes in the above-mentioned chronic diseases *in vitro* model.

During our work, we seeked solutions to the following problems and wanted to answer the next questions:

Experiments with the purified anthocyanin fraction

1. Aware that anthocyanins have a variety of therapeutic potentials, including immunmodulatory effects, we aimed to answer how the anthocyanin fraction (PAF) purified from sour cherries affects the biological processes of endothelial cells induced by lipopolysaccharide (LPS) -induced model.

Experiments with the allithiamine

1. For *in vitro* studies, we had to perform the synthetic preparation of allitiamine, which is not commercially available, and then its analytical verification of molecular structure.

2. Clarification of the hypothesis that allitiamine accumulates in peppers besides the genus *Allium*
3. Investigation of the physiological effect of allithiamine under hyperglycemic conditions in endothelial cell culture.
4. Investigation of possible mechanisms responsible for the positive biological effect.

2. Materials and Methods

2.1. Preparation of the Active Agents

2.1.1. The Purified Anthocyanin Fraction

The PAF tested during the cell culture experiments was the result of the doctoral work of Dr. Judit Rita Homoki (Institute of Food Technology of the University of Debrecen)

2.1.2. Preparation of the Reference Substance Allithiamine

The synthesis of allithiamine was carried out with minor modifications based on the experiments of Matsukawa et al. (Matsukawa et al., 1953). The reaction mixture was fractionated by HPLC and the structure of the purified allithiamine was validated by MALDI-TOF MS and NMR methods.

2.2. Examination of Allithiamine in Pepper Seeds

The ethanolic extract of pepper seeds was fractionated on a Strata-X-C cation exchange column and analyzed by HPLC and HPLC / MS.

The reagents were bought from Sigma-Aldrich (MilliporeSigma, St. Louis, Missouri, USA), and Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

2.3. Isolation, Culture and Flow Cytometric Characterization of HUVEC Cells

The investigation of the effect of bioactive compounds was performed on HUVEC (human umbilical vein endothelial cells) cell culture. The umbilical cords were obtained from the Gynecology and Obstetrics of Clinical Centre of the University of Debrecen. Tests on umbilical cords of human origin were performed in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the

University of Debrecen. (registration number: RKEB/IKEB 3712-2012). Endothelial cells were isolated from the umbilical cord vein using collagenase enzyme and cultured in M199 medium at 5% CO_2 at 37 ° C in Galaxy 170 R incubator (Eppendorf, Hamburg, Germany).

To model inflammation 100 ng/mL LPS was applied and the markers was examined after 24 hours of incubation. For hyperglycemic 30 mMol/L glucose was used and the intracellular responses was evaluate after 6, 12, 24 hours and one-week incubation.

Cells were labelled with fluorophore-conjugated monoclonal antibodies produced in mice (FITC-CD31, PE-CD54, APC-CD106, PerCP-Cy5.5-CD45) and were characterised with Becton Dickinson FACSAriaIII Cell Sorter flow cytometer (Becton Dickinson, Mountain View, CA, USA).

2.4. Determination of Cell Viability

The effect of the applied active agent on cell viability was examined by MTT assay and combined DilC1 (5) -SYTOX Green fluorescent labelling. Cells were plated in 96-well plates ($2x10^4$ cells/well) and then treated with different concentrations of the respective drugs for 24 and 48 hours for PAF and 24, 48 and 72 hours for allithiamine. Absorbance values and fluorescence intensities were detected using a Clariostar micro plate reader (BMG Labtech, Ortenberg, Germany).

2.5. Measurement of Intracellular ROS

To measure intracellular ROS, cells were incubated with 2',7'-dichlorofluorescein diacetate dye for 1 hour at 37 ° C in dark. While cells were monitored in every 5 minutes during H_2O_2 studies (for PAF treatments, the fluorescence readings were taken at 60th minutes and for allithiamine treatments fluorescence readings were taken at 120 minutes), intensities obtained after 24 hours were used to study LPS-induced ROS formation. Fluorescence intensities were detected by using a micro plate reader (BMG Labtech, Ortenberg, Germany).

