Luxemburg,
March 25 - 27, 2019

Venue:
Park Hotel, Luxemburg

Natural Products and the Hallmarks of Chronic Diseases
COST Action 16112 - Personalized Nutrition in ageing society: redox control of major age-related diseases

Proceedings and Program

Organizers:
Marc Diederich and Linda Giblin
(Working group 4: Intracellular Diagnostics)

Mustapha Cherkaoui Malki (Coordinator)

This meeting is organized by:
Recherches Scientifiques Luxemburg asbl
for COST Action 16112

This abstract book is based upon work from COST Action NutRedOx-CA16112 supported by COST (European Cooperation in Science and Technology).

Preface

Welcome to Natural Products and the Hallmarks of Chronic Diseases

In 1998, we organized the first specialized meeting in the field of signal transduction and gene expression in Luxemburg. This type of meeting was originally thought to teach doctoral students of the **molecular and cellular biology master training program** of the University of Nancy I (France).

Since 2008, over 6500 participants came to Luxembourg to attend our meetings. In 2018, a first meeting was organized at Seoul National University in Seoul with over 150 registered participants.

This is the **14th meeting** we organize in Luxemburg and for this year's **COST Action 16112 meeting,** we could gather 90 consortium members. From a scientific point of view, we will focus on recent progress in natural compound research related to the hallmarks of chronic diseases and aging. Based on the outstanding research of our COST consortium partners, we selected **30 oral presentations** divided in **8 sessions** as well as 12 posters.

Our topics include novel advances in oxidant and antioxidant research combined with the main hallmarks of aging and chronic diseases including epigenetic and metabolic modulation. We also cover specific chronic pathologies including cancer, musculoskeletal aging combined to advanced technological improvements. A considerable part of the program will investigate old and novel compounds of natural origins with their effects on redox modulation, health and disease.

Welcome to Luxemburg!

Marc Diederich
Luxemburg

What is NutRedOx

The main aim of the NutRedOx network is to gather experts from across Europe and neighboring countries, and from different disciplines that are involved in the study of biological redox active food components and are relevant to the ageing population, its health, function, and vulnerability to disease.

Get more information here:

http://www.cost.eu/COST_Actions/ca/CA16112

http://blog.u-bourgogne.fr/cost-nutredox/

https://www.researchgate.net/project/Personalized-Nutrition-in-aging-society-redox-control-of-major-age-related-diseases

Dissemination measures for this meeting

"Proceedings"

We will collect the submissions from the authors, and send all the accepted papers, after peer review, to be included in the Proceedings in one package.

The basic idea is that you submit your meeting abstract already formatted for "Proceedings", together with your registration.

- 1-4 pages
- Editing fee: 12 Euro per paper paid by COST
- No other publication fees
- Novelty – do not copy and paste previous papers or abstracts - Unpublished abstracts
- Adhere to the abstract guidelines
- Tight turnaround after the conference is 1 week
- A local Scientific Committee (native English speakers) has been formed to fast track abstracts for submission

"Special number in Biochemical Pharmacology"

Biochem Pharmacol accepted a special number with contributions (reviews or original data) from the participants of this COST meeting.

- Tentative title: "Natural compounds with pharmacological applications in health, disease and ageing"
- Expected deadline June 2019 - No publishing fees - Peer-review according to the standards of the journal

Acknowledgments

This meeting has been organized by: Recherches Scientifiques Luxemburg asbl

This meeting was organized onsite by the research team of the "Laboratoire de Biologie Moléculaire et Cellulaire du Cancer (LBMCC)"

The organizers thank the help of the following colleagues who edited and formatted the participants for publications in this abstract book and the special number of "Proceedings" (https://www.mdpi.com/journal/proceedings):
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Dr. Mairead Coakley, Teagasc
Dr. Alina Kondrashina, Teagasc

The organizers thank Teagasc, the Irish Agriculture and Food Development Authority (www.teagasc.ie), for sponsoring a prize for the best presentation of our Early Career Scientists.

This abstract book is based upon work from COST Action NutRedOx-CA16112 supported by COST (European Cooperation in Science and Technology).

General Information

Meeting Venue
Hotel Park Alvisse
120, Route d'Echternach
L-1453 Luxemburg
Telephone: +352 43 56 43
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1.5 km from Dommeldange train station;
3.6 km from the Luxexpo event venue in the Kirchberg;
7 km from the Luxemburg Airport.

Registrations will take place at the registration desk (9h00-19h00) at the entrance of the main meeting room "Salle Europe C".

Coffee breaks will be served at the bar area of the "Salle Europe C".

A lunch buffet will be served at the bar area of the "Salle Europe C".

Networking dinner will be organized at the "Salle Wiltz-Vianden".

How to reach the Meeting venue:
http://www.parc-hotel.lu/en/contact/

Scientific Program

Monday, March 25th, 2019

11h00: Marc Diederich, Mustapha Cherkaoui Malki, Claus Jacob: Welcome address and dissemination activities

12h30: Lunch break

Session 1. Oxidative stress (Chairman: Marc Diederich)
14h00: Michael J. Davies (Dept. of Biomedical Sciences, Panum Institute, University of Copenhagen, Denmark): Quantification and mechanisms of oxidative stress in chronic disease
14h30: Mustapha Cherkaoui Malki (Laboratory BioPeroxIL, University of Bourgogne-Franche Comté, Dijon, France): Cytoprotective and antioxidants in peroxisomal neurodegenerative diseases
15h00: Andriy P. Cherkas (Lviv National Medical University/Sanofi-Aventis Deutschland GmbH): Helicobacter pylori in health and disease: its contribution to gastrointestinal disorders and systemic metabolic effects

Session 2. Epigenetic alterations (Chairman: Mustapha Cherkaoui Malki)
15h15: Michael Schnekenburger (LBMCC, Luxemburg): Natural compounds as epigenetic modulators in cancer
15h45: Wim Vanden Berghe (PPES Epigenetic Signaling, Wilrijk (Antwerpen), Beligum): Blood surrogate epigenetic biomarkers of atherosclerosis reveal common signature of inflammaging-disorders
16h15: Carsten Carlberg (University of Eastern Finland, Kuopio, Finland): Epigenome-wide effects of vitamin D
16h45: Patrycja Jakubek (Faculty of Chemistry, Gdańsk University of Technology, Poland): DNA methylation changes induced by redox-active compounds – choosing the right PCR-based method

17h00: Coffee break & Posters

Session 3. Metabolic alterations (Chairman: Claus Jacob)
17h30: Shlomo Sasson (Institute for Drug Research, Faculty of Medicine, The Hebrew University, Jerusalem Israel): Fatty Aldehyde Dehydrogenase (ALDH3A2)-dependent neutralization of Advanced Lipid Peroxidation End Products (ALEs) at the bifurcation of hormetic and degenerative pathways in pancreatic beta cells
18h00: Lars-Oliver Klotz (Inst Nutritional Sciences, University of Jena, Germany): FOXO transcription factors: regulators of metabolism and stress resistance
18h30: Niki Chondrogianni (Institute of Biology, Medicinal Chemistry and Biotechnology, National Hellenic Research Foundation, Athens, Greece): Quest for anti-ageing compounds in our diet: potential anti-ageing and anti-aggregation applications

19h00: End of the scientific day

Tuesday March 26th, 2019

Session 4. Innovative approaches (Chairman: Elke Richling)
9h00: Patrick Chaimbault (University of Lorraine, France): Diagnosis of biological activities by Mass Spectrometry
9h30: Claudia Cerella (LBMCC, Luxemburg): Targeted anticancer strategies with garlic derivatives
10h00: Caroline Gaucher (CITHEFOR EA3452/Université de Lorraine, Nancy, France): Antioxidant Properties of *S*-nitrosoglutathione and nanotechnologies
10h30: Yi Zhou (Université de Lorraine, CITHEFOR, F-54000 Nancy, France): Nano or micro: 3 different particles to deliver and protect *S*-nitrosoglutathione for oral route administration

10h45: Coffee break & Posters

Session 5. Cancer (Chairman: Wim Vanden Berghe)
11:15: Muriel Cuendet (School of pharmaceutical sciences, University of Geneva, University of Lausanne, Geneva, Switzerland): Targeting the resistance in multiple myeloma
11h45: Sungmi Song (LBMCC, Luxemburg and College of Pharmacy, Seoul National University, South Korea): Hydroquinone-derivatives induce apoptotic and necrotic cell death in chronic myelogenous leukemia
12h00: Behrouz Hassannia (Inflammation Research Center-University of Gent, Belgium): Nano-targeting of ferroptosis eradicates high-risk neuroblastoma
12h15: Tomris Ozben (Department of Medical Biochemistry, Faculty of Medicine, Akdeniz University, Antalya, Turkey): Effects of N-Acetylcysteine on Multidrug Resistance-Associated Protein 1 (MRP1) Involved Multidrug Resistance

12h30: Lunch break

Session 6: Redox in health and disease (Chairman: Norbert Latruffe)
14h00: James R Edwards (Botnar Research Centre, University of Oxford, UK): Musculoskeletal Ageing – Dietary Modification of Longevity Mechanisms to Improve Skeletal Health
14h30: Jedrzej Antosiewicz (Medical University of Gdansk, Department of Bioenergetics and Physiology of Exercise, Poland: Effects of diet and exercise on endocrine function of skeletal muscle
15h00: Armen Trchounian (Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University, Yerevan, Armenia): Herb extracts in treatment and prevention of experimental metabolic disorders: Synergistic hypoglycemic activity of ethanol extracts of a mixture of *Hypericum alpestre* and *Rumex obtusifolius*

15h30: Coffee break & Posters

Session 7: Natural compound redox modulators 1 (Chairman: Agnieszka Bartoszek)
16h00: Elke Richling (University of Kaiserslautern, Germany): Bioactive metabolites in humans – the example of coffee
16h30: Norbert Latruffe (Laboratory BioPeroxIL, University of Bourgogne-Franche Comté, Dijon, France): Resveratrol-dependent stimulation of mitochondrial fatty acid oxidation in deficient cells. Implication of miRNAs
17h00: Nina Hermans (Research Group NatuRA / University of Antwerp, Belgium): Protective effects of dietary polyphenols on arterial stiffness

17h30: Zuzanna Koziara (Department of Food Chemistry, Technology and Biotechnology, Gdansk University of Technology, Gdansk, Poland): Comparison of redox properties of flavonoid aglycones and corresponding glycosides and their mixtures in the cellular model

17h45: End of the scientific day

19h30: COST networking dinner
(Alvisse Park Hotel)

Wednesday, March 27th, 2019

Session 8: Natural compound redox modulators 2 (Chairman: Linda Giblin)
9h00: Martin C.H. Gruhlke (Department of Plant Physiology (Bio III), RWTH Aachen University, Germany): Thiol-modification as Important Mode of Action for Allicin from Garlic (*Allium sativum*)
9h30: Kateřina Valentová (Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic): 2,3-Dehydroderivatives of Silymarin Flavonolignans: Prospective Natural Compounds for the Prevention of Chronic Diseases
9h45: Christine Fuchs (Technische Universität Kaiserslautern, Germany): Effects of extracts from winery by-products on mitochondrial functions in HepG2 cells
10h00: Claus Jacob (Saarland University, Saarbrücken, Germany): Inspired by Nature: Redox modulators and natural nanoparticles

10h30: Coffee break

11h00: Olivier Mortelé (NatuRA, University of Antwerp, Belgium): Chlorogenic acid as a model compound for optimization of an *in vitro* gut microbiome-metabolism model
11h30: Mustapha Cherkaoui Malki (Chair NutRdOx CA16112), NutRedOx Action activities: Running and Next Grant Periods
12h00: Marc Diederich: Award ceremony and closing NutRedOx Meeting

Oral Presentations
(in the order of the scientific program)

proceedings

Abstract

Quantification and mechanisms of oxidative stress in chronic disease †

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† Presented Natural Products and the Hallmarks of Chronic Diseases – COST Action 16112, Luxemburg 25-27 March 2019

Abstract: There is now strong evidence that the redox environment inside cells is very different to that outside the cell, and that many extracellular environments are both more oxidizing and also subject to extensive oxidation. This difference in redox environments results in significant changes in oxidation chemistry and biology, altered redox equilibria, and antioxidant defense mechanisms. It is also increasingly apparent that oxidation events both inside and outside cells (extracellular oxidation) play a critical role in driving many diseases. Many extracellular proteins are highly abundant, long-lived and relatively poorly protected against damage. They can therefore accumulate high levels of modification during ageing and chronic disease, resulting in their use as biomarkers of long-term oxidative stress. Furthermore, increasing evidence supports the hypothesis that oxidized extracellular matrix materials play a key role in determining cell function and fate.

Keywords: Protein oxidation, post-translation modification, extracellular matrix, cardiovascular disease, atherosclerosis, peroxynitrous acid, nitration, hypochlorous acid, chlorination

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Biological systems are continually exposed to endogenous and exogenous free radicals and two-electron oxidants [1]. The formation and reactions of these species are limited by defense systems within cells and organisms, including low-molecular-mass scavengers (e.g. ascorbic acid, thiols, quinols, tocopherols, carotenoids, polyphenols, urate), enzymes that remove oxidants (e.g. superoxide dismutases), enzymes that remove oxidant precursors (e.g. peroxiredoxins, glutathione peroxidases and catalases), and enzyme systems that repair or remove damaged materials (methionine sulfoxide reductases, disulfide reductases / isomerases, sulfiredoxins, proteasomes, lysosomes, proteases, phospholipases, DNA repair enzymes) [1].

Despite the presence of these mechanisms, elevated levels of oxidative damage have been detected in multiple human, animal, microbial and plant systems [1]. This may be due to increased oxidant levels, a decrease or failure of defense systems, or both. In many cases both may be important, as defense systems are themselves subject to damage or depletion of critical co-factors. Ageing also results in declines in enzyme activity, and lower levels of essential trace elements and metabolites, with this decline often accelerated by disease or environmental factors.

A large number of oxidants can be generated *in vivo*, and these vary markedly in their reactivity and specificity [1,2]. Both one-electron (radical, e.g. hydroxyl radicals, HO•) and two-electron oxidants (e.g. peroxynitrous acid, ONOOH; hypochlorous acid, HOCl; singlet oxygen, $^1O_2$; peroxides) can be formed, with these having markedly different reactions kinetics and mechanisms of reaction [2]. Whilst many radicals react with high rate constants (i.e. very rapidly), this is not always true, with some radicals being relatively long-lived, and poorly reactive [2]. A similar pattern is seen with two-electron oxidants, with some having very high rate constants for reaction with particular targets (e.g. HOCl, $^1O_2$ with sulfur-containing and aromatic amino acids), and others low rate constants [2]. The damage generated by these species is therefore highly variable and complex [2]. HO• is unselective and causes widespread indiscriminate damage, whilst

other oxidants (e.g. H_2O_2) may react slowly and with high selectivity (i.e. with very specific targets) [2]. The products generate by these reactions are also numerous and diverse [2-5]. In some cases, the products are generic (e.g. protein carbonyls [6-8]) and are formed by multiple different pathways, whereas in some other cases, the product is specific and diagnostic for a particular type of reactive species. Thus, the species 3-chlorotyrosine formed from the amino acid tyrosine (Tyr) is a specific product generated by the powerful two-electron oxidant, HOCl [9-11]. Highly reactive oxidants only diffuse short distances before reaction, whereas less reactive species have longer half-lives, can diffuse longer distances, and induce damage at remote locations [2,12-14]. Reactive oxidants therefore usually give rise to site-specific damage at, or near, their sites of formation. Many oxidants can also give rise to secondary oxidants (e.g. radicals formed by the decomposition of lipid peroxides), of different reactivity and lifetimes when compared to the initial species, complicating the analysis of damage [2]. Understanding the nature and reactivity of potential oxidants, and the patterns and extents of damage that they induce is therefore critical.

Oxidant formation can occur both *within* cells and *external* to them [1]. Electron leakage from mitochondria (and to a lesser extent other electron transport chains) is a major source of oxidants within cells, with this resulting in significant fluxes of superoxide radical anions ($O_2^{-}\bullet$), and subsequently H_2O_2 as a result of the rapid spontaneous, and enzyme-catalyzed dismutation (by superoxide dismutases) of $O_2^{-}\bullet$ [1]. Acute and chronic inflammation is a major source of extracellular oxidant formation, as stimulation of neutrophils, monocytes and macrophages can result in the enzymatic formation of multiple oxidants including $O_2^{-}\bullet$, H_2O_2, HOCl and ONOOH. HOCl is generated from H_2O_2 and chloride ions by the heme enzyme myeloperoxidase, which is released from intra-cellular storage granules of neutrophils, monocytes and some tissue macrophages [9-11]. The precursor of ONOOH, NO•, is formed via the inducible nitric oxide synthase of macrophages [12]. Lower levels of NO• are also generated by the constitutive nitric oxide synthase isoforms present in many cells. Concomitant with the formation of NO•, $O_2^{-}\bullet$is generated at relatively high levels by plasma membrane NADPH oxidases (at the expense of intracellular NADPH), with subsequent rapid (diffusion-controlled) reaction of NO• with $O_2^{-}\bullet$ giving peroxynitrous acid, ONOOH [12,15].

Proteins are major targets for many oxidants due to their high abundance and the high rate constants for reaction with many oxidants, both within cells and in extracellular compartments (e.g. the extracellular matrix, and in plasma and other fluids) [2,16]. With reactive species, such as HOCl and ONOOH, both side-chain (mainly at Cys, Met, Trp, Tyr and His) and backbone damage can be detected, whereas with less reactive species, such as $O_2^{-}\bullet$ and H_2O_2, damage is both limited and highly selective [2,12-14,16]. Thus, H_2O_2 primarily modifies Cys, and to a lesser extent Met, residues on proteins.

Protein damage *within* cells is often rapidly repaired (but *only* in the case of damage to Cys and Met), or removed via catabolism [2]. External to cells the situation is somewhat different, as extracellular materials are usually poorly protected against damage [17], and there is limited capacity for repair. The high abundance of extracellular proteins, their proximity to sites of oxidant formation, their (in general) long half-lives, and poor repair mechanisms, are likely to result in an accumulation of damage on these materials, particularly with increasing age, with this enhanced by disease.

Elastin is the most abundant extracellular matrix protein in elastic tissues, including the lungs, skin and arteries, and comprises 30-57 % of the aorta by dry mass. Most elastin synthesis occurs during the early years of life, with limited synthesis in adults, therefore most elastin is as old as the host. It is therefore believed to accumulate high levels of modifications over time. Mature elastin is synthesized from monomeric tropoelastin (TE), with the latter undergoing complex processing to form mature elastic fibers. As considerable evidence supports ONOOH formation in the inflamed artery wall (e.g. [18-22]), we hypothesized that TE would be highly susceptible to modification and structural alteration by ONOOH, with consequences for the function of TE. This damage to TE may play a role in the development of cardiovascular disease, as modified matrix species have been implicated in atherosclerotic lesion development and rupture [18-28].

We have shown that TE is highly sensitive to ONOOH, with extensive dimerization and fragmentation (detected by SDS-PAGE with silver staining or Western blotting) and nitration of tyrosine (Tyr) residues to give 3-nitroTyr, detected by both amino acid analysis and mass spectrometry (MS) peptide

mass mapping [22]. This damage can be detected with equimolar or greater levels of oxidant and increases in a dose-dependent manner [22]. Quantification of Tyr loss and 3-nitroTyr formation indicates extensive Tyr modification with up to two modified Tyr per protein molecule, and up to 8% conversion of ONOOH to 3-nitroTyr [22]. These effects were modulated by bicarbonate, a competitive target for ONOOH. 3-nitroTyr formation was detected at 12 of 15 Tyr sites in TE treated with equimolar or higher levels of ONOOH. Label-free MS quantification has revealed extensive nitration (> 50% modification) at some sites. Four Tyr residues have also been shown to be involved in the formation of inter- and intra-molecular di-tyrosine cross-links, with these characterized using an ^{18}O labelling MS approach [22,29,30].

TE treatment with ONOOH lowered the concentration at which TE assembles to give larger structures (as determined by turbidity and sedimentation experiments; Lorentzen et al., unpublished data), increased the rate of this process, and markedly impaired the reversibility of this process (as determined by dynamic light scattering; Lorentzen et al., unpublished data)). Scanning electron microscopy indicated the presence of abnormal TE structures (Lorentzen et al., unpublished data). Studies on human atherosclerotic lesions using immunohistochemistry showed colocalization of 3-nitroTyr with elastin epitopes, consistent with the occurrence of these modifications *in vivo*, and also an association of 3-nitroTyr-containing proteins and elastin with lipid deposits [22]. Together these data suggest that exposure of TE to ONOOH gives marked chemical, structural and functional changes to TE and alters extracellular matrix assembly. This damage accumulates in human arterial tissue with age, and during the development of atherosclerosis.

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Abstract

Cytoprotective and antioxidants in peroxisomal neurodegenerative diseases †

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Several of the peroxisomal neurodegenerative disorders are the consequence of a specific deficiency of an enzyme or a transporter involved in peroxisomal beta-oxidation of very long chain fatty acids [1, 2]. One of the hallmarks in these peroxisomal rare neurodegenerative diseases and in other common demyelinating disorders is the accompanying oxidative damage and neuroinflammation [5]. Compelling data indicates that oxidative stress can activate microglia leading to the overproduction of pro-inflammatory molecules [3, 4]. Thus, targeting oxidative stress to limit neuroinflammation may open a new pharmacological therapy window for these still incurable devastating peroxisomal diseases. Here, we present different natural (resveratrol) [6] and synthetic (organoselenides) [7] antioxidant compounds for their capacity of scavenging oxidative stress and in the perspective therapeutic use against oxidative damage in peroxisomal disorders.

Keywords: Antioxidant; Leukodystrophy; Organoselenides; Peroxisome; Resveratrol.

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Abstract

Helicobacter pylori in health and disease: its contribution to gastrointestinal disorders and systemic metabolic effects†

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Helicobacter pylori is one of the most common human infections worldwide and it is estimated that more than half of global population is affected. This microorganism induces oxidative stress in gastric mucosa and causes chronic gastritis, duodenal peptic ulcer, gastric cancer, and as reasonably suspected a number of extragastric diseases [1-2]. Our research was focused on both local (on the level of gastric mucosa) and systemic effects of *H. pylori* positivity in patients with duodenal peptic ulcer and healthy volunteers. It was shown that increased accumulation of 4-hydroxynonenal (HNE) persists even despite *H. pylori* eradication [3-4]. We performed an interventional study to evaluate effects of Amaranth oil on accumulation of HNE-histidine adducts in gastric mucosa of patients undergoing routine anti-*H. pylori* treatment. It was demonstrated that Amaranth oil supplementation provided significant improvement of gastric mucosa morphological pattern and also had a positive effect on heart rate variability [5]. In a separate study we demonstrated that *H. pylori* in apparently healthy sedentary young male subjects is associated with higher heart rate, sympathetic activity and insulin resistance, however, we detected no changes in parameters reflecting inflammatory profile, metabolic parameters or oxidative stress [6-8]. In summary, there is a growing evidence of systemic metabolic effects of *H. pylori* infection not only in patients with overt gastrointestinal disorders, but also in apparently asymptomatic healthy subjects. Thus *H. pylori* status should be seriously considered in human studies focusing on both pharmacological and non-pharmacological approaches.

Keywords: Helicobacter pylori; oxidative stress; gastric mucosa, metabolic syndrome, insulin resistance, Amaranth oil.

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Abstract

Natural compounds as epigenetic modulators in cancer †

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Epigenetics refers to the study of heritable changes in gene function that are mediated by mechanisms other than nucleotide alterations in the primary DNA sequence. These changes are achieved by methylation on cytosine bases in DNA, by post-transcriptional modifications (e.g., acetylation and methylation) on histone proteins or RNA transcripts by non-coding RNAs (RNA-mediated gene silencing) [1-3].

Nowadays, it is well established that the disruption of epigenetic processes plays a significant role in every step of carcinogenesis by altering gene expression profiles and protein functions. These global changes in the epigenetic landscape represent a hallmark of cancer [1,4-6].