2.6. Reverse Transcription and Real-Time Quantitative Polymerase Chain Reaction

Total RNA from the samples was isolated with Extrazole (Blirt, Gdańsk, Poland) and qualitatively and quantitatively verified with NanoDrop (Thermo Fisher Scientific,

Waltham, Massachusetts, USA). Reverse transcription was performed on a 2720 Thermal Cycler (Foster City, CA, USA). Quantitative real-time PCR (Q-PCR) reactions were performed on a Roche LightCycler 480 (Hoffmann-La Roche, Basel, Switzerland).

2.7. Investigation of Cytokine Secretion by Luminex/MagPlex Method

Cytokine concentrations were determined by using a MILLIPLEX MAP human cytokine / chemokine magnetic panel (EMD Millipore Corp., Billerica, Massachusetts, USA) using a Luminex 200 instrument (Luminex Corp., Austin, Texas, USA) according to the manufacturer's instructions.

2.8. ELISA Assays

AGEs were quantified by using OxiSelect competitive ELISA kits following the manufacturer's protocol (Cell Biolabs Inc., San Diego, California, USA). Activated NF-κB was quantified with the Human InstantOne ™ NFkB p65 (Total) ELISA kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Secretion of tPA TxA_2 and PGI_2 from cell supernatants was determined using colorimetric ELISA kits (Abcam, Cambridge, UK) according to the manufacturer's instructions.

2.9. Statistical Analysis

For statistical analyzes, One-Way variance analysis (ANOVA) was used implemented with a modified t-test according to Bonferroni.

3. Result

3.1. Analytical Examination of Active Substances

3.1.1. The Purified Anthocyanin Fraction

Based on our measurements, the main anthocyanin components of PAF are cyanidin-3-*O*-glucosyl rutinoside, cyanidin-3-*O*-glucoside, and cyanidin-3-*O*-rutinoside.

3.1.2. The Allithiamine

3.1.2.1. Synthesis of the Allithiamine

Allithiamine was separated and purified from the by-products produced during the chemical synthesis by liquid chromatography. Allithiamine fractions were pooled and MALDI-TOF MS and NMR analyzes of their structure were performed. Both methods clearly demonstrated that the chromatographically purified compound was N-[(4-amino-2-methylpyrimidin-5-yl) methyl]-N-[(2E)-5-hydroxy-3-(prop-2-en-1-yldisulfanyl) pent-2-en-2-yl] formamide, i.e. allithiamine.

3.1.2.2. HPLC-MS Analysis of Pepper Seed Extract

The ethanolic extract of pepper seeds was analyzed by HPLC-MS, during which it was determined that allithiamine is accumulated in pepper seeds.

3.2. Flow Cytometric Measurements of HUVECs

Given that we used primary cells in our experiments, rather than commercially available cell lines characterized by the manufacturer, we began our *in vitro* studies by phenotyping the cells. Positive and negative marker proteins expressed on the cell membrane (CD54; CD31; CD45; CD106) were labelled with specific antibodies and endothelial cells were characterized by flow cytometry. The data confirm and validate the efficiency and accuracy of the isolation for further studies with HUVECs.

3.3. Effect of PAF on LPS-induced Inflammatory Processes

3.3.1. Determination of the Optimal Concentration of PAF

3.3.1.1. Viability Studies

As a first step in the *in vitro* study of the drugs, we determined the concentration range of PAF, which does not significantly affect cell viability. An MTT assay and a combined $DilC_1(5)$ -SYTOX Green fluorescent label were used to rule out the possibility of early

apoptotic and necrotic events. We found that PAF in the concentration range of 1-100 μg/ml did not have a negative biological effect on cell viability even after 24 and 48 hours of sampling.