The potential reversibility of epigenetic abnormalities encouraged the development of pharmacological modulators, so-called epigenetic drugs, against the writers, eraser or readers of epigenetics marks as valuable anti-cancer therapeutic targets. Although epigenetic drugs have a relevant therapeutic potential, only a relatively limited number of molecules including DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors have been approved by the FDA and the EMEA for cancer treatment. Accordingly, there is an urgent need to develop new epigenetically active compounds for improved therapeutics in cancer therapy.

Owing to their diverse biological activities and medicinal potentials, bioactive compounds isolated from natural sources (plants, fungi, marine life forms) and their derivatives, thanks to combinatorial chemistry, represent an inexhaustible source for drug discovery leading the development of new epigenetic drug candidates [2,7-12]

In this presentation, we will focus on the significant findings regarding our research related to the characterization of new epigenetically active compounds of natural origin or their derivatives with anti-cancer activities.

Keywords: cancer; epigenetic; DNA methylation; histones modifications; natural compounds

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Abstract

Blood surrogate epigenetic biomarkers of atherosclerosis reveal common signature of inflamm-aging-disorders †

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DNA methylation is the most well-known epigenetic modification of DNA. This epigenetic mark is crucial in controlling gene expression profiles, maintaining cellular identity, genomic imprinting and X-chromosome inactivation. Furthermore, DNA methylation is plastic and can adapt to environmental stimuli, acting as a cellular memory of past events. Whereas epigenetic DNA methylation profiling in cancer diagnostics is now well established, associations with other chronic age-associated diseases, including obesity, diabetes, cardiovascular and neurological diseases have recently started to be explored for prognostic, diagnostic and therapeutic applications. Upon genome-wide DNA methylation profiling of whole blood samples from atherosclerotic patients, we characterized various atherosclerosis specific differentially methylated regions (DMRs). Interestingly, similar DMRs were also observed in other age- and inflammation-associated diseases, like obesity, cancer, Alzheimer's and Parkinson's disease, both in blood as well as in brain and tumor tissues. This suggests that inflammaging diseases share a common epigenetic signature of the immune system, which is different from the classic epigenetic clock signature. Furthermore, a cardio-protective flavanol-rich diet intervention can partially reverse this inflammaging disease associated epigenetic pattern. We found that this methylation profile mainly reflects shifts in immune cell type composition and infiltrating immune cell populations. Upon correcting for differences in immune cell composition in blood samples, we identified BRCA1 DNA methylation as an atherosclerosis-specific methylation biomarker irrespective of variations in immune cell biomarkers. How BRCA1 DNA methylation differentially promotes cancer, neurodegeneration or atherosclerosis pathologies requires further investigation. In conclusion, atherosclerosis patient blood samples reveal inflammaging and atherosclerosis-specific DNA methylation

biomarkers, which could potentially be used as lifestyle biomarkers to estimate disease risk of neurodegeneration, cardiometabolic disorders and cancer in aging populations.

Keywords: epigenetic biomarker, atherosclerosis, inflammaging

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Abstract

Epigenome-wide effects of vitamin D †

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Vitamin D_3 has, via its metabolite 1,25-dihydroxyvitaminD_3 (1,25$(OH)_2D_3$), a high affinity to the transcription factor vitamin D receptor (VDR), and thereby directly affects the epigenome of its target tissues. Changing the transcriptome results in modulation of physiological function, such as calcium homeostasis and the response of innate and adaptive immunity. Genome-wide datasets on the 1,25$(OH)_2D_3$-triggered binding of VDR, its pioneer factors PU.1 and CEPBA, histone markers and chromatin accessibility in THP-1 human monocytes compared to those obtained in peripheral blood mononuclear cells from vitaminD_3-supplemented human volunteers (VitDbol study) allow the assessment of the epigenome-wide effects of vitamin D.

Keywords: vitamin D; epigenomics; gene regulation; VDR; vitamin D intervention studies; chromatin; monocytes

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Abstract

DNA methylation changes induced by redox-active compounds – choosing the right PCR-based method†

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Abstract: The impact of catechins on the expression profile of redox-related genes in HT29 cell line has been studied recently by our groups using Oxidative Stress RT2 Profiler PCR Array. Within the examined panel of 84 genes, the down-regulation of SRXN1 gene was unique among other up-regulated genes. We hypothesized that the observed down-regulation resulted from DNA methylation and have exploited this observation to choose the proper strategy to monitor the changes in DNA methylation pattern incurred by dietary antioxidants. The current study verified two PCR-based approaches.

Keywords: catechins; DNA methylation; methylation-specific PCR; methylation-sensitive high resolution melting; redox homeostasis; epigenetics

1. Introduction

The role of phytochemicals in epigenetic processes, such as DNA methylation, has been of major interest over past years. The choice of an appropriate method for assessment of DNA methylation depends on the biological question of scientific investigation. High throughput techniques find its application in a measurement of whole genome methylation status, whereas techniques of low throughput are used to evaluate methylation level of a region of interest. The second group includes PCR-based methods: methylation-specific PCR (MSP) and methylation-sensitive high-resolution melting (MS-HRM), which differs by reproducibility, sensitivity and specificity. The initial step before any analysis is to transform DNA in a bisulfite conversion reaction in order to distinguish methylated cytosines from unmethylated ones within the investigated sequence. MSP is a simple method and to perform it, two pairs of primers are needed: one specific to methylated and the other to unmethylated CpG's. This approach requires two separate PCR reactions to be run for each sample. In MS-HRM method, the methylation level is based on the melting profile of a PCR product, which must be compared to the standards of known percentage of methylation. MSP method assesses methylation of CpG's within primer-binding sites only, while MS-HRM evaluates the ratio of methylated to unmethylated CpG's present within a sequence delineated with designed primer pair [1].

In a previous study, we reported that exposure of HT29 cell line to physiological concentration of catechins up-regulates the expression of genes associated with oxidative stress defense [2]. On the other hand, the higher – 10 μM – concentration, relevant only for the intestinal cells being in a direct contact with ingested food, seems to preserve cellular redox state, hence the expression of redox-related genes remained not affected with the exception of the down-regulation of SRXN1 gene by (-)-epigallocatechin (EGC). The SRXN1 gene encodes for sulfiredoxin (Srx) – the enzyme responsible for restoring the activity of hydrogen peroxide scavengers, namely peroxiredoxins (Prxs) that under oxidative stress conditions become oxidized and inactivated [3]. The increased expression of SRXN1 has been shown in various types of cancer, including colon cancer. In contrast, it was not up-regulated neither in mouse nor human normal colon epithelial cells nor cancer adjacent normal tissues [10–12].

The objective of this study was to choose the proper methodology for the detection of changes in methylation profile of CpG islands within the promoter area of SRXN1 gene in HT29 cell line exposed to model

dietary antioxidants. For the study, three compounds were chosen, that in previous experiments displayed different impact on this gene expression: two catechins and a major endogenous antioxidant present in many foods – glutathione.

2. Materials and methods

2.1. Cell culture and treatment with antioxidants

Human colon adenocarcinoma cell line (HT29) from the ATCC was cultured in supplemented McCoy's medium in a humidified atmosphere with 5 % CO_2 at 37°C as described previously [2]. Cells for DNA methylation analysis were seeded in 6-well tissue culture plates and treated with (+)-catechin (C), (-)-epigallocatechin (EGC) (Extrasynthese) or glutathione (GSH) (Sigma-Aldrich) for 24 h at 37°C. The experiments were performed in three independent biological replicates and with adequate control cells (treated with 3% ethanol or water).

2.2. Genomic DNA isolation and bisulfite conversion

Genomic DNA was isolated from treated and control cells using QuickDNA Miniprep Plus Kit from Zymo Research (USA) according to the manufacturer's protocol. Bisulfite conversion of isolated genomic DNA was performed with EZ DNA Methylation kit (Zymo Research, USA) according to the protocol provided by the manufacturer.

2.3. Prediction of CpG islands and design of primers

CpG island of human SRXN1 gene (SRXN1; NC_000020.11) was predicted with EMBOSS CpGplot (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) using standard parameters. The sequence of genomic DNA uploaded for the analysis comprised the region of 1,000 bp upstream and 395 bp downstream. Methylated (M) and unmethylated (U) sets of primers for MSP and MS-HRM analyses were designed using MethPrimer. The sequences of the primers (Invitrogen, USA) were as follows: forward M1 5'-GTTAGATTGGAAGTGGAATCGTT-3', reverse M1 5'-CCAAAATAAATCGACAAAACCC-3', forward U1 5'-GTTAGATTGGAAGTGGAATTGT-3', reverse U1 5'-AACCAAAATAAATCAACAAAACCC-3'.

2.4. Methylation-specific PCR

For Methylation-Specific PCR (MSP), Maxima SYBR Green/ ROX qPCR Master Mix (2X) from Thermo Scientific (USA) was used. Thermal cycling conditions of MSP were as follows: initial denaturation step at 95°C for 10 min followed by three-step protocol with 40 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. After 40 cycles of three-step protocol, a 10 min final extension step at 72°C was added to complete synthesis of MSP products. To verify the specificity and identity the PCR products, the melting curve analysis was performed at the following thermal conditions: 95°C for 15 s, 60°C for 60 s and 95°C for 1 s. MSP was performed in QuantStudio 3 Real-Time PCR System from Applied Biosystems (USA). Relative DNA methylation was calculated based on the difference of C_t values for methylated and unmethylated PCR products.

2.5. Methylation-sensitive high resolution melting

For the detection of all possible variants in DNA methylation status, each reaction contained a mix of all 4 primers (methylated and unmethylated pairs). PCR amplification and subsequent HRM analysis were performed in QuantStudio 3 Real-Time PCR System from Applied Biosystems (USA). The thermal cycling conditions included 10 min of initial denaturation at 95°C, followed by three-step PCR with 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 30 s and elongation at 72°C for 30 s. The protocol was finished with 7 min of subsequent final extension at 72°C. PCR amplification was followed by HRM that consisted of denaturation at 95°C for 15 s, annealing at 60°C for 60 s and the final 15 s of denaturation at 95°C with a ramp of 0.025°C per second. For the end-product analysis, HRM Software c3.1 (Applied Biosystems,

USA) was applied. Standard curves for Methylation-Sensitive High-Resolution Melting (MS HRM) analysis were prepared using human methylated and non-methylated DNA standards (Zymo Research, USA), which were first bisulfite-converted using EZ DNA Methylation kit (Zymo Research, USA) according to the protocol. Subsequently, the converted methylated and non-methylated DNA samples were mixed to obtain standards of 0, 50 and 100% methylation. After normalization of obtained results, the linear regression equation was used to quantitatively calculate the percentage of methylation of each sample.

3. Results

The SRXN1 gene is 6.7 kb long with the promoter area of 2.7 kb according to GeneCards (Human Gene Database). Using the EMBOSS CpGplot online tool, we identified one CpG island of 706 bp length located at -366 to +340 from the transcription starting site (TSS) and designed sets of methylated and unmethylated primers (M1/U1) that bind within this sequence as shown in Fig. 1.

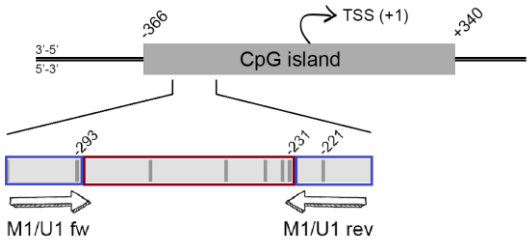

Figure 1. Schematic presentation of promoter area of the SRXN1 gene encompassing investigated CpG island. Primer binding sites are shown in the inset with CpG dinucleotides marked as short vertical lines. The position of the TSS is marked as "+1"; M1/U1 fw, methylated and unmethylated forward primers, respectively; M1/U1 rev, methylated and unmethylated reverse primers, respectively. Blue outlining indicates regions investigated with MSP, whereas red one – MS-HRM.

Methylation status of specific CpG's investigated with MSP method did not differ between control and investigated compounds (C, EGC, GSH at 10 μM concentration). On the other hand, MS-HRM analysis showed significant changes in the methylation level within the region of interest, but only after treatment with catechins. The treatment of HT29 cells with glutathione did not influence DNA methylation or expression of SRXN1 gene. As hypothesized, 10 μM EGC that was reported to down-regulate the expression of SRXN1 gene, was also able to induce significant increase in the DNA methylation level within investigated area of CpG island. However, the impact of (+)-catechin was opposite to EGC, i.e., in this case DNA methylation was significantly decreased.

4. Discussion

Based on the results of microarray analysis from the previous study [2], we expected that DNA of cells treated with EGC at 10 μM concentration would be more methylated within the examined CpG island of SRXN1 promoter, since such a treatment caused down-regulation of this gene expression. These initial assumptions were confirmed only in the experiments carried out by MS-HRM approach. In the case of (+)-catechin (parent structure of EGC) and glutathione (of similar to EGC standard redox potential), no change in this gene methylation profile was detected by any of two PCR-based methods tested. Such a result was expected, because these two compounds did not alter SRNX1 gene expression.

MSP and MS-HRM methods, at least theoretically, enable different insight into DNA methylation process as they refer not only to different CpG's, but also to a different occurrence of cytosines in the sequence analysed. In the case of our investigations, though MSP analysis did not show any changes in the methylation status of specific cytosines in the sequence of interest, the difference became clearly seen when the bigger area of CpG island was analysed by MS-HRM. The results obtained with this second approach showed that treatment of HT29 cells with 10 μM EGC resulted in elevated DNA methylation level, which is consistent with down-regulated gene expression. Based on these observations, MS-HRM method seems more appropriate for

the further investigation of changes in DNA methylation status in the promoter area of SRXN1 gene induced by phytochemicals exerting antioxidant activity.

Interestingly, discussed experiments provided additional insight into the possible mechanisms of catechin impact on DNA methylation. Up till now, catechins have been reported only to act as inhibitors of DNA methyltransferases, which was reflected by the decrease in DNA methylation demonstrated for e.g. (+)-catechin. Current results demonstrate that these flavonoids may also be capable of increasing the level of methylated cytosines.

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Abstract

Fatty Aldehyde Dehydrogenase (ALDH3A2)-Dependent Neutralization of Advanced Lipid Peroxidation End Products (ALEs) at the Bifurcation of Hormetic and Degenerative Pathways in Pancreatic Beta Cells †

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Hyperglycemia and hyperlipidemia synergistically and adversely impair insulin secretion and ultimately lead to pancreatic beta cell decomposition. We found that both nutrient overload conditions displace arachidonic and linoleic acids from membrane phospholipids and subject them to free radical-mediated peroxidation and generation of advanced lipid peroxidation end products (ALEs), of which the aldehyde 4-hydroxy-2-nonenal (4-HNE) is prominent. When present at high levels this electrophilic molecule binds covalently to nucleophilic moieties in proteins, phospholipids and nucleic acid, modifies their structure and function and leads to severe cellular dysfunction and apoptosis. However, when present at low and unharmful levels this same molecule activates the nuclear receptor PPARδ and augments insulin secretion. The level of endogenous 4-HNE is determined by the extent of lipid peroxidation on one hand, and by enzymatic neutralization of the aldehyde on the other. The latter step is mediated by enzymatic processes of which the transformation of the aldehyde to the corresponding inactive carboxylic derivative 4-hydroxy-2-nonenoic acid (4-HNA) is significant. The enzyme responsible for this transformation, which belongs to the large family of aldehyde dehydrogenases and selectively neutralizes fatty acid-derived aldehydes, is ALDH3A2, which is also known as fatty aldehyde dehydrogenase (FALDH). Consequently, we hypothesized that the expression level and function of ALDH3A2 may determine the fate of beta cells under nutrient overload conditions: insufficient neutralization of 4-HNE by the enzyme will lead to cell demise, whereas increased expression and function will extend the adaptive response of beta cells. This adaptive response that is characterized with increased insulin secretion enables effective storage of the nutrient surplus in peripheral tissues and organs while minimizing the dire consequences of the nutrient overload.

We aimed at investigating the expression pattern of ALDH3A2 in pancreatic beta cells (the INS-1E cell line) under hyperglycemic condition without or with supplementation with saturated fatty acids (e.g. palmitic acid). Our results show significant glucose- and palmitic acid-dependent induction of ALDH3A2 expression in the cells. We also found that the transformation of palmitic acid (16:1) to mono-unsaturated palmitoleic acid (16:1, *cis* 9) by the enzyme Stearoyl-CoA desaturase-1 (SCD1) decreased the burden of the lipid stress on the cells and abrogated the stimulus for the induction of ALDH3A2. Preliminary experiments indicated that the upregulation of the induction of ALDH3A2 was partly induced by PPARδ. These findings correlate to our previous discovery that the hormetic effects of 4-HNE were mediated via activation of this nuclear receptor.

In summary, this study assigns a central role to the enzyme ALDH3A2 in the protective mechanism beta cells employ to mitigate detrimental effects of ALEs, and divert them into hormetic agents, that by feedback mechanism through PPARδ increase ALDH3A2 expression.

Keywords: aldehyde dehydrogenase**,** ALDH3A2; beta cells; 2; type 2 diabetes; lipid peroxidation; 4-hydroxynonenal.

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Abstract

FOXO transcription factors: regulators of metabolism and stress resistance †

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Abstract: FOXO (Forkhead box, class O) proteins are transcriptional regulators ubiquitously expressed in mammalian cells with roles in modulating fuel metabolism, stress resistance and cell death. FOXO transcription factors are regulated by redox processes at several levels, including enzymatic and nonenzymatic posttranslational modification. Target genes controlled by FOXO proteins include genes encoding antioxidant proteins, thus likely contributing to the key role FOXOs play in the cellular response to oxidative stress. Here, an overview is provided on (i) the modulation of FOXO proteins by thiol depleting agents, (ii) consequences of thiol depletion for stress resistance and life span of a model organism, *Caenorhabditis elegans*, and (iii) the role of FOXO proteins therein.

Keywords: hormesis; diethyl maleate; glutathione; *C. elegans*, stress signaling, Nrf2

1. Introduction

FOXO transcription factors are regulated by posttranslational modification, including phosphorylation, acetylation and ubiquitinylation. In addition, nonenzymatic modification, such as thiol oxidation to generate intermolecular disulfide bonds between FOXOs and regulating proteins was described to occur under exposure to reactive oxygen species, such as hydrogen peroxide [1], constituting one of the several layers of redox regulation of these transcription factors [2].

Here, we summarize recent data on the consequences of cellular thiol depletion for FOXO activity and organismal life span in addition to reviewing the role of individual cysteine residues in controlling their transcriptional regulation by FOXOs.

2. Hormetic effects of thiol depletion in *Caenorhabditis elegans*

Exposure of a model organism, *C. elegans*, to thiol-depleting agents, such as the alkylating agent diethyl maleate (DEM), causes a moderate depletion of glutathione and a modulation of the organism's life span. Whereas growth of worms on agar containing 1 mM DEM significantly shortens life span by approx. 10% relative to control conditions, an exposure of worms to lower concentrations of DEM (up to 0.1 mM) elicits an extension of life span (by approx. 5%), pointing to an adaptive response elicited by lower DEM concentrations [3]. In fact, resistance to oxidative stress elicited by the redox cycler paraquat is also elevated in worms exposed to lower DEM concentrations. Interestingly, the same low DEM concentrations stimulate the expression of genes encoding antioxidative proteins. Additionally, Thiol depletion elicited by attenuation of glutathione biosynthesis through RNA interference with expression of *C. elegans* γ-glutamyl-cysteine synthetase confirms that glutathione depletion may cause an adaptive response eliciting life span extension [3].

DEM-induced life span extension is mediated, in part, by the *C. elegans* FOXO ortholog, DAF-16, as demonstrated by RNA interference. In addition to DAF-16, the *C. elegans* ortholog of transcription factor Nrf2, SKN-1, appears to be involved in the adaptive response, in line with the known Nrf2 activating activity of DEM [4].

3. Diethyl maleate in mammalian cells: glutathione depletion and FOXO modulation

Although DEM affects *C. elegans* life span via modulation of the nematode FOXO ortholog, no change in transcriptional regulatory activity is observed for mammalian FOXO1 in cells exposed to DEM [5]. Despite a nuclear accumulation of FOXO1 that occurs in response to exposure of human HepG2 hepatoma cells to DEM, no upregulation of FOXO1 target genes is observed – quite in contrast to Nrf2 target genes, which are upregulated at the same time. Moreover, FOXO1 remains located in the nucleus even in the presence of insulin, a stimulus eliciting FOXO inactivation and nuclear exclusion. In summary, DEM traps inactive FOXO1 in the nucleus whereas it stimulates Nrf2-dependent gene expression [5].

4. Distinct roles of FOXO1 cysteine residues in modulating transcriptional activity

Analysis of the contributions of individual cysteine residues of human FOXO1 to its transcriptional regulatory activity through site-directed mutagenesis indicates that only one of the seven cysteines is required for full basal activity [6]. Cys-612, even under basal culture conditions, mediates the stimulatory effects of transcriptional coregulators, such as CBP or PGC1α. Interestingly, the extent of Cys-612 contribution is dependent on the promoter context, as demonstrated with two different FOXO1 target promoters, those of the genes coding for glucose 6-phosphatase (catalytic subunit) and of selenoprotein P [6].

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Abstract

QUEST FOR BIOACTIVE COMPOUNDS IN OUR DIET WITH ANTI-AGEING AND ANTI-AGGREGATION PROPERTIES†

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Abstract: Ageing is a complex process affected by both genetic and environmental factors, characterized by a gradual failure of functionality, reduced stress response and resistance, leading to enhanced probability for age-related diseases and mortality. During the last decades, natural compounds have attracted the attention of researchers in the quest of bioactive phytochemicals with anti-ageing properties. For a few of these compounds an extra advantage appears; many of them have been shown to decelerate the progression of age-related diseases with emphasis on aggregation-related diseases. Using the nematode *Caenorhabditis elegans* along with the replicative senescence model of human primary fibroblasts, we have identified compounds that are part of our diet with anti-oxidation, anti-ageing and anti-aggregation activities. Some of the identified compounds promote their anti-ageing activity through activation of the proteasome, others through the activation of Nrf2 transcription factor, while others through inhibition of glucose transporters (GLUTs). Our work identifies new bioactive compounds with anti-ageing and/or anti-aggregation properties or reveals additional beneficial properties on already known bioactive compounds.

Keywords: anti-ageing, anti-aggregation, anti-oxidation, bioactive compounds, proteasome, Nrf2, glucose transporters

1. Introduction

Ageing is a multi-factorial, complex process affected by both genetic and environmental factors. It is characterized by a gradual failure of functionality, reduced stress response and resistance, leading to enhanced probability for age-related diseases and eventually mortality [1]. Natural compounds have attracted the attention of multiple labs world-wide due to their multiple beneficial properties (anti-oxidant, anti-ageing, anti-inflammatory, among others), their variety and of course their natural origin that makes them more accessible. If they are also part of the normal diet, the side-effects are further diluted out.

Human primary fibroblasts perform a certain number of population doublings before they enter a state of irreversible growth arrest where they accumulate a series of different characteristics (morphological, biochemical, physiological etc) that clearly distinguish them from their young counterparts. This is known as the replicative senescence model [2]. The nematode *Caenorhabditis elegans* is a multi-used organismal model with ideal features for aging (and age-related diseases) studies; it is inexpensive to maintain and grow in the laboratory, it has a short life cycle and lifespan, it has multiple readouts, it is frequently used for screening of compounds and ~80% of nematode genes have human homologs while numerous human diseases have been modelled and studied in *C. elegans* [3].

Using the above-mentioned model, our lab has identified compounds that are part of our diet with anti-oxidation, anti-ageing and anti-aggregation activities.

2. Anti-ageing compounds

Proteostatic mechanisms and especially the proteasome, are highly affected by aging [4] while loss of proteostasis features among the hallmarks of aging [1]. Activation of the proteasome either through genetic means or through natural compounds has been suggested as a promising anti-ageing strategy [5]. We have therefore sough to identify compounds that are part of normal diet with proteasome activating properties. Oleuropein, the olive constituent [6], quercetin [7] and 18α-glycyrrhetinic acid [8,9] are few of the natural compounds that we have identified as proteasome activators and were shown to extend cellular and organismal lifespan.

We have also identified natural compounds with anti-ageing properties that do not affect the proteasome. A minor component of silymarin that is used in a plethora of dietary supplements, namely 2,3-dehydrosilybin A/B (DHS A/B), was shown to promote cellular and organismal lifespan extension on top of its already known anti-oxidative and neuroprotective properties [10]. Using *C. elegans*, we revealed that this extension is FGT-1 (facilitative glucose transporter)-dependent; FGT-1 has been suggested as the GLUT (glucose transporters) paralogue in the nematodes.

3. Anti-aggregation compounds

Ageing is a major risk factor for the manifestation of human diseases including neurodegenerative diseases [11]. Amelioration of healthspan is predicted to exert beneficial effects on the progression of diseases as well. Specifically, if we refer to aggregation-related diseases like Alzheimer's disease (AD) where aggregates accumulate due to dysfunctional proteostatic mechanisms [12], anti-ageing compounds that have been isolated for their proteasome-activating properties are expected to exert positive action is conditions of such disease. Indeed, 18α-glycyrrhetinic acid that has been identified as a proteasome activator [8] was found to exert anti-aggregation activity in the context of a multicellular organism (*C. elegans* model for AD) but also in human and murine cells of nervous origin [9].

Similar positive action was shown for DHS A/B [10] that was identified as an anti-ageing compound in the absence of effects on proteostatic mechanisms. Its anti-oxidant activity is probably playing a major role in the observed protective effect against aggregation.

4. Conclusions

Nature offers a huge inventory of compounds with multiple structures and activities that may be used in anti-ageing and/or anti-aggregation applications. What is still missing is extensive investigation on the pathways that are involved in the observed beneficial phenotypes. Nevertheless, results to date are encouraging and suggest that cellular ageing might be decelerated.

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Abstract

Diagnosis of biological activities by Mass Spectrometry †

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Abstract: Oxidative reactions are essential for life, but they also cause important stresses and cellular damages resulting in cancers, cardiovascular or neurodegenerative diseases. Antioxidant secondary metabolites of plant can be involved in the cell defense and enter the body via food intake of vegetables, fruits or beverages. Screening natural active metabolites in plants requires different analytical techniques among which mass spectrometry has become one of the most popular, not just because of its ability to provide structural information on causative molecules but also because this technique belongs to the arsenal of diagnostic tools for the determination of biological activities.

Keywords: Mass spectrometry; Imaging Mass Spectrometry; plants; anti-cancer; host-pathogen relationship.

1. Introduction

The oxidative reaction is essential for life but is responsible of important stresses causing serious cellular damages such as cancers, cardiovascular or neurodegenerative diseases. Among the available armory against reactive oxygen species, antioxidant secondary metabolites of plant can be involved in the cell defense. One of the sources of natural antioxidants is via dietary intake.

The identification of natural antioxidants is an increasingly important subject. Their isolation from plants and their structural elucidation require the use of several techniques and the development of different analytical methods including extraction procedures. These procedures have to be adapted at each stage (extraction, separation, detection, etc) based on the physicochemical properties.

In the last 25 years, mass spectrometry (MS) has become one of the most useful tools for structural elucidation of metabolites of biological interest but also for the direct diagnosis of their activity. This presentation will provide an overview of the current techniques used to identify interesting natural products, especially secondary metabolites. Some applications will be presented showing that MS is not just appreciable because of its ability to provide structural information on involved molecules but also because this technique belongs to the arsenal of direct diagnostic tools for the determination of biological activities.

2. Monitoring and evaluation of disturbances occurring in the cell cycles and pathophysiological events.

The measurement of very small messenger (molecular weight < 50 u) such as nitric oxide (NO) is particularly difficult by MS.

NO plays a pivotal role in vascular tone homeostasis and inflammation. During aging and in case of cardiovascular diseases (CVD), endogenous NO production decreases. Its origin includes both endogenous and exogenous sources (drinking water and food intake). Oral administration of S-nitrosothiols (RSNOs) appears promising to restore the NO pool in CVD and to fight against oxidative stress and inflammation.

In the presented example, intestine permeability of RSNO and especially S-nitrosoglutathione (GSNO) are studied by using in vitro models by measuring metabolites (nitrate, nitrite ions and low- and high-molecular mass RSNOs). An analytical method relying upon liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS), able to distinguish between the exogenous origin of NO species and other

sources (endogenous synthesis and diet), was developed [1]. RSNO were labeled with the stable nitrogen isotope ^{15}N (exhibiting a very low natural abundance compared to ^{14}N). 2,3-Diaminonaphthalene (DAN) assay was selected for nitrite derivatization and the resulting product, i.e. 2,3-naphthotriazole (NAT), was examined in LC-MS/MS conditions. An interference was observed between unlabeled NAT and ^{15}N labeled one, due to the presence of ^{13}C (stable natural isotope abundance of 1%), and the interference ratio was about 3%. Besides, an original new mass fragmentation pattern offering high sensitivity was optimized by using appropriate mobile phase containing acetonitrile and detection mode with collision the HCD cell (Higher-energy Collisional Dissociation HCD).

The present analytical method was validated and applied to GSNO permeability studies. The different NO species were measured in apical/basolateral, donor-acceptor compartments of the models. The results situate this drug candidate within the middle permeability class.

3. Fishing active metabolites from plant extracts

Plants are a source of natural active compounds. They are involved in many fields of application such as medicine or cosmetic. The search for new natural active molecules against cancer (and obviously other diseases) in plant extracts can be directly obtained through the analysis their extract by Matrix Assisted Laser Desorption/Ionization - Time Of Flight MS (MALDI-TOFMS) or Fourier Transform Ion Cycloton Resonance MS (MALDI-FTICRMS). As described in Figure 1, the first step requires their contact with implied target proteins. The target protein acts as a filter which retains the candidate molecules. The complex protein-ligand is analyzed by MS and the direct detection of the ligand reflects its potential inhibition activity against the target. Screening tests have already been developed for the ligands of tubulin [2-3], dihydroxyfolate reductase (DHFR) [4] and recently for reversible and irreversible ligands of CDC25 phosphatases (isoforms A et C) [5]. This approach demonstrates its effectiveness with Madagascar periwinkle for tubulin, with Colchicum and green tea for DHFR and some promising plants are currently evaluated on CDC25s.

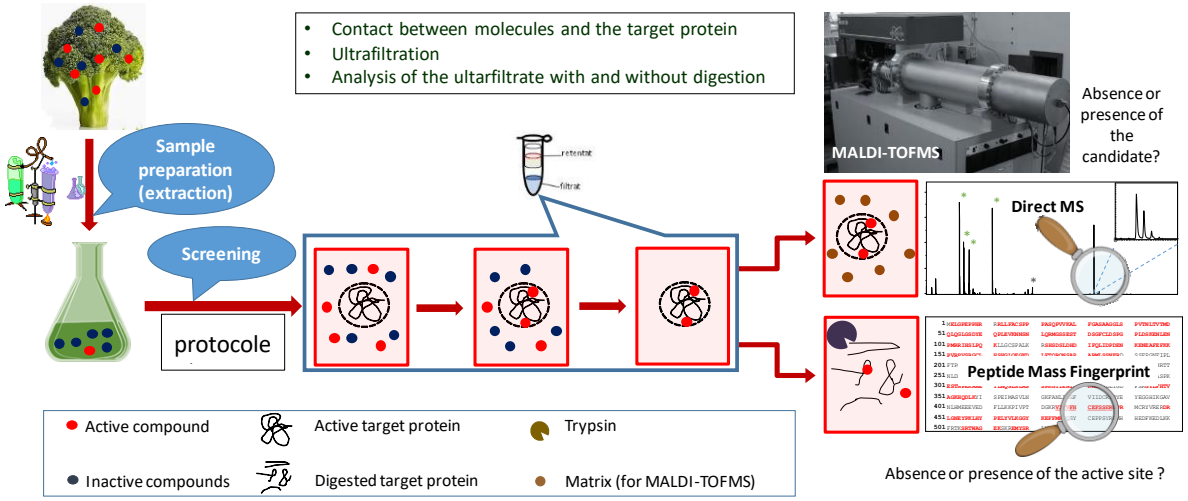

Figure 1. Principle of the direct screening protocol. The plant is first undergoing extraction with appropriate solvents. Then, the extract is put in contact with the target protein to form a complex with the active candidates. The complex is ultra-filtrated to eliminate non active compounds before MALDI-TOFMS analysis. The retentate is directly submitted to MS analysis to check if one candidate (or even several) bound to the target protein. After a protein digestion, the presence of the target active site by peptide mass fingerprint (PMF) can be determined and whether or not the interaction is reversible.

4. Screening biomarkers in tissues

The host-pathogen relationship between plant and their pathogens can also be directly studied by MS. Grapevine culture is often affected by pathogens causing severe harvest losses. Understanding host-pathogen relationships may be a key to solve this problem. Direct flow injection by electrospray - Fourier transform ion cyclotron resonance MS (ESI-FTICRMS) of leaf extracts is a rapid method for the study of grapevine response to downy mildew (*Plasmopara viticola*) attack (Figure 2) [6]. The comparison of MS profiles obtained from control and infected leaves of different levels of resistant grapevines highlights several classes of metabolites identified using high resolution MS and tandem MS (MS/MS). Statistical analysis of 19 marker ions shows a clear segregation between inoculated and healthy samples. In this study, disaccharides, acyl lipids and sulfoquinovosyl diacylglycerols emerge as possible metabolites involved in the plant defense. These results can be completed by Imaging MS (IMS), which allows the direct spatial localization of metabolites of interest (stilbenes [7], flavonoids [8] or ellagic acid) in the leaf tissues. When necessary, LC-MS and LC-MS/MS can be used to complete the information, especially regarding the quantification of specific metabolites of interest in leaf extracts.

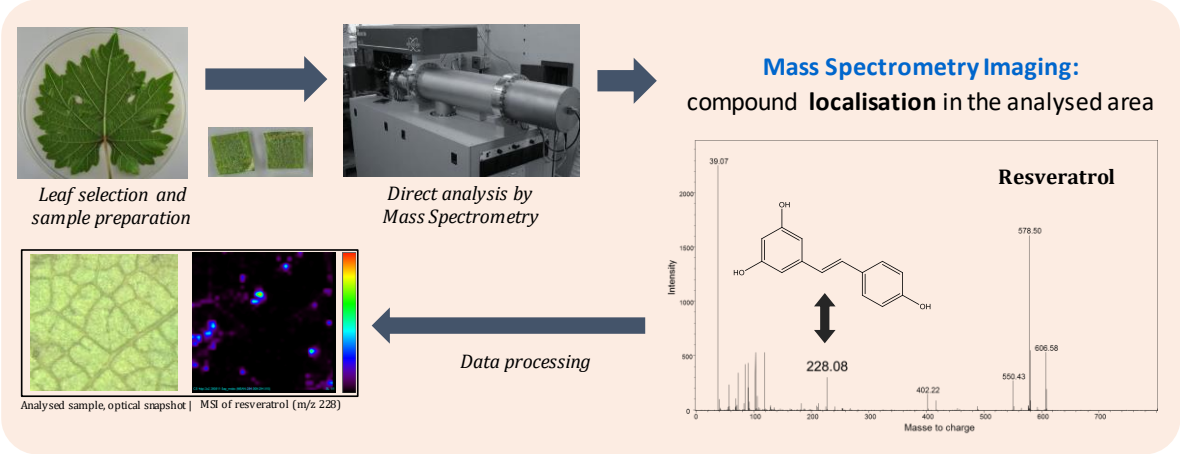

Figure 2. General scheme of Imaging MS by (MA)LDI-TOFMS. The sample, a *Plasmopara viticola*), infected vine leaf, is cut and fixed to the MALDI plate with or without matrix deposition. The MALDI plate is introduced in the MS. A grid pattern covering the surface to be analyzed is defined. The sample is then mapped; each pixel of the grid is composed of mass spectra. With the help of data processing software, the image of the distribution of each ion of interest can be built (e.g.m/z 228 for resveratrol). The image can be compared with control sample or superposed onto the initial photo.

Author Contributions: V.C, P.L and P.C. performed the research, analyzed the results and drafted the article.

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Abstract

Targeted anticancer strategies with garlic derivatives†

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Diallyl polysulfides from edible plants have been widely investigated in cancer research holding the promise of a translational application. Generally recognized as inducers of mitotic arrest and cell death, yet their activities appear broad, without specific intracellular targets. Here we suggest their potential as targeted agents and cancer types as suitable responders, taking the garlic-derived diallyl tetrasulfides (DATTS) and its most effective hemi-synthetic derivative di-benzyl tetrasulfide (DBTTS) as lead compounds [1-4]. We discovered DATTS/DBTTS as reversible tubulin binders, via redox modulation of the tubulin thiols. Translating our investigations to cellular models, we selected cancer types of the gastrointestinal tract (colorectal cancer, CRC) and the blood (acute forms of leukemia), being both highly proliferating and exposed *in vivo* to appropriate and stable concentrations of sulfur compounds. In both cell types, DATTS/DBTTS binding compromises the microtubule machinery, thereby inducing mitotic arrest and apoptosis [1-4]. Of note, a higher expression of genes coding specific tubulin isoforms in KRAS-mutated CRC SW480 and SW620 correlates with faster cell proliferation and the increased susceptibility to these compounds vs. the most resistant BRAF-mutated HT-29 [1]. The resistance in HT-29 associated with the impairment of the autophagic flux concomitant with the prolonged mitotic block and characterized by p62 protein accumulation. Genetic p62 inhibition restores sensitivity. We confirmed the translational potential of DBTTS in 3D CRC models (*in vitro*: spheroids and colony formation assay; and *in vivo*: zebrafish xenografts) [1]. In both cell types, anti-apoptotic Bcl-2 protein members undergo phospho-modulation. In hematological cancer, Bcl-2 proteolysis/inhibition promotes cell death [2-4]. In line, Bcl-2 over-expression makes the cells more resistant; vice versa, isogenic cell lines expressing Bcl-2 mutated in the phosphorylable residues are again sensitized to the treatment, suggesting Bcl-2 proteins as critical stress sensors and transducers. Overall, we recommend components of the microtubule network, differential autophagic capacities, and Bcl-2 proteins modulation as essential factors of vulnerabilities to prioritize DATTS/DBTTS treatment.

Keywords: Diallyl compounds; tubulin; Bcl-2; autophagic flux; apoptosis.

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Abstract

Antioxidant Properties of S-nitrosoglutathione and nanotechnologies †

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Abstract: Cardiovascular diseases are associated with oxidative stress and a reduced bioavailability of nitric oxide (NO). To counteract both processes, the administration of *S*-nitrosoglutathione (GSNO) can be envisaged. GSNO is able to induce protein *S*-nitrosation (Pr-SNO), which is a post-translational modification of proteins, participating in the storage of NO in tissues, and protect thiol functions from oxidation. However, GSNO antioxidant power is poorly studied, which is probably linked to its low stability. This low stability can be addressed by nanotechnologies that will increase GSNO protection and provide a sustained release of the drug.

Keywords: *S*-nitrosoglutathione, oxidative stress, protein *S*-nitrosation, nanotechnologies.

1. Introduction

Cardiovascular diseas is the main cause of mortality in the world with around 17.5 million of deaths in 2012 according to the World Health Organization. In France, it is the second one for men, after cancer, and the first one for women. Risk factors such as high blood pressure, diabetes, smoking, obesity or the lack of physical activities increase the risk of developing a cardiovascular pathology including coronary artery diseases, venous thrombosis, angina pectoris and atherosclerosis. Most of these diseases are associated with oxidative stress and a reduced bioavailability of nitric oxide (NO). Indeed, NO, a physiological gaseous messenger with a short half-live, plays a central role in vascular homeostasis and its biosynthesis decreases during physiopathological processes such as ageing, endothelial dysfunction or oxidative stress [1]. The endogenous *S*-nitrosothiols (RSNOs), one of the main transport and storage forms of NO in the bloodstream and tissues, are good candidates as therapeutics to restore the physiological concentration of NO. Among RSNOs, the *S*-nitrosoglutathione (GSNO), made by *S*-nitrosation of glutathione, was the main NO-donor investigated for its therapeutic potential as antiplatelet agent with arterioselective vasodilator effects and also with well-documented antithrombotic effects. The *S*-nitrosation process apart from its ability to modulate signaling pathways by post-translational modification of proteins (Pr-SNO) is also a protection of thiol functions from oxidation [2-4]. Indeed, GSNO was shown to be able to reduce disulfide bounds forming *S*-nitroso functions, which are a lower oxidation state of thiol functions than disulfide or sulfenic/sulfonic acid functions [2, 5]. However, the ability of GSNO to regulate NO bioavailability under oxidative stress is poorly studied. Enzymatic and non-enzymatic degradation of GSNO occurs, reducing its clinical potential to provide a long-lasting effect and to deliver appropriate NO concentrations to target tissues. Therefore, encapsulation of GSNO and other RSNOs is an interesting approach to overcome these drawbacks. The nanotechnologies may offer a wide range of solutions for RSNO protection and sustained release.

2. Antioxidant power of NO and GSNO

The protein *S*-nitrosation is a redox reversible process with high spatial and temporal specificity. One determinant that governs the specificity of protein *S*-nitrosation resides in the colocalization of NO sources: NO is provided mostly from NO synthases (NOS) and also from denitrosation of target proteins. Conversely,

the *S*-nitrosation process is also a temporal signaling event, which depends on NO synthesis by NOS and its consumption by the soluble guanylate cyclase through the nitrosylation process for example. Cells environment also influences *S*-nitrosation of proteins. Indeed, in endothelial cells, physiological shear stress promotes *S*-nitrosation of protein independently from cGMP-dependent signaling [6, 7], whereas TNF-α and oxidized LDL treatments reduce it [8]. Many proteins and transcriptional factors contain cysteine residues, which are potential targets for ROS-dependent or RNS-dependent modifications. NO shows antioxidant effects via NADPH oxidase inhibition, [9] or promotion of thioredoxin-1 activity [10, 11] by direct S-nitrosation of their cysteine residues. Transcriptional factors all contain reactive thiols in their DNA binding regions, whose modification alters their binding to DNA. AP-1 activity is altered by *S*-nitrosation [12] and by oxidation of Cys residues [13]. Indeed, H_2O_2 treatment inhibits AP-1 activity and decreases eNOS promoter activity [14].

The antioxidant power of NO and GSNO is also mediated via their ability to reduce disulfide bound forming *S*-nitroso functions. Oxidative stress causes oxidation of the thiol groups of proteins. For example, we showed, using a smooth muscle cell model of oxidative stress, that GSNO prevents oxidation of thiol functions by increasing the level of *S*-nitrosated proteins [4]. Mass spectrometry analysis revealed that these proteins were mainly implicated in cell contraction, morphogenesis and movement, meaning that the redox protection of GSNO will help to maintain smooth muscle cells contractile phenotype.

3. Nanotechnologies supply

Unfortunately, the low stability of NO and its derivatives such as *S*-nitrosothiols, constitutes a limitation for their therapeutic administration especially for chronic treatment [3]. There are several strategies to protect RSNOs from physicochemical and enzymatic degradations such as assembling of macromolecular RSNO in nanostructures (thiomers), or *S*-nitrosation of encapsulated free thiols (S-nitrosothiol-loaded carrier) [15, 16]. The most frequently used approach is the direct encapsulation of RSNOs in liposomes, inorganic or polymeric nanoparticles or films [17, 18]. A sustained release of NO is required to maintain stable and physiological NO concentrations. High concentrations should be avoided as they may lead to oxidative/nitrosative stress, especially destructive in a context of cardiovascular diseases. Moreover, in a context of chronic pathologies, oral delivery will be the most acceptable for patients. Here, RSNO will encounter unfavorable environment all along the gastrointestinal tract, ranging from a low pH in the stomach to the difficulty for crossing of the intestinal barrier. We showed that RSNOs have a low intestinal permeability using a passive mode and following a paracellular pathway [19]. In order to design such an oral delivery formulation for RSNO, which can provide protection and sustained release, GSNO loaded nanoparticles made of Eudragit® with a water in oil in water double emulsion method have been developed [20]. These nanoparticles were then embedded into an alginate and chitosan matrix forming a nanocomposite particle (GSNO-acNCP). The nanocomposite protected NO donor from degradation, and the matrix enhanced intestinal absorption of GSNO [21, 22]. Indeed, alginate and chitosan were chosen as mucoadhesive polymers increasing the residence time of the formulation on the intestine mucus layer. As alginate has the capacity to penetrate the mucus layer, this will bring the drug closer to the intestinal cells, while chitosan exerts the property to open cells tight junction, helping the drug to cross the intestinal epithelium. Therefore, their combination leads to increased drug permeability through the intestinal barrier.

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Abstract

Nano or micro: 3 different particles to deliver and protect *S*-nitrosoglutathione for oral route administration †

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Abstract: As a physiological nitric oxide donor, *S*-nitrosoglutathione (GSNO) is a promising candidate for several diseases (e.g. stroke and atherosclerosis). However, its clinical application has been limited by its low stability. In order to protect GSNO suitable for oral route administration and to achieve sustained release, 3 different particles from nano-size to micro-size were obtained by a water-in-oil-in-water (W/O/W) or solid-in-oil-in-water (S/O/W) double emulsion/solvent evaporation method. The 3 different particles tuned out to have similar encapsulation efficiency while the microparticles showed longer release time. Finally, the 3 formulations have been successfully lyophilized for long term stability.

Keywords: *S*-nitrosoglutathione; nitric oxide donor; oral delivery; sustained release; storage stability.

1. Introduction

Nitric oxide (NO) is a gaseous radical, which plays important roles in different physiological activity of human beings [1-2]. The imbalance of NO may result in several disfunction and diseases (e.g. stroke and atherosclerosis). S-nitrosoglutathione (GSNO) is a promising candidate as a physiologic NO donor [3-4]. However, with a short half-time GSNO needs to be protected especially for oral administration. In a previous study, an oral delivery system made of nanoparticles (NP) has been found to protect and provide a sustained release of GSNO [5]. NP are then embedded into an alginate/chitosan matrix to form GSNO-nanocomposite particles (GSNO-acNCP). The GSNO-acNCP showed high encapsulation efficiency, a sustained release effect and led to the formation of a NO store in the aorta wall after a single oral administration to rat [6]. However, this formulation has several limitations 1) insufficient batch-to-batch reproducibility (due to the complex structure); 2) multi-step and time-consuming preparation; 3) immediate use only (no stability as suspensions). Our previous work also showed that *S*-nitrosothiols intestinal permeability (*in vitro* model) is passive and follows paracellular pathway [7]. So, by increasing the amount of GSNO brought to the intestine and by opening intestinal cells tight junctions, the GSNO-acNCP is perfectly adapted. The formulation protocol needs modification to get a higher reproducibility, longer stability during storage and a higher encapsulation efficiency (EE) with sustained release.

2. Materials and Methods

Two different GSNO loaded microparticles (GSNO-MPs) types were prepared by a water-in-oil-in-water (MP-W) or a solid-in-oil-in-water (MP-S) double emulsion solvent evaporation method and compared to the previously developed GSNO-NP. These particles were characterized as regards to their size (dynamic light scattering/laser diffraction and scanning electron microscopy), zeta potential, GSNO EE, *in vitro* release kinetic, cytocompatibility and intestinal permeability using Caco-2 cells monolayers [7]. Moreover, the three formulations (GSNO-NP, GSNO-MP-W and GSNO-MP-S) were obtained as dried powders by optimized lyophilization using sucrose as cryoprotectant. These lyophilized particles were also characterized.

3. Results and Discussion

Through the modification of nanoparticles (NP) preparation, two types of GSNO-MPs were obtained in micro-size (GSNO-MP-W: 80.2 ± 10.2 µm, n=9; GSNO-MP-S: 118.3 ± 16.4 µm, n=7) with similar GSNO EE. However, these two GSNO-MPs exhibited a longer release time and a slower release profile during the 2 first hours compared with GSNO-NP or free GSNO. The freeze-dried powders had similar EE, size and zeta potential to fresh preparations. Freeze-dried particles maintain all their physico-chemical characteristics for more than 1 month (3 months for NP, 4-6 weeks for the two MP) of storage at 4°C under inert atmosphere.

All formulations were cytocompatible with Caco-2 cells for up to 8.5 mg/mL of freeze-dried powder (corresponding to 2.8 mg/mL of polymer and 0.017 mg/mL of GSNO, i.e. 50 µM of GSNO) after 24 h. The apparent permeability coefficient (*in vitro* intestinal barrier model) of NOx species released from the particles was higher than for free GSNO meaning that these GSNO delivery systems are enhancing GSNO intestinal permeability.