3.3.1.2. Antioxidant Capacity of PAF

To determine the optimal concentration of PAF, further experiments were performed to exclude concentrations that no longer have a significant antioxidant effect. PAF at concentrations of 500 and 1000 μg/ml, which significantly reduced cell viability, was not used in these experiments. The cells were treated with H_2O_2, which resulted enhanced increase in ROS in the cells after 60 mins. PAF at 50 and 100 μg / ml was the most effective in reducing this increase. As the drug was able to eliminate a significant part, then all, of the H_2O_2-induced ROS production even at a concentration of 50 μg/ml, we used this concentration for our further research.

3.3.2. The PAF Alleviates Oxidative Stress Caused by LPS

A commonly used method to characterize oxidative stress is to measure changes in ROS and glutathione (GSH) levels. LPS resulted in more than twice the ROS production in our culture compared to the control, which PAF was able to significantly reduce. Although LPS alone did not change GHS levels, the added PAF significantly elevated GSH levels. All of these indicate an increase in the antioxidant capacity associated with PAF.

3.3.3. Anti-inflammatory Effect of the PAF

The cardinal part of inflammation – the means of initiating the complex process, limiting it in time and space, regulating the participating cells – is the production of different autocrin-endocrine molecules according to the given scenario (bacterial infection, tissue injury, etc.) (Chen et al., 2017). In the next step of our experiments, we examined how PAF affects LPS-induced cytokine and chemokine secretion. By examining the amounts of IL-6, IL-8, TNF-α, RANTES, GM-CSF, and tPA produced in the HUVEC model, we wanted to get a comprehensive picture of the inflammatory processes affecting the endothelium. Concentrations of secreted cytokines were determined from cell supernatant after 24 hours of treatment. PAF significantly reduced the increased cytokine release following LPS treatment.

3.3.4. Effect of PAF on Eicosanoid Synthesis

Besides proteins essential for the regulation of inflammation (cytokines, chemokines) arachidonic acid derivatives and eicosanoids also play an important role in the local regulation of inflammation (Imig, 2020). In our inflammatory model, we examined TxA_2 and PGI_2 levels. PAF significantly affected PGI2 levels, while TxA2 secretion was not significantly altered.

Inhibition of the increase in PGI2 concentration can be realized in several ways in practice (reduction of transcription of synthesizing enzymes, post-translational modification, direct inhibition, etc.). According to our measurements, the expression of the genes of the synthesizing enzymes (COX-1, COX-2 and PGI2 synthase) changes. This is true at the transcriptional level based on the specific mRNA examined under the influence of both LPS (increase) and PAF (decrease).

3.4. The effect of Allitiamine on Hyperglycaemia-induced Endothelial Dysfunction

3.4.1. Determination of the Optimal Concentration of Allithiamine

The anti-hyperglycaemic effects of allithiamine were studied in a HUVEC model. For allithiamine, its effect on cell viability was first assessed by using MTT assay and $DilC_1(5)$ and SYTOX Green fluorescent labelling. HUVECs were treated with various concentrations of allithiamine (0.1-50 μg/ml) for 24, 48, 72. The highest allithiamine concentration that did not yet reduce cell viability was 5 μg/ml at each sampling time point.

3.4.2. Allithiamine Reduces the Formation of AGEs

In our experiments, we modelled chronic hyperglycaemia by exposing endothelial cells to persistently high glucose concentrations (30 mMol/L). Persistent hyperglycaemia causes increased development of AGEs (advanced glycation end-products) in living cells. Effects of allithiamine on AGE formation were investigated by evaluating AGEs levels after exposure to high sugar concentrations for one day and one week. After one week incubation, 30 mMol/L glucose increased the level of AGEs in endothelial cells. Allithiamine was able to significantly inhibit the almost twofold increase induced by hyperglycemia.

3.4.3. Allithiamine Affects Inflammatory Processes Caused by Hyperglycaemia

In order to gain a deeper insight into the cellular events caused by hyperglycaemia, we searched for marker proteins whose expression levels change in the short term in a hyperglycaemic environment. Several studies have reported a strong association between hyperglycaemia and the inflammatory response of the endothelium (Funk et al., 2012; Hoffman, 2015). Therefore, the activation of the transcription factor NF-κB, which plays an important role in the inflammatory processes activated during hyperglycaemia, was evaluated after 6 and 12 h of hyperglycaemic incubation in HUVEC lysate. We found that the hyperglycaemic state activated NF-κB at an early stage, which allithiamine was able to significantly reduce.