In summary, two different GSNO-MP were obtained through the modification of nanoparticles preparation process. All three GSNO loaded particles were successfully converted into dried and stable powders through the freeze-drying method, which facilitated their storage and mode of application. Among these particles, the S/O/W microparticles is an attractive avenue as it's EE and release kinetics may be improved by reducing the size of GSNO powder (currently 40 µm). This challenging procedure is in progress using supercritical fluid technology.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract

Targeting the resistance in multiple myeloma †

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Abstract: Multiple myeloma is a hematological cancer characterized by the clonal proliferation of malignant plasma cells in the bone marrow. That disease has a rather low incidence but displays a high rate of relapse and resistance to conventional therapies. It is therefore necessary to find new therapeutic strategies to overcome this resistance, which is partly attributed to a subpopulation of cells known as cancer stem cells. Withanolides and HDAC6 selective inhibitors were identified as promising compounds in various resistant multiple myeloma models.

Keywords: multiple myeloma; 3D co-culture model; cancer resistance

1. Introduction

Multiple myeloma (MM) is a disorder characterized by the clonal proliferation of malignant plasma cells in the bone marrow. One of the main complications in this type of tumor is the lytic lesions in the bone aroused by the activation of osteoclasts and suppression of osteoblasts in a very complex network of interactions between the cancer cells and the bone marrow niche [1]. This malignancy represented 0.8% of all cancers worldwide in 2012, counting for 1% of cancer deaths [2]. Regardless of the improvement in overall survival observed over the past decades, treatment strategies still represent a huge challenge mostly for patients with relapsed and refractory disease [3,4]. It is therefore necessary to find new therapeutic strategies to overcome this resistance.

2. Withanolides

Withaferin A (WFA) is a steroidal lactone isolated from the leaves of *Withania somnifera* and has been shown to exert an array of biological activities relevant to various disorders such as cancer [5,6]. In multiple myeloma, WFA induced cell death and was capable of stimulating cell differentiation at relatively low doses as demonstrated by clear morphological changes and alterations in gene expression levels reminiscent of hematopoietic stem cell differentiation [7]. Access to a library of withanolides led to the identification of withanolide D that showed stronger activity than WFA and exerted similar cytostatic effects between MM-sensitive and -resistant cell lines, which were independent of P-glycoprotein efflux [8]. The antiproliferative activity of drugs currently used to treat MM was also evaluated in MM-cancer stem cells (CSCs), RPMI 8226, MM1.S and MM1.R cells. Most of them did not show any activity against the resistant MM-CSCs (IC_{50} > 50 μM) and displayed a wide range of IC_{50} values in the other cell lines.

3. Histone deacetylase inhibitors

Some of the new agents that have displayed great potential in the past years are histone deacetylase (HDAC) inhibitors [9]. HDACs have been reported as dysregulated in MM and the overexpression of HDAC1 and HDAC6 has been associated with poor prognosis [10]. The pan-HDAC inhibitors presently used in MM treatment, such as panobinostat, display high toxicity despite being very effective in overcoming resistance to bortezomib [11]. Therefore, using selective HDAC6 inhibitors could be as active as non-selective inhibitors and at the same time decrease the global toxicity. Ricolinostat is the first HDAC6 inhibitor to reach clinical trials and the first reports showed an improved safety profile when compared with pan-HDAC inhibitors [12].

HDAC inhibitors alone do not display a huge clinical benefit but when combined with other therapies, they showed a great value, with a better outcome in cases of refractory MM [13].

HDAC6 appears to function at various cellular crossroads between two cellular signaling systems, which each involve protein lysine acetylation and ubiquitination [14]. It is a crucial factor in the coordination of the cell response, and it plays an important role in the formation or degradation of cytotoxic protein aggregates in the course of various diseases such as cancer. HDAC6 recruits polyubiquitinated protein aggregates via the ZnF-UBP domain and loads misfolded proteins onto dynein to form the aggresome. The modulation of this pathway represents a strategy to overcome resistance to proteasome inhibitors in MM [15]. The HDAC6 inhibition of several compounds was tested, as well as their antiproliferative activity.

4. 3D co-culture spheroids

Some compounds active in 2D cultures failed during development because of their lack of efficiency in co-culture conditions, due to the supportive function of stromal cells. Therefore, 3D co-culture spheroids, including malignant plasma cells and cells from the microenvironment, were used to evaluate the activity of compounds having shown activity in monolayer cultures [16]. MM-CSCs were also introduced in the model in a way that the total amount of CSCs present in the spheroid would correspond to 20% of cancer cells to mimic resistant cancers. By screening compounds in this model not only the individual response of the malignant cells is considered, but also how the spheroid, as an entity, behaves when exposed to the treatment. Therefore, this model better reflects the cellular and molecular complexity found *in vivo* and was used to study the activity of various compounds with the aim to a more efficient transition between pre-clinical experiments and clinical trials.

Author Contributions: Conceptualization, M.F., M.E.I and M.C.; methodology, M.F. and M.E.I.; validation, M.F., M.E.I and M.C.; resources, M.C.; writing—original draft preparation, M.C.; writing—review and editing, M.F., M.E.I and M.C.; supervision, M.C.; project administration, M.C.; funding acquisition, M.C. All authors read and approved the final manuscript.

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Abstract

Hydroquinone-derivatives induce apoptotic and necrotic cell death in chronic myelogenous leukemia

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Abstract: Hydroquinone (HQ) is a phenolic metabolite of benzene, which is used as a skin whitener. Insects synthesize this natural compound as a deterrent and mushrooms as a toxin. Pro-apoptotic effects of HQ were previously documented on various cancer cell types. Here we investigated the cell-death inducing mechanisms of this compound in chronic myeloid leukemia cell models.

Keywords: chronic myeloid leukemia; apoptosis; necroptosis; autophagy.

1. Introduction and Results

Chronic myeloid leukemia (CML) results from a t(9;22)(q34;q11) translocation, also called Philadelphia chromosome (Ph). This reciprocal translocation causes a constitutively-activated tyrosine kinase BCR-ABL fusion gene [1]. Imatinib (STI571, Gleevec) is targeting the oncogenic BCR-ABL protein to treat patients with CML [2]. However, this drug triggers resistance in CML patients and does not entirely eradicate BCR-ABL-expressing cells [3].

Necroptosis is known as type III programmed cell death that has been explained in many pathological contexts [4]. Necroptosis is regulated by ligand binding to receptors of the tumor necrosis factor (TNF) family [5]. The main molecular signaling pathway involves a multi-protein complex called necrosome, including the receptor-interacting kinases RIP-1 and -3 and the mixed lineage kinase-like domain (MLKL) executioner protein [6]. Necrostatin -1 (Nec-1) is known as a specific inhibitor of necroptosis which targets to RIP1/3 necrosome complex activation [7]. Recently, induction of necroptosis has been described as an alternative therapeutic approach to trigger programmed cell death in apoptosis-resistant CML. For this reason, novel drugs are still required to improve CML therapies.

Here we investigated various tetrahydrobenzimidazole derivatives and determined their cytotoxic potential against hematopoietic cancer cell lines including Jurkat, Raji, K562 and U937 compared to peripheral blood mononuclear cells (PBMCs) from healthy donors. Some of them, especially TMQ153 exhibited significant cytotoxicity against cancer cells [8]. Our studies then aimed to clarify the molecular mechanisms by which TMQ0153 concentration-dependently triggered caspase-dependent apoptosis at lower concentrations whereas autophagy-independent necroptosis was activated at higher concentrations in human K562 CML cells.

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Abstract

Nano-targeting of ferroptosis eradicates high-risk neuroblastoma †

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Despite extensive multi-agent therapy, there is a high need to exploit potential novel treatment strategies to circumvent therapy-resistance and/or disease relapse in high-risk neuroblastoma patients. In this regard, plants can provide a valuable source for drug discovery. Here, we identified the superior efficacy of withaferin A, derived from the plant *Withania Somnifera*, in killing a heterogeneous panel of high-risk neuroblastoma cells. We implicate that withaferin A triggers a form of non-apoptotic cell death coined ferroptosis, which is an iron-catalyzed lipid peroxidation cell death. Mechanistically, withaferin A activates the nuclear factor–like 2 (NRF2) pathway through targeting of Kelch-like ECH-associated protein 1 (KEAP1), which leads to excessive activation of heme oxygenase-1 and an ensuing increase of intracellular labile Fe^{2+} referred to as non-canonical ferroptosis induction. Additionally, withaferin A inactivates the lipid repair enzyme glutathione peroxidase 4 (GPX4) in a canonical ferroptosis induction. We further show that withaferin A suppresses high-risk neuroblastoma tumor growth and relapse rates compared with etoposide. By performing a liquid chromatography-mass spectrometry-based redox-lipidomics analysis, we indicate that tumor regression by withaferin A occurs through lipid peroxidation. Finally, to cope with the poor aqueous solubility of withaferin A and sever weight loss-related adverse effects upon systemic application, we used a nano-medicinal approach. Withaferin A was formulated in a novel amphiphilic degradable pH-sensitive nanocarrier and was tested in a neuroblastoma xenograft model. The nano-targeting of withaferin A allowed systemic administration and suppressed tumor growth due to an enhanced accumulation at the tumor site. In conclusion, our findings suggest a novel therapeutic approach to efficiently eradicate cancer cells by ferroptosis.

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Abstract

Effects of N-Acetylcysteine on Multidrug Resistance-Associated Protein 1 (MRP1) involved Multidrug Resistance †

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Abstract: Resistance of cancer cells to anticancer agents is caused partly by Multidrug Resistance-Associated Protein 1 (MRP1). The exact mechanism of MRP1-involved multidrug resistance has not been clarified yet, though glutathione (GSH) is likely to have a role for the resistance to occur. N-acetylcysteine (NAC) is a pro-glutathione drug. DL-Buthionine (S,R)-sulfoximine (BSO) inhibits GSH synthesis. The aim of our study was to investigate the effect of NAC and BSO on MRP1-mediated doxorubicin and vincristine resistance in Human Embryonic Kidney cells (HEK293) and its MRP1 transfected 293MRP cells.

Keywords: MRP1, doxorubicin, vincristine, HEK293, N-acetylcysteine, BSO, GSH.

1. Introduction

Multiple drug resistance (MDR) may develop against chemotherapeutic agents with unrelated chemical structures and mechanisms of action used for the treatment of cancer, reducing the efficacy of the drugs, and remains a major challenge in the treatment of cancer. A complex redox pattern underlies MDR problems. Natural product modulators of MDR are used as low toxicity chemosensitizers to enhance the efficacy of anticancer protocols and to overcome MDR [1-11]. Redox active drugs could provide a valid and promising way to overcome MDR in cancer therapies by targeting an axis consisting of drug transporters, aryl hydrocarbon receptor, phase I/II metabolic enzymes, and the inducible Nrf2-linked pathway [1-3]. The mechanism underlying the MDR inhibition by natural products obtained from plants and fungi lies in the blockade of the drug binding site, interference with the ATP hydrolysis process, alteration in integrity of cell membrane lipids, and decrease in Pgp or/and MRP1 expression. During co-administration, natural modulators compete with cytotoxic agents for binding to the active site of the transporters and reduce drug efflux, interference with the ATP hydrolysis process, alteration in integrity of cell membrane lipids, and decrease in Pgp or/and MRP1 expression [1-3]. However, beneficial versus deleterious effects of these substances must be well evaluated in chemoresistance and cancer therapy. Resistance of cancer cells against anticancer agents is caused partly by Multidrug Resistance-Associated Protein 1 (MRP1). The exact mechanism of MRP1 involved multidrug resistance has not been clarified yet, although glutathione (GSH) is likely to have a role for the resistance to occur [7-11].

Doxorubicin is an anthracycline type antitumor agent and produces free radicals. MRP1-mediated drug resistance occurs against doxorubicin, although the mechanisms have not been exactly understood. GSH is the most abundant non-protein intracellular thiol containing compound that is a key molecule in MRP1-mediated MDR. N-acetylcysteine (NAC) is a pro-glutathione drug. DL-Buthionine (S,R)-sulfoximine (BSO) inhibits GSH synthesis [9-11]. We investigated the effect of NAC and BSO on MRP1-mediated doxorubicin and vincristine resistance in MRP1-transfected and control Human Embryonic Kidney (HEK293) cells.

2. Methods

Human Embryonic Kidney (HEK293) cells were transfected with a plasmid encoding the whole MRP1 gene. Both cells were incubated with doxorubicin and vincristine in the presence or absence of NAC and/or BSO. The viability of both cells was determined under different incubation conditions.

3. Results

NAC increased the resistance of both cells against doxorubicin and vincristine (Figures 1 and 3). BSO decreased NAC-enhanced MRP1-mediated doxorubicin and vincristine resistance, indicating that induction of MRP1-mediated doxorubicin and vincristine resistance depends on GSH synthesis (Figures 2 and 4). N-acetylcysteine at both concentrations significantly increased the viability of 293MRP and HEK293 cells pretreated with BSO against doxorubicin and vincristine, but these increases were lower in comparison to the corresponding cells untreated with BSO.

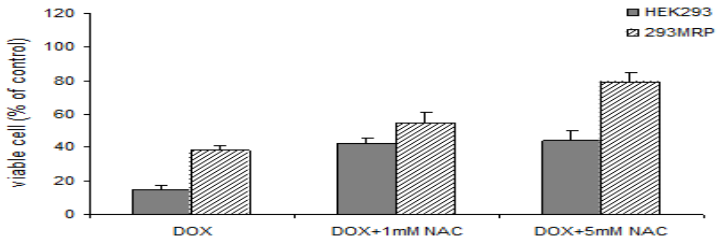

Figure 1. Effect of NAC on doxorubicin cytotoxicity in HEK293 and 293MRP cells.

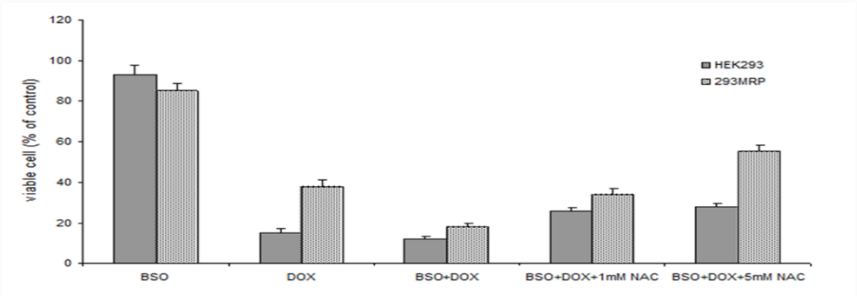

Figure 2. Effect of BSO on doxorubicin cytotoxicity and survival promoting effect of NAC in HEK293 and 293MRP cells.

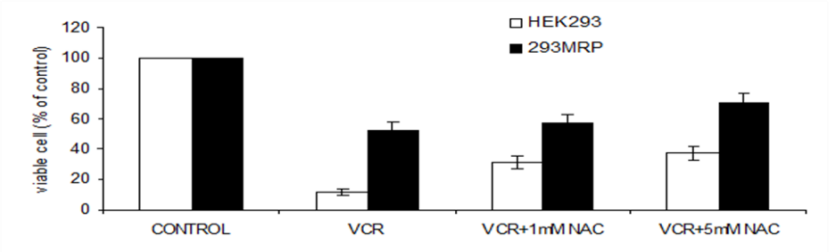

Figure 3. Effect of NAC on vincristine cytotoxicity in HEK293 and 293MRP cells.

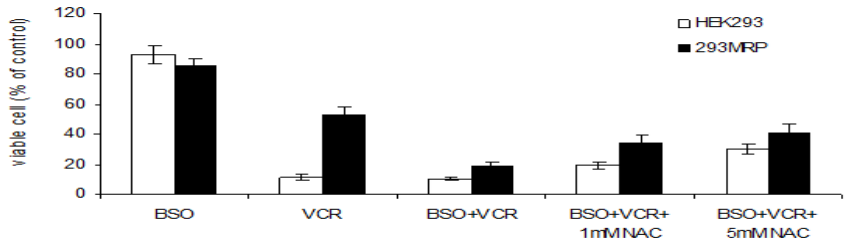

Figure 4. Effect of BSO on vincristine cytotoxicity and survival promoting effect of NAC in HEK293 and 293MRP cells.

4. Discussion and Conclusion

Our results demonstrate that NAC enhances MRP1-mediated doxorubicin and vincristin resistance and that this effect depends on GSH synthesis. NAC did not increase the viability of both cells pretreated with BSO as much as the cells treated with only NAC against doxorubicin and vincristine. This might be explained in that BSO counterbalances the effect of NAC as a precursor of GSH. This is further evidence that the survival promoting action of NAC depends on GSH synthesis.

We conclude that NAC and BSO have opposite effects in MRP1-mediated doxorubicin and vincristine resistance and that BSO seems a promising chemotherapy improving agent in MRP1-overexpressing tumor cells.

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Abstract

Musculoskeletal Ageing – Dietary Modification of Longevity Mechanisms to Improve Skeletal Health†

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Ageing is inextricably linked to a deterioration of the musculoskeletal system. This suggests factors governing lifespan might also impact the maintenance of skeletal integrity throughout life. The Oxford Musculoskeletal Ageing group studies the causes and consequences of skeletal ageing. Recent findings indicate dietary constituents (polyphenols, omega 3 fatty acids, polyamines) have the potential to activate longevity mechanisms in vitro and prevent the onset of age-related disorders in vivo. Alterations in RedOx mediators, autophagic flux, sirtuin enzymes and senescence all contribute to an inter-linked ageing nexus manipulated by diet, to maintain health throughout life.

Keywords: Ageing; Nutrition; Skeleton; Polyphenols; Polyamines

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Abstract

Effects of Diet and Exercise on Endocrine Function of Skeletal Muscle

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Keywords: myokines; IL-10; inflammation; antioxidants; iron

Skeletal muscle has been recognized as an endocrine tissue that releases appreciable amounts of circulating proteins, called myokines. Currently, we know that the skeletal muscles synthesize several hundreds of peptides classified as myokines, and muscle contraction stimulates their release [1,2]. Myokines can act in autocrine, paracrine or endocrine mode and there is an increasing number of data showing that they can affect different organs and tissues, e.g. the brain, bones, adipocyte tissue, heart artery, and many others [3]. For instance, the interleukins IL-6 and IL-10, released by the muscles during exercise, exert powerful local and systemic anti-inflammatory effects. Furthermore, IL-10 has been shown to provide cardio- and neuroprotection, which is mediated by the activation of anti-apoptotic protein kinase B (PKB or Akt) [4,5]. In addition, myokines like SPARC and oncostatin M show inhibitory activity against colon and breast cancer cells, respectively. Skeletal muscles represent the largest organ of the human body (the muscles constitute approximately 40% of total body mass), thus their role in the regulation of metabolic processes *via* myokines appears to be very important. Unfortunately, there is a limited amount of data demonstrating the effects of nutraceuticals on exercise-induced release of myokines. It has been shown that release of IL-6 from skeletal muscle was inhibited in persons supplemented with vitamin C and E. We hypothesize that natural compounds may exert their protective activity against some human diseases by modulating myokine synthesis.

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Abstract

Herbs extracts in the treatment and prevention of experimental metabolic disorders: Synergistic hypoglycemic activity of ethanol extracts of *Hypericum alpestre* and *Rumex obtusifolius*†

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Abstract: Hyperglycemia is a metabolic condition characterized by high blood glucose level due to the disturbances of carbohydrate, lipids and protein metabolism. These disorders lead to impaired of insulin secretion and β-cell dysfunction. It is known that continuous and long-term use of synthetic anti-diabetic drugs can have side effects including hepatic impairment. Thus, the identifications of potent hypoglycemic agents from natural sources of both medicinal and edible plants with minimum side effects, is desirable. Synergetic hypoglycemic activity in experimental animals (rabbits) was shown with ethanol extracts of a mixture of the plant species *Hypericum alpestre* and *Rumex obtusifolius*. The chemical composition of these extracts was identified by gas chromatography-mass spectrometry analysis. The plant extracts mixture and natural products extracted from medicinal plant sources have potential for treatment and prevention of diabetes mellitus and metabolic disorders.

Keywords: Immobilization stress**;** Hyperglycemia; Plant extract; Armenian folk medicine

1. Introduction

The diversity of plants in Armenia is due to the singularity of the natural environment. Previous studies have reported that *Rumex obtusifolius* separately possesses significant antihyperglycemic and antihyperlipidemic activities. However, we are interested in synergistic hypoglycemic activity of ethanol extract of a mixture of *Hypericum alpestre* and *Rumex obtusifolius* (HR). Our findings showed that *H. alpestre* and *R. obtusifolius* have high concentration of phenolic compounds, flavonoids and tannins, which might be responsible for hypoglycemic effects [1]. In Armenian folk medicine these species are used as antimicrobial agents [2]. Therefore, the purpose of this study was to investigate the biochemical properties and antihyperglycemic activities of the ethanol extract of the herbal mixture in hyperglycemia induced by immobilization stress in rabbits after 21 days of oral treatment.

2. Materials and Methods

2.1. Plant material and preparation of the extract

Plants have been collected in Armenia according to recommendations. Briefly, *H. alpestre* subsp. *polygonifolium* was harvested in the flowering period. *R. obtusifolius* L. seeds were harvested after maturation. Plant materials were identified and deposited in the herbarium of Yerevan State University. A voucher specimen for *H. alpestre* was ERCB13206, for *R. obtusifolius* - ERCB13208. The *R. obtusifolius* seeds and *H. alpestre* aerial part were extracted with 40% ethanol for 20 min at 60°C. After that the extract was filtered and was orally administrated after cooling.

2.2. Study design and induction of hyperglycemia in experimental rabbits

Hypoglycemic activity of HR ethanol extract was carried out in rabbits (*Oryctolagus cuniculus domesticus*) with the same sex (1800-2000 g). The experiments were performed in accordance with the ethical norms authorized by "International Recommendation on Carrying out of Biochemical Researchers with use of Animals" and study plan has been approved by the National Center of Bioethics (Armenia).

The animals were kept under standard environmental conditions (temperature 22±2°C, light/dark cycle of 12 h) and were randomly divided into three groups; I- normoglycemic; II – hyperglycemic control (putting immobilization); III – hyperglycemic experimental (received HR extract). Hyperglycemia was induced by immobilization stress in rabbits for 21 days (5 h daily). Immobilization stress leads to disorder of the endocrine system and lipid metabolism. Moreover, prolonged immobilization stress increases the risk of cardiovascular diseases and atherosclerosis [3].

2.3 Biochemical analysis

The biochemical analysis was performed to measure the serum level of glucose, total cholesterol (TC), high-density lipoprotein (HDL), law-density lipoprotein (LDL), triglycerides (TG). All parameters were assayed using enzymatic kit. Serum was collected and liver enzymes markers (ALT, AST) were determined by kinetic UV assay using kit. Liver and muscle glycogen contents were also carried out by histopathological examination of tissue samples.

2.4 GC-MS analysis

Gas chromatography and mass spectrometry (GC-MS) technique (HP 5890 Series II gas chromatograph with HP 5972 Series MS detector) was used for identification of the bio-active constituents from the plants.

3. Results

Our findings indicated that oral administration of HR ethanol extract (300 mg/kg body weight) significantly decreased fasting glucose levels of treated group of animals (66.2%, $p<0.05$), improving glucose tolerance (29.5%), increasing liver and muscle glycogen contents and corrected of the lipid profile, liver function enzymes compared to the hyperglycemic control group.

After 21 days of oral treatment with the HR ethanol extract, blood lipids parameters demonstrated significantly decreased total cholesterol (66.6%), LDL-cholesterol (64.6%) and TG (70.8%) levels compared to the hyperglycemic control group. There were no differences of HDL- cholesterol level between the treated and the normoglycemic group of animals. Treatment with HR reduced liver enzymes level; ALT and AST (31.8% and 48.6 %, respectively) in comparison with hyperglycemic control group. As the result of immobilization stress, liver and muscle glycogen levels were reduced which might be caused to decrease of glycogen synthase activity due to low insulin level. However, treated animals had increased liver and muscle glycogen level (1.4, 1.2-fold, respectively, $p<0.01$) compared to the untreated rabbits. The rabbits in the hyperglycemic control group also demonstrated loss of body weight (21.6%, $p<0.01$) in comparison with the treated group of animals (8.6%).

The *R. obtusifolius* seeds have significant concentration of phenolics, particularly, flavonoids and tannins [1,2]. These biologically active compounds may be responsible for the hypoglycemic effects of HR. Treatment with ethanol extract of herbal mixture single dose for 21 days can correct a carbohydrate and lipids metabolism.

In the extracts of selected tested plant materials, various biologically active compounds were identified by GC-MS technique, which could play important role in total hypoglycemic effect. GC-MS analysis of the extract of *H. alpestre* revealed the presence of 24 compounds. The major compounds with potential biological activities were several phenolic compounds (Catechol (2.9%), guaiacol (0.56%), vanillic acid (3.70%)), some fatty acids (tetradecanoic acid (7.69%)), palmitic acid (9.46%), linolenic acid (17.44%), octadecanoic acid (2.15%), two terpenoids (phytol (0.52%), trans-farnesol (0.48%)), furfural (1.44%) and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (8.62%) compounds. In the extract of *R. obtusifolius* 32 compounds were identified. Some of the identified compounds are considered to possess biological activities. These were linoleic acid (41.32%), cis-vaccenic acid (24.43%), palmitic acid (12.25%), oleic acid (3.65%) 1,2,4-benzenetriol (0.46%), N-[4-bromo-n-butyl]-2-piperidinone (0.64%), (Z) - 9-octadecenal (0.53%), methyl linoleate (0.45%), cis-9-hexadecenoic acid (0.40%) and supraene (0.21%). The combination of these compounds is suggested to be responsible to the hypoglycemic effects determined. Possible mechanisms would be revealed.

4. Conclusions

In conclusion, the present study has shown a positive effect of the synergetic action between *H. alpestre* and *R. obtusifolius* plants that contributes to higher antihyperglycemic activity in comparison to *R. obtusifolius.*

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Abstract

Fast, sensitive and robust determination of nicotinic acid (vitamin B_3) contents in coffee beverages depending on the degree of roasting and brewing technique †

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Abstract: The vitamin B3 (niacin) is present in various foods. During roasting of green coffee beans, niacin is formed from the alkaloid trigonelline. Therefore, we established a novel fast and sensitive HPLC-MS/MS method to determine niacin in coffee brews from five commercially available coffee samples. Additionally, we investigated the influence of the brewing method, brewing temperature, and degree of roasting on niacin contents. In the respective coffee brews, we were able to show that the content of niacin in coffee beverages is not only affected by the degree of roasting, but also by the extraction performance of different brewing methods to a lesser extent.

Keywords: niacin; coffee; HPLC-ESI-MS/MS; vitamin B_3; stable isotope dilution analysis (SIDA)

1. Introduction

Vitamin B_3 (nicotinic acid (NA) and nicotinamide (NAM), known as niacin) is present in various foods. NA and NAM play an important role in physiology. Niacin is a part of the cofactors nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate [1,2]. The recommended daily intake of niacin is about 15 mg/day [3], but it is a semi-essential vitamin. It can be formed from the amino acid tryptophan in the human body, with approximately 60 mg of tryptophan being equivalent to 1 mg NA. [4] During roasting of green coffee beans, NA is formed from the alkaloid trigonelline. Powdered coffee contains up to 300 mg/kg of NA depending on the degree of roasting. Nevertheless, in literature, information on the NA contents of commercially available coffee samples as well as the influence of brewing techniques on the NA content in prepared beverages is rather scarce. Therefore, we developed a fast and sensitive HPLC-MS/MS method to determine NA in coffee beverages. Additionally, the influence of brewing methods, degree of roasting, and brewing temperature was investigated.

2. Materials and Methods

2.1 Materials

Nicotinic acid (NA) and d_4-nicotinic acid (d_4-NA) were obtained from Toronto Research Chemicals (Toronto, Canada). Acetonitrile HPLC grade was acquired from VWR (Darmstadt, Germany). Formic acid p.a. was purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2 Preparation of the coffee beverages

Five commercially available coffee pad samples of ground coffee from the same manufacturer, containing approximately 7.4 g, (mild (taste intensity = 2), classic (taste intensity = 3), extra (taste intensity = 4), Espresso (taste intensity = 5), and decaffeinated (taste intensity = 4)) were purchased in a local supermarket in Germany. The "taste intensity"-value on a scale of 1 to 5 were provided on the packing box from the manufacturer to characterize the consumer expectation and is mainly influenced by the degree of roasting. All coffees were

100% arabica. After opening coffee powders were completely emptied in all cases, except in tests with the pad machine, and weighted. Coffee beverages were prepared in agreement with the described methods [5] using the following machines: a standard 'drip' coffee maker (Tchibo, Hamburg, Germany) fitted with filter paper, a Senseo coffee pad machine (Philips, Netherlands) and an automatic coffee maker (Bosch, Germany). Other samples were also prepared using: a manual plastic coffee filter with filter paper and boiling water; a pot for the 'Turkish' coffee (powder and water heated together); a 'French press' (Bodum AG, Triengen, Switzerland) and additionally, a so-called cold brew was prepared in the refrigerator for 24 h at +4 °C. For each method, triplicates were prepared and stored at −20 °C until the analysis. To investigate the influence of brewing temperatures, the ground coffee from the ‚classic' pads was emptied and brewed by hand filter at temperatures of 40, 60, 80, 90, and 100 °C.

2.3 Sample preparation

Aliquots (n = 3) of the coffee beverages were diluted 1:100, in the case of the coffee brew prepared from ‚mild' or ‚decaffeinated' coffee, as well as prepared at a temperature of 40 °C, the samples were diluted 1:50. The diluted coffee samples were spiked with an isotopically labelled standard d_4-NA to achieve a final concentration of 800 nM in each sample. After equilibration, the samples were passed through a syringe filter (Chromafil AO-45/25, Polyamid, 0.45 µm, Macherey-Nagel, Düren, Germany) and a 2 µL aliquotes were analyzed.

2.4 HPLC-ESI-MS/MS analysis

HPLC-ESI-MS/MS system (1200 series, Agilent, Waldbronn, Germany and API 3200 triple quadrupole mass spectrometer from Applied Biosystems, Darmstadt, Germany) was used. NA was analyzed using a VDSpher PUR HILIC-Z column (100 x 2.0 mm, 100 Å, 5.0 µm, VDS optilab, Berlin, Germany) at 20 °C using 0.01% aqueous formic acid (eluent A) and acetonitrile (eluent B) at a flow rate of 1000 µL/min. The injection volume was 2 µl. Gradient started at 90% eluent B, isocratic for 0.5 min, then eluent B was reduced to 10% within 0.1 min. From minute 0.6 to 4.0, the system was kept isocratic at 10% eluent B. Afterwards the column was washed and equilibrated. The ESI-source was operated in positive ion mode (5.500 V), and nitrogen was used as nebulizer gas (55 psi), heater gas (65 psi, 350 °C), curtain gas (25 psi), and collision gas (2.0 psi). Nicotinic acid and d_4-NA were detected in multiple reaction monitoring mode. MS transitions were: *m/z* 124 → 80 as qualifier and *m/z* 124 → 78 as quantifier and the isotopically labelled analogue *m/z* 128 → 84, respectively.

2.5 Quantitative analysis and method validation

To quantitate NA mixtures at eight analyte/internal standard mole ratios in the range of concentrations from 80 to 10000 nM for each analyte and at 800 nM for the associated internal standard d_4-NA were analyzed by HPLC-MS/MS. The ratios of the peak areas of the ions selected for quantitation were plotted versus the weight ratio of analyte/internal standard. The linear regression coefficient (R^2) of the calibration curve was > 0.99. Limits of detection and quantification, defined as signal to-noise ratios of 3:1 and 10:1 [6], were 14.7 nM and 77.5 nM, respectively. Recovery rates were determined using 640, 800, and 960 nM of NA as well as the respective IS and were between 91 and 96%. The established HPLC-ESI-MS/MS method was fast and sensitive enough to determine nicotinic acid in coffee brews within 4 min.

Data were processed by Analyst 1.6 (AB Sciex, Darmstadt, Germany). Experimental results are reported as means of at least three independent preparations with ± SD as error bars.

3. Results

From the five commercially available coffee samples under study the respective brews were prepared by a pad machine and NA was quantitated. To observe the influence of the brewing method, a Senseo coffee pad machine using pads named ‚classic', and the coffee powder from those pads was used to brew coffee by traditional filter, standard 'drip' coffee maker, automatic coffee maker, manual plastic coffee filter with filter

paper, 'Turkish' coffee pot, 'French press', and so called cold brew method. Figure 1 represents the calculated quantities of NA extracted from the powdered coffee [µg/cup of coffee].

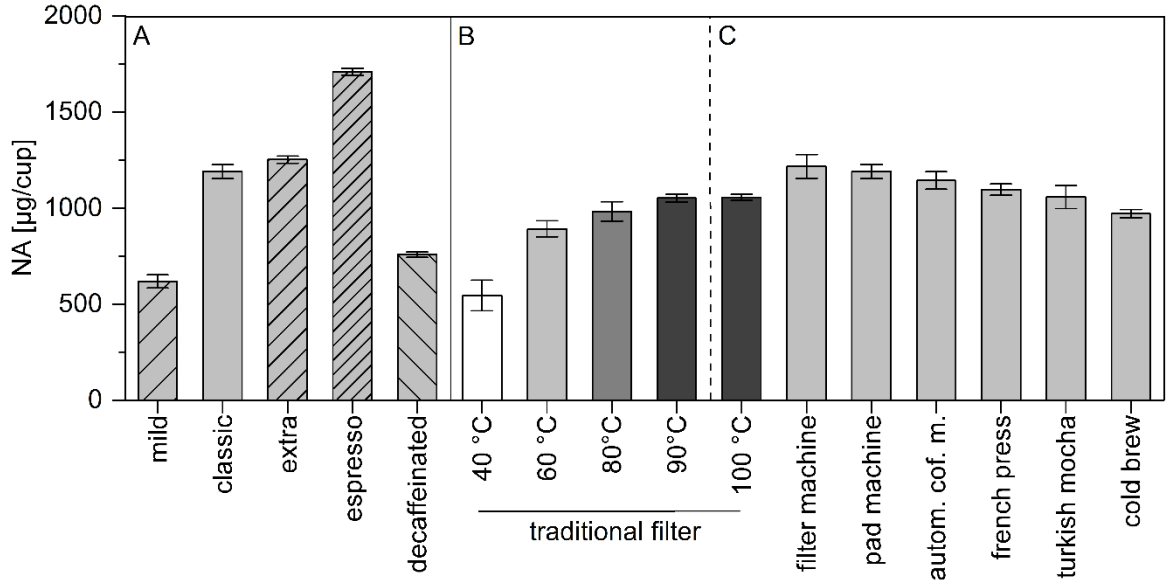

Figure 1: Nicotinic acid (NA) contents [mg/cup] of (**A**) five different coffee samples (all 100 % arabica) prepared by pad machine (Ø cup volume 112 mL); (**B**) coffee sample brewed at different temperatures with traditional filter method (all n = 3); (**C**) the sort ‚classic' prepared by different brewing techniques: filter machine (Ø cup volume 112 mL), automatic coffee maker (Ø cup volume 110 mL), French press (Ø cup volume 98 mL), Turkish mocha (Ø cup volume 71 mL), cold brew (Ø cup volume 96 mL) and traditional filter (Ø cup volume 116 mL).

It is demonstrated that the degree of roasting seems to influence the amount of NA: with higher roasting degree, the amount of NA is increased. Therefore, ‚espresso' showed the highest NA content with 1711 ± 18 µg/cup of coffee. The influence of the preparation seems to be minor. Here the automatic coffee maker and filter machine revealed highest amounts of NA (1144 ± 46 and 1217 ± 62 µg/cup), respectively. The lowest amount was determined in the cold brew with 972 ± 21 µg/cup NA.

One sample, namely the ‚classic' pad was used to observe the influence of brewing temperature on the NA transfer into the coffee brews. Water temperatures of 40, 60, 80, 90 and 100 °C were used to prepare traditional filtered coffee. Whereas at 40 °C the amount was only 545 ± 80 µg/cup, it increased with higher brewing temperatures: 892 ± 41 at 60 °C, 983 ± 41 at 80 °C, 1052 ± 20 at 90 °C and 1056 ± 17 µg/cup at 100 °C. At temperatures higher than 60 °C, the NA content of coffee beverages was almost independent of the brewing temperature reaching a plateau at 90 to 100 °C.

4. Discussion

We were able to show, that the NA amount in coffee is dependent on the degree of roasting. These results are in agreement with the study by Lang *et al*., who investigated the influence of different roasting conditions on NA formation. The authors found that hotter or longer roasted coffee beans contain more NA. [7] Additionally, the brewing method seems to influence the amount of NA transferred into the respective brews. This can depend on the water temperature used to prepare the coffees. Here it was shown that the maximum amount of NA is liberated at water temperatures above 80 °C. In contrast, it was shown that the duration of extraction has an influence on the amount of extractable NA. With the cold brew method (4 °C for 24 h) it is possible to obtain about 972 ± 21 µg/cup NA in contrast to 545 ± 80 µg/cup NA with traditional filter at 40 °C. However, even the choice of brewing method itself can have an influence on the extractable amount of NA. Thus, mechanical methods that allow continuous extraction (e.g. pad, filter and automatic coffee maker) show a higher yield than discontinuous methods (e.g. French press and Turkish mocha).

The results are presented in µg NA extracted per cup of coffee, accounting for up to 11 mg NA per liter, present in the coffee brew prepared by the coffee pad machine from the ‚classic' coffee sample. This corresponds to 1.2 mg per 125 ml cup covering about 9 % of the recommended daily intake (RDA) of 15 mg NA. [3] It could be shown that coffee consumption contributes to the daily niacin (vitamin B_3) intake.

Author Contributions: conceptualization, E.R.; investigation, L.B.; validation, L.B.; formal analyzes, L.B.; methodology, L.B., J.I.K. and S.S; project administration, E.R.; supervision, E.R.; writing – original draft preparation, E.R.; writing – review and editing, J.I.K., S.S. and E.R.

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Abstract

Resveratrol-dependent stimulation of mitochondrial fatty acid oxidation in deficient cells. Implication of miRNAs†

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The mitochondrial-located enzyme Carnitine palmitoyltransferase-2 (CPT2) is involved in long-chain fatty acid transport into mitochondria for β-oxidation to give energy production. It has been observed two phenotypes associated with a reduced CPT2 activity in genetically deficient patients: showing neonatal lethality or, in milder forms, myopathy. Resveratrol (RSV, trans-3,5,4'-trihydroxystilbene) is a phytophenol produced by grape plants in response to biotic or abiotic stresses that displays anti-oxidant properties. This polyphenol protects humans against various diseases (cardiovascular and inflammation-associated pathologies, like infection, cancer, neurodegenerescence, aging, etc.) through the modulation of several signaling pathways, including those mediated by transcription factors AP-1, NFκB, and STAT-3 or the COX enzyme. RSV can enhance residual CPT2 activities in human fibroblasts derived from *CPT2*-deficient patients and restores normal fatty acid oxidation rates. We reported changes in miRNA expression linked to *CPT2*-deficiency and identified miRNAs whose expression changed following RSV treatment of control or *CPT2*-deficient fibroblasts isolated from patients. Our findings suggest that RSV consumption might exert beneficiary effects in patients with *CPT2* deficiency.

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Abstract

Protective effects of dietary polyphenols on arterial stiffness†

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Cardiovascular diseases are the major cause of mortality, with 17.9 million deaths/year worldwide and 3.9 million deaths/year in Europe, representing a cost to the EU economy of €210 billion/year [1,2]. Arterial stiffness has been shown to increase cardiovascular morbidity and mortality [3,4]. It is a complex phenomenon characterized by decreased vascular distensibility [5]. This degenerative process is influenced by ageing and several risk factors but is mainly associated with changes in the extracellular components of elastic arteries [5,6]. Several factors, including vascular function, oxidative stress, inflammation, glycation and autophagy contribute to the pathophysiology of arterial stiffness. Considering that the structural degeneration of the extracellular matrix of the vascular wall is practically irreversible with current therapies, it is extremely important to evaluate the impact of preventive interventions, for example reducing the impact of aging on increasing stiffness [5]. Most cardiovascular diseases can be prevented by addressing behavioral risk factors, of which dietary factors make the largest contribution [2]. Polyphenols are a widespread class of plant secondary metabolites that are found in several foods and possess a diverse range of biological activities. Dietary polyphenols display pleiotropic effects, interacting with most mechanisms involved in arterial stiffness etiology. Therefore, they could constitute an interesting option to target vascular stiffening. *In vivo* activity of polyphenols or polyphenol containing foods is known [7]. For several polyphenols or polyphenol containing foods, including cocoa, grapes, berries and olive, intervention studies point to a beneficial effect on vascular stiffness [8-12]. With regard to olive polyphenols specifically, our previous intervention study has shown blood pressure lowering effects [12-15]. In order to further elucidate mechanisms of action, we recently focused on specific studies investigating the potency of olive polyphenols as autophagy-inducing compounds, and the contribution of this mechanism to their atheroprotective effects.

Keywords: Arterial stiffness, dietary polyphenols, olive polyphenols, autophagy

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Abstract

Comparison of redox properties of flavonoid aglycones and corresponding glycosides and their mixtures in the cellular model †

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Abstract: Flavonoids are polyphenolic compounds commonly found in plants. As dietary components, they have been shown to exhibit numerous pro-health properties that are believed to be associated with their antioxidant properties. In this study, the antioxidant activity of four flavonoid compounds was determined by cellular antioxidant activity assay using HT29 cells as a model of the alimentary tract. The strongest impact on cellular redox status was observed for aglycones which acted as both antioxidants (quercetin) and prooxidants (naringenin). Interestingly, mixtures of tested compounds displayed only antioxidative properties.

Keywords: cellular antioxidant activity, redox properties, quercetin, rutin, naringin, naringenin

1. Introduction

Flavonoids are one of the largest groups of secondary metabolites in the plant kingdom. They are phenolic compound, which contain in their main structure 15 carbon atoms that form a characteristic carbon skeleton composed of two aromatic rings connected by either tri-carbon bridge or heterocyclic ring. At the highest concentrations, they occur in the epidermis of leaves, flower petals and fruit peel. Therefore, some plant foods are a rich source of these phytochemicals, e.g. fruits, vegetables, tea, coffee, red wine, herbs, spices or chocolate. The results of *in vitro* and *in vivo* studies documented the significant impact of flavonoids on redox status of cells. Most data indicate antioxidant properties of these compounds results in anti-inflammatory, anticancer and hepatoprotective properties. However, flavonoids may also act as prooxidants. This prooxidative effect is responsible for the proapoptotic and cytotoxic effects of some compounds belonging to this group.

Flavonoids occur in nature and thus in food products in the form of aglycones or O-glycosides. Very common aglycones are quercetin and naringenin whose glycosides are, respectively, rutin (quercetin-3-rutinoside) and naringin (naringenin-7-rhamnoglucoside). The presence of the sugar residue is known to affect bioactivity, bioavailability and metabolism of flavonoids in the human body [1-3].

The aim of this study was to compare the impact of selected pairs of flavonoids on modulation of cellular antioxidant barrier in the colon adenocarcinoma cell line (HT29) used as model of alimentary tract to learn how important the sugar fragment is in the core structure for redox properties of the compounds.

2. Materials and methods

2.1. Chemicals and reagents

The following compounds were used: quercetin, rutin, naringin and naringenin from Sigma-Aldrich (USA), analytical grade ethanol from POCH (Poland). The OxiSelect™ Cellular Antioxidant Assay Kit was purchased from Cell Biolabs, Inc. (USA). All tested compounds were dissolved in 10% ethanol.

2.2. Cell culture

HT29 cells (human colon adenocarcinoma) from the ATCC were used for these studies. Cells were maintained in McCoy's medium with sodium pyruvate (200 g/L), L-glutamine (2 mol/ L), antibiotics (100 U/mL streptomycin and 100 g/L penicillin) and fetal bovine serum (100 mL/L). All cell culture chemicals and biochemicals were derived from Sigma-Aldrich (USA). Cells were kept at 37 °C under 5% CO_2 atmosphere in a cell incubator (Heal Force).

2.3. CAA (Cellular Antioxidant Activity) assay

The cellular antioxidant activity of studied compounds in HT29 cells was investigated using CAA assay (The OxiSelect™, Cell Biolabs, Inc., USA). Cells were seeded in 96-well tissue culture black plates with transparent bottoms for fluorescence measurements (3×10^4 cells per well in 200 µL of medium). The cells were incubated to settle for 24 h at 37 °C and then were treated with 50 µL of different concentrations of flavonoids for 1 h. Treatments were performed as three technical replicates and three independent repetitions of each treatment were carried out. Final concentrations of studied compounds ranged from 1 to 100 µM. The control cells were treated with 10% ethanol. Further experimental steps were performed according to the procedure available from the manufacturer's website (https://www.cellbiolabs.com). The emission of fluorescence (at 538 nm) was measured every 5 min for 1 h after excitation (at 485 nm) using a TECAN Infinite M200 plate reader. CAA units were calculated according to the equation:

$$\textbf{CAA units} = \mathbf{100} - \frac{\mathbf{SA}}{\mathbf{CA}} \times \mathbf{10}$$

where SA means the area under the fluorescence curve plotted as a function of time corresponding to each concentration of tested compound, while CA is the area under the control fluorescence curve as a function of time for cells treated only with the appropriate solvent [4].

3. Results

The CAA assay is a biological test, which is said to effectively mimic the redox conditions found in the human body. It encompasses such aspects as metabolism, bioavailability and distribution of antioxidants in the cell. For this reason, it is believed to better reflect antioxidant activity *in vivo* than chemical tests. This method relies on the ability of redox-active substances to oxidize or inhibit the oxidation of a fluorescent probe that is absorbed by cells. The intensity of fluorescence is directly proportional to the degree of oxidation of the probe, which allows to assess the anti- or pro-oxidative capacity of the test substance in a cell model used. The results of fluorescence measurements are presented as CAA units. Higher values of CAA units indicate stronger antioxidant properties, while negative values suggest prooxidative activity [4, 5].

In this study, CAA assay was performed for four flavonoid compounds which constituted pairs aglycone – glycoside. These were quercetin and its rutinoside – rutin and naringenin and its rhamnoglucoside – naringin. The results obtained revealed that naringin negligibly influenced cellular redox properties within the concentration range studied (Fig. 1). All remaining compounds affected cellular redox status in a dose dependent manner but differed substantially in their effects: quercetin was the strongest antioxidant, rutin displayed weak antioxidant properties at the highest concentration tested, while CAA units for naringenin decreased with increasing concentration reaching negative values, which is indicative of prooxidative properties.

In the next series of experiments, the cellular redox activity of two mixtures of compounds studied was examined (Fig. 1). The mixture M1 contained aglycones (quercetin and naringenin); the mixture M2 included glycosides (rutin and naringin). In contrary to expectations, such a combined treatment of HT29 cells did not result in averaged effects and CAA values determined tended to reflect only antioxidant activity of mixtures' components.