In the next step of our experiments, the release of TNF-α, IL-6 and IL-8 cytokines was measured from the cell supernatant after 6, 12, 24 h of incubation. While IL-6 secretion was significantly increased during all sampling times after 30 mMol/L glucose treatments, TNF-α secretion was significantly only after 6 and 12 h. After 24 hours, no significant but marked biological change was observed. IL-8 secretion was statistically significantly increased after both 6 and 24 h of incubation. Allithiamine was able to significantly moderate the increased cytokine release at all sampling times. Allithiamine was able to significantly moderate the increased cytokine release at all sampling times.

3.4.4. Presumed Origin of the Positive Biological Effect of Allithiamine

3.4.4.1. Allithiamine does not increase the activity of the enzyme transketolase

The question arises what is the molecular mechanism of action behind the above-presented positive effects of allithiamine. We hypothesized that allithiamine could activate the transketolase enzyme similarly to benfothiamine (Hammes et al., 2003). Therefore, we examined in our culture whether allithiamine is able to exert this effect. Allithiamine did not significantly affect, while benfothiamine almost doubled transketolase activity. The results suggest that the positive effects of allithiamine are independent of transketolase enzyme activation.

3.4.4.2. Allithiamine Has a Strong Antioxidant Capacity

Mechanisms independent on transketolase activity enhancement have been described for benfotiamine (Balakumar et al., 2010), including antioxidant effects (Schmid et al., 2008). Similarly, the strong antioxidant capacity of garlic sulfur compounds having an

allyl disulfide moiety (ajoene, S-allyl cysteine, etc.) is known (Jang et al., 2017; Kay et al., 2010; Lu et al., 2017). Taking all this into consideration, we examined the ROS-eliminating ability of allithiamine. Allithiamine was able to significantly reduce the increased ROS production caused by H2O2, emphasizing the strong antioxidant property of allithiamine.

4. New Scientific Results

In our experiments, on the one hand, we performed various plant drug tests in an *in vitro* model system, and on the other hand, we used instrumental analytical tests to identify allithiamine from pepper seeds. The new scientific results of the experiments presented in the dissertation, which are basically based on two different methodologies (*in vitro* experiments, studies on the analysis of active substances) can be summarized in three points.

1. Based on experiments with anthocyanins of sour cherry, we found that PAF can simultaneously relieve oxidative stress: ROS (73.9%; $p < 0.005$), GSH (97% $p < 0.0001$), reduce the secretion of IL-6 (33.4%; $p<0.01$), IL-8 (48.1%; $p<0.005$), TNF-α (52.8% $p<0.005$), RANTES (35.9%; p<.,005), GM-CSF (51.6%; $p<0.0001$) pro-inflammatory cytokines, positively influence tPA levels (43.6%; p<0,0001) and influence the expression of enzymes involved in PGI_2 synthesis: PGI_2 synthase (70.5%; $p < 0.005$), COX-1 (50%; $p < 0.01$), COX-2 (52.3% $p < 0.001$ in the inflammatory HUVEC model. Therefore, the pleiotropic effect of PAF can be hypothesized, during which its antioxidant, anti-inflammatory, vasoactive and haemostasis-modulating role may prevail in acute inflammation.

2. Allithiamine was identified in the pepper seed of "kápia" (*Capsicum annuum* L.). To the best of our knowledge, the occurrence outside the genus *Allium* was confirmed for the first time.