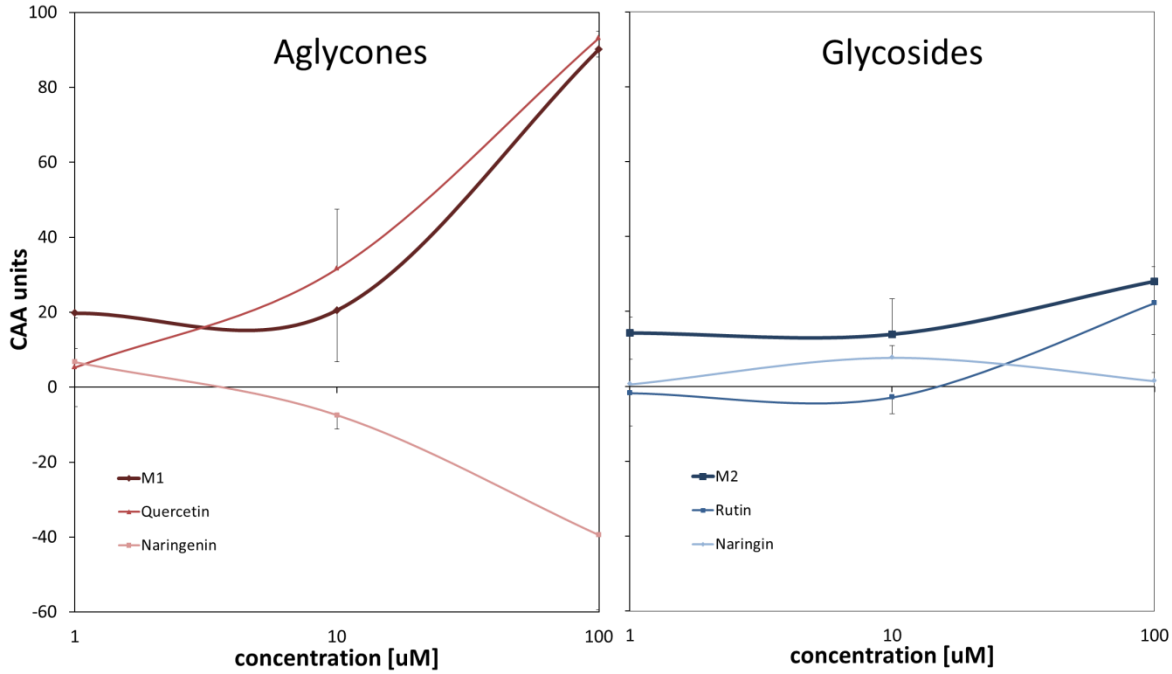

Figure 1. The dependence between cellular antioxidant activity (expressed as CAA units) and the concentration of rutin, quercetin, naringin, naringenin and their mixtures. The results are the average of three biological replicates.

4. Discussion

This study determined the effect of selected flavonoids, aglycones and corresponding glycosides, and their mixtures on cellular antioxidant activity in the colon adenocarcinoma cell line (HT29) model. In general, it was observed that aglycones are stronger modulators of cellular redox homeostasis. The results obtained for rutin and quercetin revealed the dose dependent antioxidant activity of these compounds. Their physiological concentrations (1-10 μM) did not significantly affect the antioxidant status of cells. However, higher concentration (100 μM), which may reflect intestinal epithelium cells being in contact with food ingested, strongly enhanced cellular antioxidant protection, especially in the case of quercetin. For naringin, differences in CAA units for all tested concentrations are small and oscillate around zero, while naringenin exhibited prooxidative properties at higher concentrations tested. Therefore, consumption of these two flavonoids in a purified form, e.g. isolated from citrus fruits, may not offer expected protection against oxidative stress. The presence of sugar residue seemed to quench these strong reducing or oxidative responses leading to the maintenance of proper cellular redox homeostasis. Alternatively, the lower activity of glycosides may result from their more hydrophilic nature, which decreases their bioavailability, thus effective concentration inside cells.

Interestingly, neither of mixtures showed cellular antioxidant activity that would indicate the additive effect of two components. The presence of the substance with prooxidant activity (naringenin) did not diminish the impact of the antioxidant (quercetin). The response of cells to the treatment with the combination of flavonoids seemed to be only affected by their antioxidant properties, while the prooxidant ones did not influence cellular redox status. This observation suggests that the cellular redox system, when supported by exogenous antioxidants may successfully resist the exposure to prooxidants.

Author Contributions: conceptualization, Agnieszka Bartoszek; methodology, Monika Baranowska; investigation, Zuzanna Koziara and Monika Baranowska; writing—original draft preparation, Zuzanna Koziara; writing—review and editing, Agnieszka Bartoszek, funding acquisition, Jacek Namieśnik

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract

Thiol-modification as Important Mode of Action for Allicin from Garlic (*Allium sativum*) †

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Abstract: Garlic is a common ingredient in food, normally used as spice but is also used since ancient times for its health beneficial activity. The thiosulfinate allicin is the first active compound in freshly damaged garlic tissue and reacts with thiol-groups. Hence, allicin is able to modify thiol groups, both of protein cysteine-residues and low-molecular weight thiols like glutathione. This thiol-modification is supposed to be an important mechanism for allicin's biological activity. Here, the mechanisms and possible targets for allicin in cells are discussed.

1. Introduction

Garlic (*Allium sativum* L.) is used as spice in food since ancient times and it is well known for its health-beneficial and even therapeutic properties. Its health properties have been documented in many scientific studies [1]. Therefore, garlic is potentially interesting for its nutriceutical use. However, the modes of action of garlic compounds are still not fully understood.

Allicin is the first compound that is produced in freshly damaged garlic tissue from the non-proteinogenic amino acid alliin. One clove of garlic produces about 50 mg allicin. However, allicin has a Janus-face. On the one hand, there are many different beneficial effects ascribed to allicin but, on the other hand, allicin is a broad-spectrum biocide, killing microbial and human cells [2]. Allicin is a very reactive thiosulfinate that is able to oxidize thiol groups in a reaction that is similar to the thiol-disulfide exchange reaction. Thus, the former thiol is oxidized to an S-thioallyl adduct, the process is called S-thioallylation [3]. In a second step it is possible this this adduct reacts with a second thiol within the protein to form a disulfide bridge; the S-thioallyl residue is reduced to allylmeracptane.

Both protein thiols and low-molecular weight thiols like glutathione are targets for *S*-thioallylation. The thioallylated glutathione (*S*-mercaptoglutathione) cannot be used as redox-buffer, although S-mercaptoglutathione is also a substrate for glutathione reductase, which recycles S-mercaptoglutathione to GSH and allylmercaptane using NADPH [4]. Consequently, the pentose-phosphate pathway (PPP), which is a primary source of NAPDH in the cell, is crucial to resist against allicin [5].

Furthermore, allicin reacts with Protein thiol-groups. In a proteome-wide study of human cells 232 proteins were found that are thioallylated upon exposure to sublethal concentration of allicin. Most of these proteins were related to the cytoskeleton. Also, enzymes involved in glycolysis were modified, which suggests that allicin impairs the primary metabolism of the cell directly. These are two examples how S-thioallylation might influence the cellular physiology and, thus, explain allicin's mode of action.

Keywords: garlic; Allium sativum; allicin; thiol; *S*-Thioallylation.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract

2,3-Dehydroderivatives of Silymarin Flavonolignans: Prospective Natural Compounds for the Prevention of Chronic Diseases†

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Abstract: *Silybum marianum* fruit extract silymarin displays various biological activities, which are attributed mostly to its major component silybin. However, silymarin contain several other isomeric flavonolignans (isosilybin, silychristin, silydianin) and their oxidation products, the 2,3-dehydroflavonolignans (2,3-dehydrosilybin, 2,3-dehydrosilychristin, 2,3-dehydrosilydianin). The latter compounds were found to be 1-2 orders of magnitude more efficient radical scavengers, reducing, chelating, cytoprotective, anti-aging, anti-cancer and anti-angiogenic agents than the parent flavonolignans. Although 2,3-dehydroflavonolignans occur in silymarin as minorities, they seem to be responsible for the majority of the biological activity and therefore have potential for the prevention of chronic diseases.

Keywords: silymarin; flavonolignan; chemoprevention.

1. Introduction

Food supplements containing *Silybum marianum* (L.) Gaertn. (Asteraceae) fruit extract, silymarin, are used especially by the elderly population. Silymarin displays many biological activities such as antioxidant, anti-inflammatory, immunomodulatory and hepatoprotective properties. Depending on the plant cultivar and extraction method used [1], silymarin contains a mixture of structurally closely related flavonolignans (flavonoids with fused lignan part). The main silymarin constituents are silybin A, silybin B, isosilybin A, isosilybin B, silychristin A, silydianin, the flavonol taxifolin (Figure 1) [2] together with minor flavonolignans and approximately 30% of undefined polymeric fraction. The most abundant and hence easily isolated silymarin component [3] is silybin (mixture of diastereomers A and B) and therefore, it is considered as the major active principle of silymarin. Therefore, the literature mostly focuses on silybin and ignores all other components. However, other flavonolignans are likely to contribute to, or even be responsible for distinct beneficial effects of silymarin. In particular, minor flavonolignans of the silymarin complex, *e.g.* 2,3-dehydroflavonolignans, which occur as a result of bio-oxidation in the plant itself or due to oxidation during extraction and processing [4] have been neglected for a long time. The aim of the present study was to evaluate the chemopreventive potential of all silymarin constituents including the minorities and their potential metabolites.

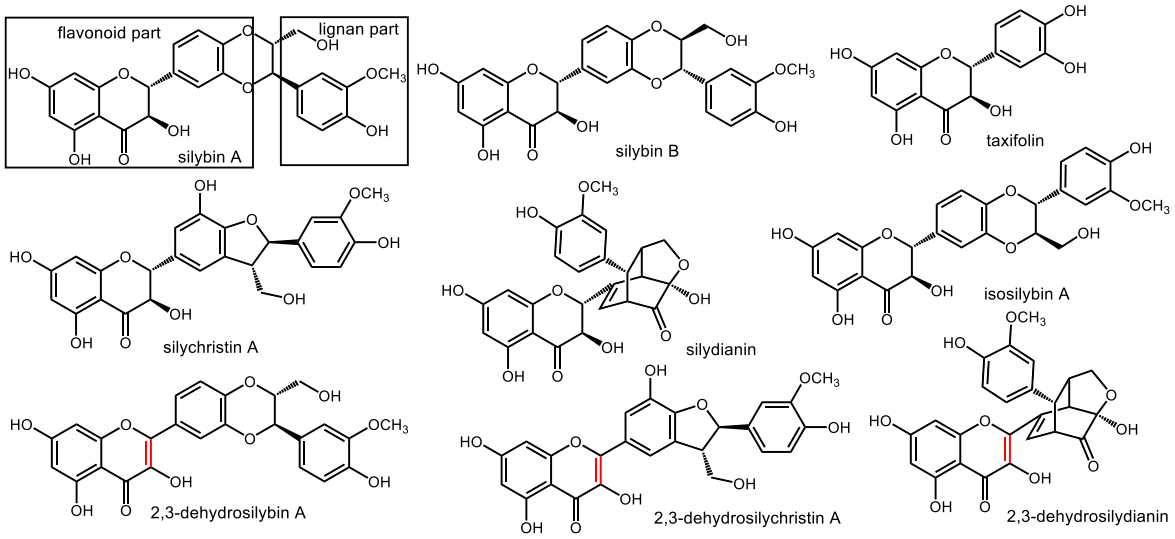

Figure 1. Selected silymarin components

2. Materials and Methods

Silybin A, silybin B, silychristin A, and silydianin were isolated from silymarin (Liaoning Senrong Pharmaceutical, Panjin, China; batch No. 120501) as described previously [5,6]. 2,3-Dehydrosilybin, 2,3-dehydrosilychristin and 2,3-dehydrosilydianin were prepared from respective flavonolignans by optimized oxidative methods [4,7,8] in the presence of organic bases. 2,3-Dehydroanhydrosilychristin was prepared by treatment of 2,3-dehydrosilychristin by HCl [7]. Sulfated metabolites of (2,3-dehydro)flavonolignans were prepared using arylsulfotransferase from *Desulfitobacterium hafniense* heterologously expressed in *E. coli* [9]. Reducing, radical-scavenging [4], metal chelating [10], cytotoxic [7] and cytoprotective [4] activities of all compounds were measured and compared to those of parent flavonolignans.

3. Results

All the compounds were successfully prepared in multimiligram up to gram amounts, enabling a detailed study of their properties. The 2,3-dehydroflavonolignans proved to be 1-2 orders of magnitude more active radical scavengers, reducing and cytoprotective agents than their parent compounds [4,7,8]. Significant reducing and antioxidant activity remained even after sulfate conjugation of the 2,3-dehydroderivatives [9]. While silybin A, silybin B and silychristin A were quite weak chelators, 2,3-dehydrosilybin was found to be a potent iron and copper chelator [10]. Moreover, 2,3-dehydrosilydianin (but not silybin, silychristin, silydianin, 2,3-dehydrosilybin, or 2,3-dehydrosilychristin) was found to activate Nrf2 and upregulate NAD(P)H:quinone oxidoreductase 1 in Hepa1c1c7 cells [11]. 2,3-Dehydrosilybin and its gallates were also more efficient inhibitors of angiogenesis that silybin and silybin derived gallates [12,13]. In more a complex model, 2,3-dehydrosilybin (and especially the A enantiomer) displayed lifespan-extension effect superior to that of silybin, isosilybin, silychristin and silydianin in *Caenorhabditis elegans* [14]. Moreover, 2,3-dehydrosilybin inhibited basal cell carcinoma allograft tumor growth more than silybin in mice [15].

4. Conclusion

Although the 2,3-dehydroflavonolignans occur as minorities in silymarin, their biological activity is superior to that of other silymarin constituents and they therefore may be responsible for certain biological activities of this complex plant extract. These natural compounds have therefore a great potential for the prevention of chronic diseases.

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Abstract

Effects of vine shoot extract on riboflavin-induced DNA damage in HepG2 cells†

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Abstract:

Winery by-products, including pomace, stem, vine leaves and shoots, arise in high amounts during the wine-making process. Due to high contents of secondary plant metabolites, such as polyphenols, their usage in the food, cosmetics and pharmaceutical industry is possible. In this study, we investigated effects of an extract prepared from vine shoots (*Vitis vinifera* L. cv. Riesling) on riboflavin-induced DNA damage in human hepatocellular carcinoma cells HepG2 by comet assay. Significant decreases of DNA damages were detected after 2 h and 24 h extract incubation at concentration ranges of 1 to 30 µg/ml (2 h) and 1 to 10 µg/ml (24 h), respectively. These effects may be attributable to polyphenolic compounds, which has to be further investigated.

Keywords: vine shoots, *Vitis vinifera* L. cv. Riesling, polyphenols, DNA damage, riboflavin, ROS

1. Introduction

During the wine-making process winery by-products, such as pomace, stem, vine leaves and shoots, arise. Vine shoots for example occur in huge amounts at pruning in springtime and are traditionally used as natural fertilizer [1]. Due to secondary plant metabolites, such as polyphenols, the usage of winery by-products as sources of bioactive compounds offers an opportunity to obtain value-added products for the food, cosmetics, and pharmaceutical industry [2]. It is known that polyphenols protect cells against oxidative stress due to their antioxidative and radical scavenging abilities and are associated with numerous beneficial health effects. A broad spectrum of biological activities is described, including antioxidant, anticancer and anti-inflammatory activities, amongst others [3]. Vine shoots are rich sources of phenolic acids, flavonoids and especially stilbenoids, such as resveratrol [4]. The aim of the present study was to investigate the effects of an extract from vine shoots of *Vitis vinifera* L. cv. Riesling on riboflavin-induced DNA damage by the comet assay. Additional treatment of DNA with formamidopyrimidin glycosylase (FPG) enhanced the sensitivity towards reactive oxygen species (ROS) induced DNA lesions and thus enabled the detection of oxidative DNA damage.

2. Materials and Methods

2.2 Chemicals, cells and media

Vine shoots of *Vitis vinifera* L. cv. Riesling were provided from a vineyard (conventional production) in Rhineland-Palatinate (Grünstadt-Sausenheim). They were cut-off before pruning of the vines in springtime 2016 and stored at room temperature till extraction. Chemicals and solvents were all of analytical grade or compliant with standards required for cell culture experiments. Formamidopyrimidine glycosylase (FPG) was provided by Prof. A. Collins (University of Oslo, Norway). HepG2 cells were obtained from DSMZ (Braunschweig, Germany), riboflavin, catalase, dimethyl sulfoxide (DMSO) 99.6 %, Amberlite® XAD16N, ethanol absolute(≥ 99.8 %), trypan blue solution and ethidium bromide were purchased from Sigma Aldrich (Munich, Germany), hydrochloric acid from CHEMSOLUTE®, Th. Geyer GmbH & Co. KG (Renningen, Germany), low and normal melting agarose from Bio-Rad (Munich, Germany), Trypsin from Serva

(Heidelberg, Germany). Cell culture media (RPMI 1640) and supplements (fetal calf serum (FCS), penicillin/streptomycin) were purchased from Invitrogen (Karlsruhe, Germany). Cell culture materials (petri dishes, flasks, etc.) were from Greiner Bio-One (Essen, Germany).

2.2. Vine shoot extract preparation

Lyophilized and grinded vine shoots were extracted twice at the ratio of 1 g of solid per 25 ml of extraction solvent (methanol / water / 1 N HCl: 80 / 19 / 1 (v/v/v)) at room temperature under constant stirring (1st: 60 min, 2nd: 30 min). After sterile filtration (0.2 µm) the extraction solvent was removed under reduced pressure at 40 °C and transferred into the water phase. To remove sugars, salts and organic acids solid-phase extraction was carried out using Amberlite® XAD16N resin. After conditioning with ethanol (90 %) and equilibration with double distilled water, the column was loaded with the aqueous extract and washed with 5 fold bed volumes of double distilled water. Retained substances were eluted with 5 fold bed volumes of ethanol (90 %). The eluate was evaporated under reduced pressure at 40 °C, transferred into water phase and then lyophilized. The powdery extract was homogenized and stored at -20 °C in the dark until used.

2.3. Cell culture and incubation procedure

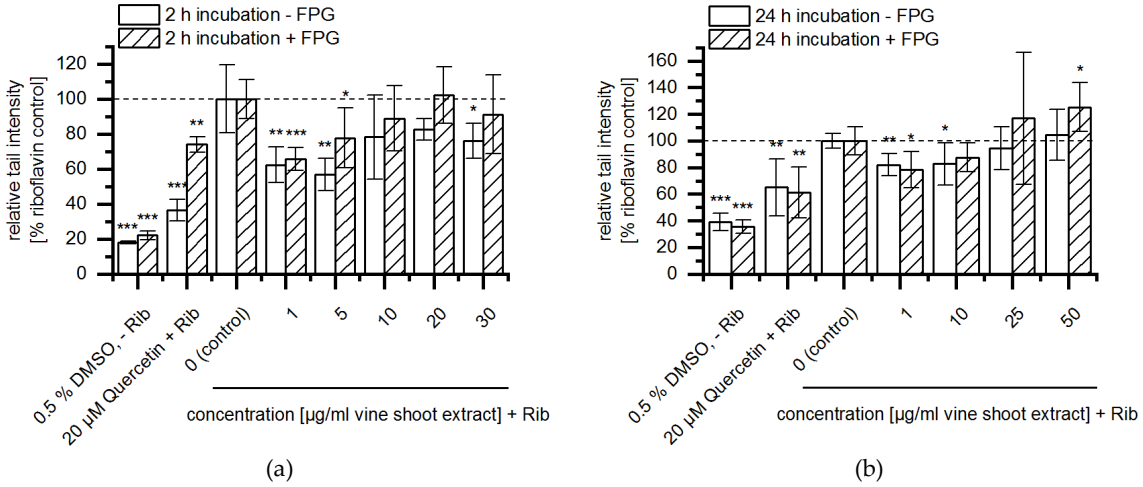
HepG2 cells were cultivated in 175 cm^2 flasks in RPMI 1640 medium with addition of 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (humidified atmosphere with 5 % CO_2 at 37 °C). The cells were seeded in 6 cm Petri dishes, cultivated for 24 h, washed with PBS and in the first step incubated with vine shoot extract in incubation medium containing 5 % FCS when performing 24 h incubation and FCS-free medium when performing 2 h incubation. Vine shoot extract was dissolved in DMSO, with a final DMSO-concentration of 0.5 % in media (2 h: 1 to 30 µg/ml; 24 h: 1 to 50 µg/ml). Secondly, cells were kept on ice, washed twice with PBS and treated with the photosensitizer riboflavin (2 h preincubation: 100 µM; 24 h preincubation 250 µM; in PBS) in combination with irradiation with a halogen lamp (distance to the bottom of the Petri dishes: 33 cm) for 10 min. Afterwards the cells were washed three times with PBS and isolated by trypsination (0.5 mg/ml trypsin). To inhibit the formation of extracellular hydrogen peroxide (H_2O_2) by pro-oxidative interaction of phenolic compounds with cell media constituents [5], incubations were performed in presence of catalase (100 U/ml).

2.6. DNA damage measurement (comet assay)

Alkaline single cell gel electrophoresis was performed according to Collins *et al.* and Bakuradze *et al.* [6,7], with slight modifications. First aliquots of suspended cells were examined for cell viability by trypan blue exclusion. Absolute viability was determined (in %; viable cells in percent of total cells). To exclude DNA damage resulting from cell death only cell suspensions with viability > 80 % were tested in comet assay. Briefly, 4 x 10^4 cells per preparation were centrifuged (10 min, 2000 rpm, 4 °C), the pellets were resuspended in 65 µl low melting agarose (0.5 %) and applied on microscope slides pre-coated with normal melting agarose (1.5 %) (2 gels per slide, 2 slides each treatment, with and without FPG, respectively). The cells were lysed over night at 4 °C. Afterwards the slides were washed in enzyme buffer three times. Either FPG in enzyme buffer or enzyme buffer (without FPG) were applied on the slides (50 µl per gel) and incubated for 30 min at 37 °C. After DNA unwinding (pH 13.5, 20 min, 4 °C) and horizontal gel electrophoresis (20 min, 25 V, 300 mA), the slides were neutralized by washing with neutralization buffer three times, fixed with ethanol (95 %), dried and stored at room temperature in the dark. Analysis was performed after labeling of DNA with ethidium bromide using fluorescence microscope (Zeiss, Germany). Overall 2 x 50 cells per slide were analyzed. DNA damage was calculated as tail intensity (TI %, intensity of DNA in the comet tail, as percent of total DNA) [6,8,9]. Results were expressed as percent of riboflavin treated control. They are presented as mean ± SD of 3 to 5 independent experiments. Differences were determined by independent one-sided t-test by Origin®, version 2019 (OriginLab Corporation, Northampton, USA).

3. Results

Comet assay was used to study the effects of vine shoot extract on riboflavin-induced DNA damage in HepG2 cells (Figure 1). An additional treatment with FPG led to an enhanced sensitivity towards ROS induced DNA lesions allowing a distinction between riboflavin-induced DNA damage and oxidative DNA damage. Treatment of HepG2 cells only with DMSO (0.5 %) led to basal levels of DNA damage of 18 to 39 % TI. After 2 h vine shoot extract preincubation significant decreased riboflavin-induced DNA damage was detected within the concentration range from 1 to 30 µg/ml, whereby lower vine shoot extract concentrations showed more distinct effects. Oxidative DNA damages were significantly reduced from 1 to 5 µg/ml vine shoot extract. For instance, 2 h preincubation with 1 µg/ml vine shoot extract showed a reduction of oxidative DNA damage down to about 66 % compared to the riboflavin control.



(a) (b)

Figure 1. Modulation of riboflavin-induced DNA damage in HepG2 cells after (a) 2 h and (b) 24 h vine shoot extract preincubation (2 h: 1 to 30 µg/ml vine shoot extract, 100 µM riboflavin; 24 h: 1 to 50 µg/ml vine shoot extract, 250 µM riboflavin); quercetin (20 µM) was used as positive control. Results were calculated as percent of riboflavin treated control; 2 h: n = 3 – 5, 24 h: n = 4 - 5 (mean ± SD). Differences were determined by independent one-sided t-test related to riboflavin treated control: *p < 0.05, **p < 0.01, ***p < 0.001.

Only preincubations with vine shoot extract concentrations of 1 – 10 µg/ml led to significant reduced riboflavin-induced DNA damage after 24 h. Oxidative DNA damage was decreased after incubation with 1 µg/ml vine shoot extract, whereas preincubation with 50 µg/ml vine shoot extract led to significant increase of oxidative DNA damage.

4. Discussion and conclusion

In previous studies, polyphenol rich extracts have been shown to protect against oxidative DNA damage. After preincubations of Caco-2 cells with apple juice and bilberry extract and subsequent induction of oxidative DNA damage by menadione, "U-shaped" forms of concentration curves were observed [10,11]. DNA-protective effects for example were described after 24 h incubation with 50 µg/ml bilberry extract and 5 µg/ml bilberry extract with FPG-treatment, respectively. In contrast, prooxidative effects were detected at high concentrations of bilberry extract (250 – 500 µg/ml) [10]. Furthermore, several polyphenolic compounds were described to be protective against oxidative DNA damage. Relatively low concentrations of grape seed polyphenols, namely catechin and gallic acid, have led to decreased H_2O_2-induced DNA damage in mice spleen cells [12]. In addition, resveratrol protected against arsenic-trioxide-induced oxidative damage at simultaneous exposure to 20 µM As_2O_3 and 5 µM resveratrol in human bronchial epithelial (HBE) cells [13].

Taken together, vine shoot extract showed protective effects against riboflavin-induced DNA damage, which was significantly reduced after 2 and 24 h preincubation with vine shoot extract at concentrations of 1 – 30 µg/ml and 1 – 10 µg/ml, respectively. These effects may be attributable to contained polyphenolic substances, which has to be further investigated.

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proceedings

Abstract

Inspired by Nature: Redox modulators and natural nanoparticles†

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Abstract: Numerous secondary metabolites found in edible plants modulate intracellular redox processes and are suggested to prevent certain diseases, especially in ageing organisms. Since such nutraceuticals provide the basis for new and innovative designer diets and therapies, extracting these substances and their potential from plants has become a focus of research, with nanotechnology and natural nanoparticles at the centre of some of these developments.

Keywords: nanoparticles, prevention, redox modulation, secondary metabolites.

In recent decades, it has become apparent that Oxidative Stress plays a major role in the formation and progression of many human diseases, in particular those related to ageing and the elderly population. Traditionally, medications have been administered to prevent or slow down these ailments. In societies affected by demographic changes, such (multi-)medication is not always feasible and, in any case, places a massive burden on the individual, the society, the environment and economy.

Nutrition provides a promising alternative. Plants and mushrooms tend to be rich in phytochemicals and many secondary metabolites are able to modulate intracellular redox processes, including Reactive Sulfur Species (RSS) and Reactive Selenium Species (RSeS) [1-3]. Compounds such as allicin and polysulfides found in many *Allium* plants, for example, can interact with the "cellular thiolstat" [4]. Such agents are moderately reactive, affect numerous cellular processes, exhibit pronounced antioxidant and also cyto-toxic activities and, therefore, have been linked to certain preventive or even therapeutic actions [5-10]. Together, a balanced cocktail of such biologically active nutritional components may therefore provide a promising and also more applicable alternative to extensive medication. Additionally, the global market place - for food - also implies that it is now possible to design diets which may address specific needs, for instance for specific sub-populations and age groups.

Despite these advantages, there are still some challenges associated with this strategy. One major obstacle, for example, is the low solubility and hence bioavailability of many secondary metabolites, such as polyphenols. Rather than simply consuming the relevant food, complicated extraction and formulation methods are required to produce adequate food supplements. Here, nanotechnology provides potential solutions. It is now possible to produce nanoparticles of the relevant plant materials with comparable ease, for instance by milling or fermentation. These nanosized materials are entirely "natural" as far as their chemical composition is concerned, and often exhibit an amazing release profile for active ingredients and therefore also considerable biological activity [11-14]. Nanosized plant materials rich in biologically active ingredients may therefore unlock the considerable potential of many food items, and possibly also of materials which so far have been considered as "waste", such as the spent coffee ground, grape seeds and various shells, leftovers and peels [15]. At the same time, nanoscopic particles of sulfur and selenium generated by and in microorganisms may represent interesting preparations for agricultural applications [16-18].

In both cases, the combination of phytochemistry and nanotechnology promises access to new biological activities and innovative applications in various areas, from nutrition and medicine to agriculture and cosmetics.

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Abstract

Chlorogenic acid as a model compound for optimization of an *in vitro* gut microbiome-metabolism model †

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Abstract: It had been believed that the metabolism of xenobiotics occurred mainly by the cytochrome P450 enzyme system in the liver. However, recent data clearly suggest a significant role for the gut microbiota in the metabolism of xenobiotic compounds. This microbiotic biotransformation could lead to differences on activation, inactivation and possible toxicity of these compounds. *In vitro* models are generally used to study the colonic biotransformation as they allow easy dynamic and multiple sampling over time. However, to ensure this accurately mimics communities *in vivo*, the pre-analytical phase requires optimization. Chlorogenic acid, a polyphenolic compound abundantly present in the human diet, was used as a model compound to optimize a ready-to-use gut microbiome biotransformation platform. Samples of the *in vitro* gastrointestinal dialysis-model with colon stage were analyzed by liquid chromatography coupled to high resolution time-of-flight mass spectrometry. Complementary screening approaches were also employed to identify the biotransformation products.

Keywords: Gut microbiome; *in vitro* gastrointestinal dialysis model; 16S rDNA sequencing, liquid chromatography-mass spectrometry

1. Introduction

The microorganisms that live inside and on the human body outnumber the human cells by a factor of ten [1-6]. The gastrointestinal tract (GIT) accommodates between 10^{12}-10^{13} microorganisms with the colon harboring the majority of bacteria of the GIT. *Firmicutes* and *Bacteroidetes* are the two dominant phyla present in the human GIT, accounting for 90 % of the gut diversity [5, 7-10]. The gut microbiota is responsible for various developmental, immunological and nutrition host functions [11, 12].

The gut microbiome, the cumulative genome content of the gut microbiota, is estimated to contain 100 to 300-fold more genes than the human genome [1, 3, 5, 13, 14] and therefore is considered an additional metabolic organ [3, 7, 15, 16]. Important differences between hepatic and bacterial metabolism have been described. Metabolic pathways by the liver cytochrome P450 enzymes (CYP450) lead to the conversion of lipophilic compounds into more hydrophilic compounds, while the majority of biotransformations encoded for by the gut microbiome are reductive and hydrolytic reactions. In addition, the gut flora is responsible for demethylation, deacylation, decarboxylation, acetylation, dealkylation, dehalogenation, dehydroxylation, deamination, and oxidation/dehydrogenation [1, 17-20]. Direct and indirect interactions can influence the activity and toxicity of xenobiotics [20]. Conversion of a prodrug to the active compound, inactivation, detoxification, change of efficacy and direct binding to xenobiotics or dietary molecules are examples of direct interferences [11, 21]. Examples of indirect interferences are competition and/or inhibition of host enzymes by microbial metabolites, the alteration of host genes expression and reactivation of drugs by deconjugation of the phase II metabolite after enterohepatic circulation [11, 21]. Varying health-promoting compounds present

in the human diet such as polyphenols are characterized by a poor absorption in the small intestines, leading to extensive biotransformation of these xenobiotics by the gut microbiota [7, 17].

In vitro GIT models offer an alternative approach to *in vivo* studies to investigate the colonic biotransformation of xenobiotics as they allow multiple sampling overtime. *In vitro* models of the GIT can simulate the different parts of the digestive system by adjusting pH, temperature, enzymes and peristalsis. The gut flora is mimicked using a fecal suspension [22-24]. However, *in vitro* models are not fully representative of *in vivo* conditions. Therefore, it is important to optimize the pre-analytical phase to mimic the *in vivo* situation in order to obtain a high level of physiological significance [22, 25]. During this study, the gastrointestinal dialysis model with colon (GIDM-colon), developed and validated by Breynaert et al., was used to investigate the colonic biotransformation of the polyphenolic compound chlorogenic acid [26]. As chlorogenic acid is a polyphenol abundantly present in the human diet of which the gastrointestinal metabolism has already been extensively investigated by multiple studies, it was selected as model compound to optimize a ready-to-use setup to study the colonic metabolism of xenobiotics [26-34].

2. Materials and Methods

The colonic biotransformation of chlorogenic acid was investigated using the GIDM-colon [26]. A blank (no chlorogenic acid) and negative control (no fecal suspension) sample were included as controls in each experiment. The pH was set at 2, 7.5 and 5.8 for the gastric, small intestinal and colon-stage respectively, together with the appropriate enzymes. Temperature was kept at 37 °C and during the colon-stage, dialysis cells were placed in an anaerobic environment.

In order to further optimize the *in vitro* GIDM-colon system, a thorough investigation was conducted to determine effects on the bacterial composition of the fecal samples used to inoculate the colon system, aiming to minimize changes in bacterial composition between initial donation of fecal samples by individuals (meeting certain inclusion criteria) and final biotransformation experiments in the *in vitro* GIDM-colon system. Thus, the processing procedure of the fecal suspension, used during the colon-stage, was optimized. Two incubation media, Wilkins-Chalgren Anaerobe Broth (WCB) and a sterile phosphate buffer (0.1 M, pH 7) were investigated. The fecal slurry was incubated for 17 h in the media and samples were taken before and after incubation and analyzed for their bacterial composition by 16S rRNA gene sequencing. Furthermore, the effect of incubation time in the chosen medium on the bacterial concentration was also investigated. A pooled fecal slurry was incubated 24 h in the chosen medium and samples were taken every 2 h whereafter the anaerobic CFU/g was determined.

In view of optimal preparation of samples for accurate analysis of colon metabolites by liquid chromatography coupled to high-resolution mass spectrometry, the sample preparation procedure after GIDM-colon experiments was optimized. Four different sample preparation methods (SPP) on the samples received from the colon-stage were evaluated: all methods included the addition of methanol (MeOH) combined with centrifugation steps (14 000 rpm, 8 min), freeze-drying (reconstituted in MeOH, sonicated 45 min), sonication (60 min) or extraction by vortex mixing (60 s). Sample preparation methods were evaluated based on the number of identified biotransformation products with MS/MS fragmentation, and the time and effort needed for sample preparation. Samples were analyzed by liquid chromatography coupled to high-resolution accurate-mass mass spectrometry (LC-QTOF-MS), including both complementary suspect and non-targeted screening workflows.

3. Results and Conclusions

Phosphate buffer was chosen as preferred incubation medium for this study. The medium demonstrated a closer resemblance to the initial *in vivo* bacterial composition, while WCB was found to introduce substantial changes. Furthermore, the anaerobic bacterial concentration remained stable during 24 h of incubation in phosphate buffer. In future experiments, no incubation is needed prior to the GIDM-colon which will lead to a reduction in total time of the experiment and reduced risk of introduction of changes to the initial bacterial composition.

Results of the investigation of different SPP prior to LC-QTOF-MS analysis, clearly showed freeze-drying to be the inferior, as lower relative peak areas of the identified biotransformation products were produced. Centrifugation, sonication and extraction SPPs showed similar relative peak areas. Furthermore, only 53 % of the identified compounds showed MS/MS fragmentation by data dependent MS/MS using freeze-drying, while 63 % was reached using the centrifugation or extraction method and 69 % using the sonication method. Extraction was chosen as SPP for future experiments as a shorter time period is needed to obtain LC-suitable samples. In total, 23 colonic biotransformation products of chlorogenic acid were identified after digesting 78 mg chlorogenic acid in the optimized GIDM-colon system using liquid chromatography coupled to high resolution mass spectrometry.

This study contributed to the development of a ready-to-use platform that can be used to investigate the gastrointestinal fate of different xenobiotic compounds and/or to compare interindividual differences in colonic biotransformation between different populations (e.g. fecal samples of patient groups).

Conflicts of Interest: The authors declare no conflict of interest.

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Poster Presentations
(In alphabetical order of the first author)

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Abstract ***Poster 1***

Lipid Profiles of Human Breast Cancer Cell Membranes: Effects of Somatostatin †

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Abstract: Fatty acids in phospholipids are the major structural unit of biological membranes. There is no study in literature regarding the effects of somatostatin on the membrane lipid profile of human breast cancer cells. We investigated the fatty acid profile of the cell membranes of two human breast cancer cells incubated with or without somatostatin.

Keywords: somatostatin, fatty acids, breast cancer

1. Introduction

Polyunsaturated fatty acids (PUFA) are the major components of phospholipids, the principal structural unit of biological membranes. Unsaturated fatty acids have one or more double-bonds in a *cis* or *trans* configuration. The *cis* geometry of double bonds in lipids is a crucial structural feature for the functionality of cell membranes. *Trans* isomers are able to perturb both cell membrane arrangements and lipid enzymatic cascades. Endogenous *trans* fatty acid isomers are formed by the isomerization of fatty acids in cell membranes due to an endogenous free radical process. Cell membranes, which are structurally made up of large amounts of PUFA, are highly susceptible to oxidative attack, and consequent changes result in altered membrane fluidity, permeability, and cellular metabolic dysfunction [1-4].

Somatostatin (SST), also known as growth hormone–inhibiting hormone (GHIH) and by several other names, is a peptide hormone that regulates the endocrine system and affects neurotransmission and cell proliferation via interaction with G protein-coupled somatostatin receptors and inhibition of the release of numerous secondary hormones. The cellular actions of SST are mediated by five somatostatin receptor subtypes (termed SSTR). The direct antiproliferative effect of SST is by binding to seven transmembrane G-protein coupled receptors (GPCRs) namely somatostatin receptors 1–5 (SSTR1-5). This direct effect of SST is either cytostatic (cell cycle arrest) or cytotoxic (apoptosis). SSTRs which are widely distributed in a variety of tumors and cancer cell lines, including small cell lung cancer, neuroendocrine tumors, prostate cancer, breast cancer, colorectal carcinoma, gastric cancer and hepatocellular carcinoma [1,2]. There is no study in the literature investigating the effects of somatostatin on membrane lipid profile in human breast cancer cells. The purpose of our work was to investigate fatty acid profile in the cell membranes of different human breast cancer cell groups incubated with or without somatostatin.

2. Materials and Methods

The sensitivity of human breast cancer cells to somatostatin were determined using cytotoxic assays. Viability analysis were carried out in somatostatin incubated cells in order to determine the IC50 for the breast cancer cell lines. *In vitro* cultured cells were incubated with somatostatin up to 24 hr. The inhibition of cell proliferation was determined using the MTT assay.

Cells incubated with somatostatin were cultured at 5 × 10^5 cells/mL. The cells were washed from the medium and concentrated to at least 4 x 10^6 cells (counted) in PBS (1 mL). This amount was transferred to a 1.5 mL centrifuge microtube, to which pure water was added (0.5 mL), shaken and centrifuged in order to obtain the membrane pellet. Lipid extraction was executed on the pellet by adding chloroform:methanol (2:1). The membrane phospholipid isolation and purity were checked by TLC using appropriate elution systems.

The membrane fatty acid profile analysis in control and other samples was performed using gas chromatography (GC) analysis with external reference standards. Lipid extraction and *trans*-esterification were performed to obtain corresponding fatty acid methyl esters.

3. Results

Incubation of the cells with somatostatin caused significant changes in membrane total ω-3 fatty acid levels in MCF7 cells compared to the control cells ($p < 0.05$). There was no significant difference in other fatty acid profiles in MCF7 cells incubated with somatostatin compared to the control MCF7 cells ($p > 0.05$), Table 1.

Table 1. Fatty acid profile of the MCF7 breast cancer cells incubated with or without somatostatin (SST).

MCF7	CONTROL	SST
	Average±SD	Average±SD
Total SFA	39,70±2,55	38,85±3,10
Total MUFA	40,53±1,00	42,13±2,11
Total PUFA	19,77±3,18	19,02±1,00
Total ω6 FA	12,63±3,45	10,81±0,82
Total ω3 FA	6,86±1,41	7,85±0,17*
Total TRANS FA	0,77±0,31	0,52±0,15

Incubation with somatostatin did not cause any change in the fatty acid profile in the MDA-MB231 cells compared to the same cells not incubated with somatostatin (Table 2).

Table 2. Fatty acid profile of the MDA-MB231 breast cancer cells incubated with or without somatostatin (SST).

MDA-MB231	CONTROL	SST
	Average±SD	Average±SD
Total SFA	39,29±5,42	40,06±2,11
Total MUFA	33,06±0,90	33,29±0,67
Total PUFA	27,65±4,80	26,65±2,73
Total ω6 FA	14,62±2,46	14,42±1,39
Total ω3 FA	12,93±2,56	12,02±1,69
Total TRANS-FA	0,60±0,36	0,80±0,26

4. Discussion

Omega-3 fatty acids constituting the major fatty acids of cell membranes have important roles in membrane structure and functions. They have many powerful health benefits for the whole body. Omega-3 fatty acids reduce the production and release of some substances during inflammatory response. Cancer is one of the leading causes of death in the Western world, and omega-3 fatty acids have long been claimed to reduce the risk of certain cancers. It was reported that people who consume omega-3 fatty acids in large amounts have up to a 55% lower risk of colon cancer. It was found that omega-3 consumption is linked to a reduced risk of prostate cancer in men and breast cancer in women. However, there are conflicting results in the literature, demonstrating that not all studies give the same results. The hormone somatostatin exerts antiproliferative, antiangiogenetic, proapoptotic, anti-nociceptive and other effects through binding to its receptors. The data of our study confirmed the protective effect of somatostatin, raising the omega-3 fatty acids in MCF7 breast cancer cells, but not in the MDA-MB231 breast cancer cells. Based on these preliminary results, we will perform further studies incubating both breast cancer cells with somatostatin for longer periods to determine the time-dependent effects of somatostatin.

Author Contributions: conceptualization, C.F., T.O. and C.C.; methodology, A.H., E.K. and F.H.; data analysis, A.H., C.F. and E.K.; writing A.H. and T.O.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract ***Poster 2***

Role of a polyphenol-rich dietary pattern in the modulation of intestinal permeability in older subjects: The MaPLE study†

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Keywords: intestinal permeability; zonulin; inflammation; microbiota; polyphenols; older subjects

1. Introduction

The inevitable rise of the proportion of people aged > 65 years worldwide is paralleled by an increased burden of chronic diseases often associated with low-grade systemic inflammation. Recent findings suggest a link between inflammation and intestinal permeability (IP), a condition characterized by an impairment of intestinal barrier function which enables the translocation of dietary and bacterial factors into the blood activating the host immune system [1-2]. Dietary components can be significant modulators of inflammation and IP, and can also affect the intestinal microbial ecosystem. In the context of a diet-microbiota-IP axis in older subjects, dietary bioactives such as polyphenols may play a significant protective role due to their widely reported antioxidant and immunomodulatory properties and potential to regulate IP [3-6].

2. Material and methods

The MaPLE project involves a multidisciplinary approach developed to ascertain the impact of a polyphenol-rich dietary pattern on a large number of markers in a target group of older subjects living in a controlled setting (i.e. nursing home).

A controlled, randomized cross-over dietary intervention study (8-week polyphenol-rich diet *versus* 8-week control diet) was undertaken. Markers of IP, inflammation, oxidative stress and vascular function and assessments of gut microbiota structure and function were quantified in serum, urine and/or fecal samples. In addition, bacterial DNAemia, and serum/urine metabolomics were assessed. In vivo with a dietary mixture similar to the human study and in vitro studies with isolated polyphenols were carried out to investigate mechanisms of action.

3. Results & discussion

The dietary intervention has been completed and as expected, IP was relatively high in this cohort of older participants, as assessed by serum levels of zonulin at baseline. Quantification of changes in various markers in response to the high polyphenol diet compared to the normal polyphenol diet are being completed and will provide evidence of the putative beneficial effect of increased polyphenol consumption in this target population.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results".

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Abstract ***Poster 3***

Effect of Bioavailable Whey Peptides on C2C12 Muscle Cells †

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Abstract: Whey proteins are highly valued food ingredients. This study examines the health benefits to muscle cells of six individual whey peptides known to cross the intestinal barrier. Results indicate that peptides KVPQ, NLPPL, VAGT, VGIN and PVPQ protect undifferentiated C2C12 myoblasts from free radical damage.

Keywords: whey peptides, bioavailable, free radicals, C2C12 cell line

1. Introduction

By 2050, 22% of the global population approximately 2 billion people will be over 60. Frailty is a common aliment of the elderly and a large proportion of this cohort have considerable loss of sketelal muscle mass and function, impairing their mobility. Muscle loss can be reversed or delayed by a combination of dietary protein and exercise [1]. Bovine whey proteins (β-Lactoglobulin, α-Lacalbumin, Bovine Serum Albumin (BSA) and Lactoferrin) are high quality proteins that contain all essential amino acids, are rich in branched chain amino acids, are noted for their bioactive peptides and are readily digested [2]. Branched chain amino acids play an essential role in muscle cells not least of which includes metabolism, protein synthesis, mitochondrial biogenesis and redox balance [3]. There is a plethora of evidence from intervention trials that consumption of whey has positive benefits on muscle [4]. Recent studies in our laboratory have identified 31 peptides from whey capable of surviving the hydrolytic conditions of the upper gastrointestinal tract and crossing the intestinal barrier *in vitro*. Of these, 6 peptides (TKIPA, NLPPL, PVPQ, VGIN, VAGT and KVPQ) were selected for cellular assays with the murine myoblast cell line, C2C12. The objective of this study was to investigate the effects of these 6 bioavailable whey peptides on cell growth and protection of the murine myoblast cell line, C2C12 from free radical damage.

2. Materials and Methods

2.1 Materials

Chemicals were purchased from Sigma-Aldrich (Ireland) unless otherwise specified. The peptides TKIPA, NLPPL, PVPQ, VGIN, VAGT and KVPQ were synthesized and purified by the method previously described [5]. The murine myoblast cell line C2C12 (ATCC CRL-1772™) was sourced from the American Type Culture Collection (ATCC, USA).

2.2 Cell Lines

C2C12 cells were grown to 80% confluency in a 75 cm^2 tissue culture flask with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin at 37ºC with 5% CO_2. Experiments were performed at passage number 8-17.

2.3 Alamar Blue Assay

C2C12 were seeded at 8 x 10^4 cells/well in 96 well plates in DMEM supplemented with 10% FBS and 1% antibiotics. Cells were incubated for 24 hr. Cells were then washed twice with phosphate buffered saline (PBS) and treated with 100 µL of each peptide (final concentration 2.5 mM or 5 mM). Cells were then incubated for a further 24 hr. Alamar Blue reagent (10 µL) was added. The plate was incubated for 4 hr at 37°C. Absorbance was read at 570 nm and 600 nm. Fluorescence was registered with excitation wavelength of 560 nm and emission at 590 nm. Controls included untreated cells with media alone and cells treated with leucine (final concentration 2.5 mM and 5 mM).

2.4 Cellular Antioxidant Activity

C2C12 were seeded at 8 x 10^4 cells/well in 96 well plates in DMEM supplemented with 10% FBS and 1% antibiotics. Cells were incubated for 24 hr. The cells were then washed twice with PBS. Cells were treated with 50 µL of each peptide (final concentration 2.5 mM or 5 mM, reconstituted in Hanks Balanced Salt Solution (HBSS)) and 2′,7′-dichlorofluorescin di-acetate (DCFH-DA) (50 µL, final concentration 25 µM) for 1 hr. Cells were washed twice with PBS. 2,2′-azobis (2-methylpropionamidine) dihydrochloride (ABAP) (100 µl final concentration 600 µM) was added. The plate was immediately placed at 37°C, in a Synergy HT BioTek micro-plate reader. Fluorescence was read every minute for 1 hr with excitation at 485 nm and emission at 535 nm. Cells treated with HBSS; and DCFH-DA (untreated) acted as the negative control. Another control included cells treated with free radical in the absence of peptides (Radical). N-acetylcysteine (NAC) (2.5 mM and 5 mM final concentration) acted as the positive control.

2.5 Statistical Analysis

Cellular assays were performed in triplicate on 2 different days. One way ANOVA using a Bonferroni's comparison test was used to compare results using SigmaPlot software. P value <0.5 determined statistical significance. Results were expressed as mean ± standard deviation.

3. Results and Discussion

3.2. Alamar Blue

Six whey peptides present in the basolateral side of differentiated CacCo2-HT-29 co-cultures after exposure to simulated gastrointestinal digestion whey protein isolate were selected for further investigation. Peptides TKIPA and VAGT were derived from β-Lactoglobulin, VGIN from α-Lactalbumin, NLPPL and KVPQ from BSA and PVPQ from β-casein. Each of the 6 peptides contained at least one branched chain amino acid. To investigate if these whey peptides promoted cell growth in muscle cells, undifferentiated C2C12 myoblasts were exposed to synthesized peptides at 2.5 mM and 5 mM concentration for 24 hr and Alamar Blue test performed (Figure 1). Alamar Blue functions as a health indicator by using the reducing capacity of living cells to quantitatively measure cell proliferation. Only peptide VAGT (5 mM) significantly ($p<0.05$) increased the growth of undifferentiated C2C12 compared to leucine at 2.5 mM but not at 5 mM. Peptides TKIPA (2.5 mM), NLPPL (2.5 mM and 5 mM), VGIN (2.5 mM) and KVPQ (5 mM) retarded growth compared to leucine at 2.5 mM and 5 mM ($p<0.05$).