3. The biological activity of allithiamine under hyperglycemic conditions was examined in HUVEC cell culture. In our experiments, we demonstrated that allithiamine at a concentration of 5 mg/ml reduces the hyperglycemia-caused increased formation of AGEs (43.9% for 7 days; $p < 0.0001$), NF-kB activation (6 h: 53, 5%, $p < 0.0005$; 12 h 45.3% $p < 0.005$), secretion of investigated proinflammatory cytokines: IL-6 (6h: 56.5%, $p < 0.01$; 12h: 34.6%, $p < 0.01$; 24h: 47%, $p < 0.01$), IL-8 (6h: 43.2%, $p < 0.0001$; 12h: 22.9%, $p < 0.01$; 24h: 34 , 1%, $p < 0.01$), TNF-β (6h: 72.2%, $p < 0.005$; 12h: 60%, $p < 0.005$; 24h: 61.5%, $p < 0.05$). It has been shown that the observed effects are not due to the enhancing of activation of the transketolase enzyme but to the antioxidant effect of the compound.

5. Practical Applicability of the Results

Given the basic research-like of our investigations , although our results carries with themselves the possibility of future use, but we can only guardedly extrapolate to a practical extent. Based on the results of further animal and human studies, the practical significance of our active ingredients can be judged objectively.

However, given that the endothelium plays an important role in mediating inflammation, the process of hemostasis, maintaining the balance of vasodilation and vasoconstriction, circulating blood and interstitial metabolism, and considering that the PAF can influence the synthesis of several important molecules involved in these processes, the PAF may alleviate inflammatory symptoms in diseases associated with endothelial dysfunction.

In our analytical studies related to allithiamine, we successfully perform the pure allithiamine production in laboratory-scale, which can serve as a basis for higher volume production. The identification of allithiamine in pepper seeds has expanded our knowledge of the chemical composition of a plant intended for consumption, and emphasizes the importance of qualitative analysis of its foods as can be a source of invaluable new information and knowledge. Our experiments also contribute to a more accurate understanding of the chemical composition of peppers.

We investigated the biological effect of allithiamine in hyperglycaemic HUVEC culture. We concluded that the compound is advantageously able to influence the pathological biological processes caused by hyperglycaemia in HUVEC. Our results contribute to a better understanding of this relatively less researched compound, especially with the respect to its beneficial effects on hyperglycaemia.

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7. List of Publications

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Candidate: Attila Biró
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List of publications related to the dissertation

Hungarian book chapters (1)

1. **Biró, A.**, Homoki, J., Pesti-Asbóth, G., Horváth, B., Cziáky, Z., Máthé, E., Gálné Remenyik, J.: Természetes eredetű antioxidánsok.
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2. **Biró, A.**, Markovics, A., Stündl, L., Gálné Remenyik, J.: Effect of anthocyanin-rich sour cherry extract on the level of IL-8 in LPS-induced endothelial cell.
   Agrártud. Közl. 2, 27-30, 2020. ISSN: 1587-1282.
   DOI: http://dx.doi.org/10.34101/ACTAAGRAR/2/7101
3. **Biró, A.**, Gálné Remenyik, J.: Effect of allithiamine on the level of hyperglycaemia-induced advanced glycation end products.
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Foreign language scientific articles in international journals (3)

4. **Biró, A.**, Markovics, A., Fazekas, M., Fidler, G., Szalóki, G., Paholcsek, M., Lukács, J., Stündl, L., Gálné Remenyik, J.: Allithiamine Alleviates Hyperglycaemia-Induced Endothelial Dysfunction.
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7. **Biró, A.**, Hegedűs, C., Gyémánt, G., Stündl, L., Paholcsek, M., Gálné Remenyik, J.: Effect of allithiamine in neuropathic pain sensation.
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8. Jevcsák, S., **Biró, A.**, Gálné Remenyik, J., Lehoczki, G., Murányi, E., Jóvér, J., Diósi, G., Sipos, P.: Műtrágyakezelés hatása a szemescirok lisztmintáinak zsírtartalmára és zsírsavösszetételére = Effect of fertilization on the fat content and fatty acid profile of sorghum flour samples.
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IF: 3.06

Total IF of journals (all publications): 17,695
Total IF of journals (publications related to the dissertation): 7,813

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

22 February, 2021

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