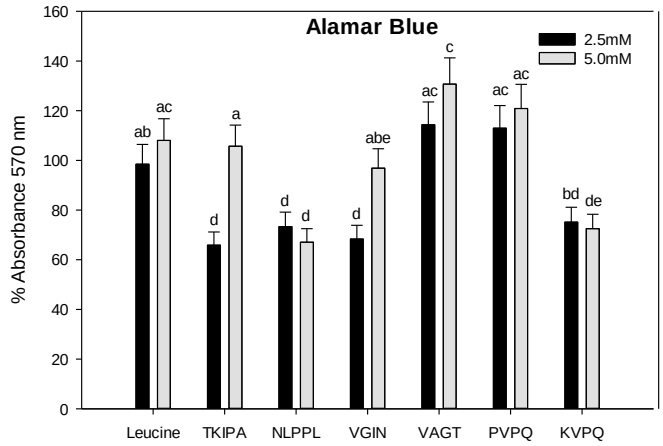

Figure 1. Cell viability of C2C12 (8 x 10^4 cells/well) treated with synthesized peptides for 24 hr, measured with the Alamar Blue assay. Experiments were performed in triplicate. Different letters indicate significant difference ($p<0.05$).

3.3. Protection against free radicals

To investigate if the peptides protected undifferentiated C2C12 myoblasts from free radicals, free radical ABAP was added to C2C12 pretreated with synthesized peptides (Figure 2). Data indicates that the peptides KVPQ (5 mM), NLPPL (5 mM), VAGT (5 mM), VGIN (5 mM) and PVPQ (5 mM) were as effective as N-acetylcysteine (5 mM) at inhibiting the free radical in C2C12 cells and significantly protective compared to radical control ($p<0.05$). In contrast, peptide TKIPA (2.5 mM or 5 mM) did not provide a protective effect.

Results indicate that 5 of the 6 peptides protect C2C12 from free radicals. In agreement, other studies have demonstrated that peptides or hydrolysates from whey have positive effects on redox balance [6,7]. However it is important to note that there are some limitations to our study namely, the peptide concentrations used are not physiological, C2C12 cells are not differentiated nor do they represent an ageing muscle. It would therefore be prudent to repeat these assays with a range of antioxidant markers (GSH, catalase), a panel of cell metabolic biomarkers and using differentiated and ageing C2C12 cells.

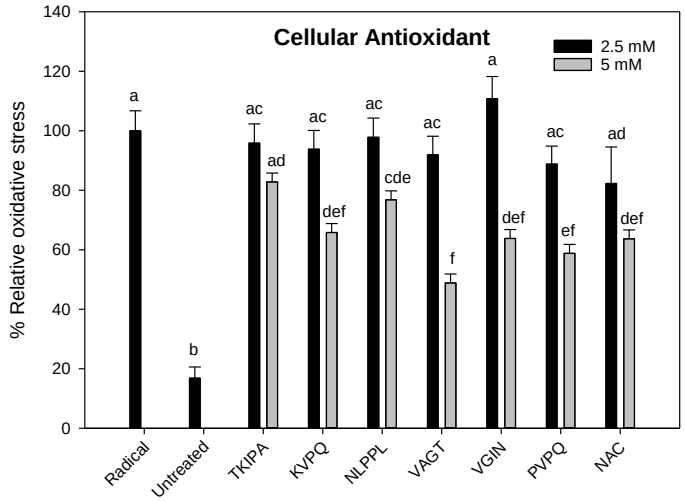

Figure 2. Relative oxidative stress in C2C12 (8 x 10^4 cells/well) after 1 hr exposure to synthesized peptides. Radical = cells treated with DMEM + DCFH-DA + ABAP, Untreated = cells treated with DMEM + DCFH-DA, NAC = cells treated with DMEM + N-acetylcysteine + DCFH-DA + ABAP. Experiments were performed in triplicate. Different letters indicate significant difference ($p<0.05$).

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Abstract ***Poster 4***

Phytochemical Combinations Modulate the Activation of Nrf2 and Expression of SOD in Pancreatic Cancer Cells more efficiently than Single Plant Components †

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Abstract: Pancreatic adenocarcinoma mainly occurs in elderly people. Thus, the management of pancreatic cancer in the aging population is becoming increasingly relevant. In this preliminary study we evaluated the effect of selected phytochemicals and their combinations on the expression and activation of Nrf2 transcription factor in the human pancreatic cancer cell line MIA-Pa-Ca-2. Treatment for 24 hr with xanthohumol (X), resveratrol (RES), indole-3-carbinol (I3C) or phenethyl isothiocyanate (PEITC) had no effect on the expression and activation of Nrf2, or the expression of the *SOD* gene controlled by Nrf2. However, combinations of these phytochemicals significantly increased Nrf2 activation and subsequently the expression of *SOD*. The most efficient were the mixtures of resveratrol and glucosinolates degradation products, I3C and PEITC**.** These results indicate that combinations of phytochemicals resembling that occurring in natural diets may efficiently modulate the signaling pathways, whose proper function is important for pancreatic cancer prophylaxis or improving the results of conventional therapy.

Keywords: Nrf2; SOD; phytochemicals; pancreatic cancer; elderly people

1. Introduction

Pancreatic cancers possess the worst prognosis, having one of the highest mortality rates [1]. Advancing age is a high risk factor for this type of cancer, and more than 60% of new cases and over 70% of cancer mortalities occur in elderly people [2]. Thus, searching for an alternative approach for both prevention and therapy of these tumors is therefore necessary. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor involved in the regulation of expression of antioxidative enzymes such as superoxide dismutase (SOD) [3]. Our previous studies have shown that naturally occurring compounds from chalcones group (xanthohumol) [4], stilbenes (resveratrol) [5], products of glucosinolates degradation (indole-3-carbinole, phenethyl isothiocyanate) [6] have the ability to modulate expression of *Nrf2* and *SOD* in HepG2 and HaCaT cells. Recent studies indicate that using combinations of naturally occurring chemopreventive agents, instead of using the individual phytochemical is a more efficient strategy of cancer chemoprevention [7]. The aim of this study was to evaluate the effect of combinations of the different classes of phytochemicals on the Nrf2 activation and expression of *SOD* in human pancreatic carcinoma cells.

2. Materials and methods

2.1. Cell culture and treatment

Human pancreatic cancer cells (MIA-Pa-Ca-2, ATCC, USA) were cultured in DMEM containing 10% fetal bovine serum, 1% antibiotic solution in a humidified atmosphere of 5% CO_2 at 37°C. Cells were treated with 1

μM phenethyl isothiocyanate (PEITC), indole-3-carbinol (I3C), resveratrol (RES), xanthohumol (X) and their combination for 24 hr.

2.2. *Preparation of RNA, cytosolic and nuclear fraction*

Extraction of total RNA from cells was performed with the GeneMatrix Universal DNA/RNA/Protein Purification Kit (EurX, Poland) according to the manufacturer's instructions. The cytosol and nuclear extracts from the MIA-Pa-Ca-2 cell line were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, USA).

2.3. *Real-time PCR analysis*

For cDNA synthesis RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA) was used. The real-time PCR assay was performed using Maxima SYBR Green qPCR Master Mix (Fermentas, USA) with specific primers for each gene. The relative change in the expression of *Nrf2* and *SOD* was calculated using the Pfaffl method. *PBGD* and *TBP* served as reference genes.

2.4. *Western Blot analysis*

Nuclear and cytosolic fractions (100 μg of protein) were separated on 10% and 12% SDS-PAGE and transferred to nitrocellulose membrane Immobilon P (Millipore). The membranes were then incubated with primary antibodies against Nrf2, SOD (Santa Cruz Biotechnology, USA) and secondary antibody with the alkaline phosphatase-labeled IgG. The amount of immunoreactive product in each lane was determined by scanning and evaluating with the Quantity One programme (BioRad, USA). Values were expressed as relative quantity (RQ) per mg of protein.

2.5. *Nrf2 activation Elisa assay*

Nrf2 activation was assessed by an enzymatic immunoassay using commercial kits (Transcription Factor ELISA Assay Kit Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. The nuclear fraction was incubated in the oligonucleotide-coated wells with oligonucleotide sequence which contain the ARE consensus binding site (5'-GTCACAGTGACTCAGCAGAATCTG-3'). Wells were then washed and incubated with the antibody against *Nrf2*. Subsequent addition of an HRP-conjugated secondary antibody allowed the colorimetric readout of the conjugate at 450 nm.

2.6. *Statistical analysis*

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's post hoc test, with $p<0.05$.

3. Results

3.1. *The effect of single phytochemicals and their combinations on the expression of Nrf2*

Quantitative analysis (Figure 1A) of *Nrf2* transcript and nuclear protein levels showed that phytochemicals alone did not affect the expression of *Nrf2* in MIA-Pa-Ca-2 cells. However, the treatment with the combinations of I3C and RES increased the amount of *Nrf2* transcript and nuclear protein by 34% (Figure 1AB). Moreover, this combination as well as the combination of RES and PEITC and to less extent X with PEITC significantly (by 80-53%, respectively) enhanced the Nrf2 activation measured in terms of the amount of *Nrf2* contained in DNA binding complex. Nrf2 consensus site-ARE was immobilized on the ELISA microplates as bait (Figure 1C).

3.2. *The effect of phytochemicals and their combinations on the expression of SOD*

The increased activation of Nrf2 as result of treatment with I3C and RES and two other combinations (RES+PEITC; X+PEITC) led to enhanced expression of the *SOD* gene. The increased expression was confirmed both on mRNA transcript and at protein level (Figure 1 D,E).

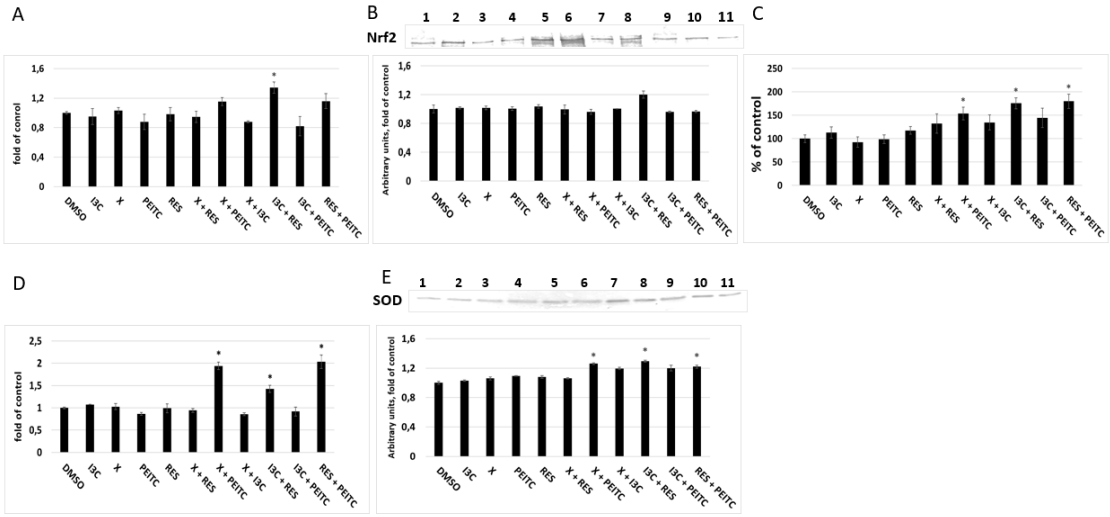

Figure 1. The effect of phytochemicals and their combinations on *Nrf2* and *SOD* expression in MIA-Pa-Ca-2 cells. (A) *Nrf2* transcript levels. The values were calculated as mRNA level in comparison with control cells (expression equals 1). (B) Representative immunoblots for the analysis of the nuclear level of Nrf2 protein. Lane 1- control; lane 2 – I3C 1μM; lane 3 – X 1μM; lane 4 – PEITC 1μM, lane 5 – RES 1μM; lane 6 – X + RES 1μM; lane 7 – X + PEITC 1μM; lane 8 – X + I3C 1μM; lane 9 – I3C + RES 1μM; lane 10 – I3C + PEITC 1 μM; lane 11 – RES + PEITC 1 μM. Results of Western blot analysis of the nuclear content of the Nrf2 protein. The values were calculated as protein level in comparison with control cells (expression equals 1). (C) Binding of Nrf2 to ARE–containing oligonucleotide, the values were calculated and compared with control level, equals 100%. (D) *SOD* transcript levels. (E) Representative immunoblots for the analysis of SOD protein. The data are presented as mean ± SEM. The asterisk (*) above the bar denotes statistically significant differences to the control group, $p<0.05$.

4. Discussion

Nrf2 regulates the expression of many antioxidant enzymes including SOD, which catalyze the dismutation of superoxide anion free radical into molecular oxygen and hydrogen peroxide. The enzyme can be considered as an anti-inflammatory agent which also prevents precancerous cell changes. SOD levels drop with aging and thus elderly people become more prone to oxidative stress-related diseases including cancer [8]. The increased expression of *SOD* found in our study as a result of treatment with phytochemicals, common diet ingredient combinations, suggest their cancer chemopreventive potential. On the other hand, in pancreatic cancer, the model that was used in this study, the increased expression of *SOD* as result of Nrf2 activation may protect against side effects of drugs such as anthracyclines, often used in pancreatic cancer treatment [9].

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract ***Poster 5***

A three-step approach to estimation of reduction potentials of natural mixtures of antioxidants based on DPPH test; illustration for catechins and cocoa

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Abstract: The aim of this study is to propose a methodology to assess electrochemical properties of complex mixtures of antioxidants, such as plant extracts, based on the results of simple and popular DPPH test. The first, most difficult step, involves determinations of standard reduction potentials (E^0) for the series of purified compounds (here catechins). The next step is the calculation of stoichiometric values (n_{10}) based on the result of DPPH test for the same compounds. Finally, a correlation equation is formulated, which is then employed to estimate "cumulative reduction potential" for the mixture of interest (here cocoa) using DPPH test results.

Keywords: standard reduction potential, stoichiometry value, DPPH test, antioxidants

1. Introduction

The enhancement of endogenous antioxidant defense system through dietary supplementation with antioxidants, such as bioactive phenolic phytochemicals, has been widely accepted and advocated as a reasonable approach to reduce the level of oxidative stress and the risk of associated diseases. Unfortunately, despite the large number of publications about antioxidants, there is still no consensus which parameters should be used as a guidance enabling reliable predictions of the impact of redox active substances on cellular redox homeostasis. It may be presumed that electrochemical properties, i.e. standard reduction potential, of a given substance could be a good predictor of this substance's ability to modulate antioxidant status of the cell. Our previous research for a series of catechins [1] supported this hypothesis. However, the precise determination of this physicochemical parameter is difficult even for pure compounds, not to mention complex mixtures, such as antioxidant rich foods.

In this study, we propose the methodology that enables estimation of E^0-like value for complex mixtures based on results of DPPH test. Initially, electrochemical properties, i.e. standard reduction potentials (E^0), were determined which express the ability of a compound to accept electrons. In the next stage, antioxidant activity of the same set of compounds was evaluated using an easy to perform popular spectrophotometric assay, employing DPPH radicals. However, the results of DPPH test were calculated so as to include kinetic aspects of the redox reaction. The created relationship was then used to assess electrochemical properties of cocoa as a model actual food product rich in antioxidants.

2. Materials and methods

2.1. Chemicals and reagents

The following redox active compounds were used for the study: (+)-catechin (C), (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG) propyl gallate (PG)

from Extrasynthese (France), L-ascorbic acid (AA), isoascorbic acid (iAA), sodium ascorbate (NaAA), calcium ascorbate (CaAA) as well as glutathione (GSH) from Sigma-Aldrich (USA). Solution of potassium hexacyanoferrate (III) from Sigma-Aldrich (USA) was applied as a titrant in potentiometric titration. For spectrophotometric tests, 1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma-Aldrich (USA), analytical grade ethanol and methanol from POCH (Poland) were used. Tablets of phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (USA). PBS solution was prepared by dissolving one tablet in 200 mL ultrapure water from a Millipore Milli-Q system.

2.2. Standard reduction potential by potentiometric titration

The determination of standard reduction potentials was performed by potentiometric titration standard as described previously [1]. Briefly, potassium hexacyanoferrate (III) was selected as a titrant. Analysed antioxidants (1 mg/mL) and the titrant were dissolved in PBS (pH = 7.4). Potentiometric titration was carried out vs. 3 M KCl Ag|AgCl reference electrode and a platinum measuring electrode at 25, 37 and 41°C using JENCO 6230N, ORP-146C Micro Oxidation-Reduction equipment (USA). Temperature during measurement was maintained by Ultra Thermostat (PolyScience, USA), while the temperature of the reaction was controlled in the range ± 0.1°C using a JENCO 6230-AST thermometer. Mixing of the reactants was ensured by bubbling inert, high purity N_2 gas. The titrant was added to the antioxidant solution in increments of 0.5 mL and potential was read after stabilisation. Each potentiometric titration was performed in three independent repeats. Obtained titration curves (E [mV] vs. $V_{titrant}$ [mL]) were analysed by non-linear regression (Marquardt-Levenberg algorithm) using SigmaPlot (Systat Software Inc., UK) software. Obtained reduction potentials of tested compounds vs. standard hydrogen electrode (SHE) were calculated.

2.3. Antioxidant activity by spectrophotometic method

The colorimetric determination of antioxidant activity was performed by standard method employing DPPH radical as described previously [1]. In short, stock solution of radical was diluted in methanol before measurement until absorbance reached 0.9 ± 0.05 at 515 nm. All reactions were carried out in 48-well plates at 25, 37 and 41 °C. Stock solutions of antioxidants were prepared in analytical grade ethanol at a concentration of 10 mM. Stock solutions of antioxidants were diluted appropriately with the same solvents to achieve concentrations falling within a linear range of the assay. The DPPH solution (1 mL) was mixed with solutions of antioxidants (30 µL) and the absorbance was measured at 515 nm after 10 min. The absorbance measurements were performed with the use of a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland).

The results of antioxidant activity evaluations obtained by DPPH test were expressed as stoichiometry values (n) using modified method [2]. The stoichiometry value was determined as a regression coefficient, and defined as the slope of concentrations of a radical scavenged vs. concentrations of the tested antioxidant present in the mixture after 10 min of reaction (n_{10}). The concentration of radicals scavenged by the tested compound in reaction media was calculated with the use of the Beer–Lambert–Bouguer law (Beer's law) according to the equation:

$$S_R = \frac{(A_0 - A_f)}{\varepsilon \times l}$$

where S_R - the concentration of scavenged radicals [M]; A_0 – the initial absorbance of the radical solution; A_f – the absorbance of the radical solution after the reaction time 10 min; ε is the molar extinction coefficient (11240 $M^{-1}cm^{-1}$ for DPPH radical at 515 nm [3]), l – optical path length (1 cm).

2.4. Statistical analysis.

All values are expressed as means ± SD of three independent experiments. Correlations between values of standard reduction potentials and antioxidant activity determined by DPPH test were examined using Pearson's coefficients. All statistical analyses were performed using Prism 4.0 software package (GraphPad Software, Inc., USA).

3. Results

The stoichiometry values n_{10} may be regarded as chemical indicators of antioxidant activity. Thus, the higher the n_{10} value is determined for a compound, the better antioxidant properties it exhibits. In turn, E^0 describes the ability of a compound to accept electrons. The lower the value of the standard reduction potential of a compound, the better an electron donor it is, which means that the compound exhibits stronger antioxidant properties. The results of antioxidant activity determinations for 9 redox active compounds expressed as either standard reduction potentials E^0 or stoichiometry values n_{10} were used to formulate the relationship described by the function $n_{10} = f(E^0)$ as shown in Fig. 1 (Panel 1 and 2) using epicatechin as an example and measurement temperature of 37°C. The two sets of values turned out to have strong inverse correlation for all temperatures tested (r=-0.894 for 25°C, r=-0.889 for 37°C, r=-0.858 for 41°C). Based on experimental data determined at 37°C, the following function equation was calculated: $n_{10} = -19.71x + 7.34$, where x denotes E^0 equivalents.

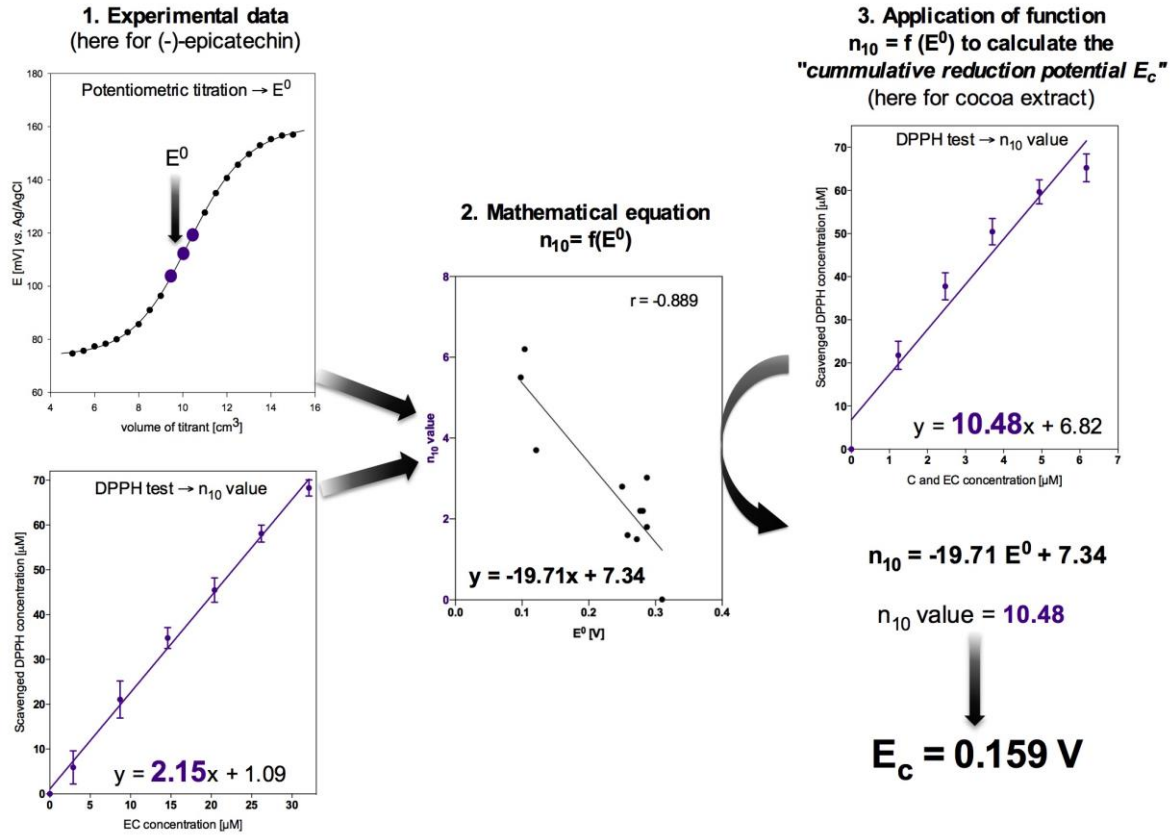

Figure 1. The three-step approach to estimate "cumulative reduction potential" of complex mixtures of antioxidants illustrated for cocoa extract.

In a separate experiment carried out under the same, corresponding to physiological, conditions (37°C, pH = 7.4), the n_{10} value was determined for cocoa extract by DPPH test (Fig. 1, Panel 3). This value was then substituted into $n_{10} = f(E^0)$ function and the "cumulative reduction potential" E_c was calculated for cocoa extract sample. The calculated E_c value of 0.159 V, falls between E^0 values determined for EGCG (0.104 V) and EC (0.277 V), two catechins that are present in cacao. The cellular antioxidant activity measured by CAA assay for cacao extract also exhibited intermediate potency when compared with these two catechins, which confirmed the expected results of the proposed approach.

4. Discussion

Standard reduction potential of redox active compounds has been shown to be a helpful, chemically defined, unambiguous predictor of their impact on a number of biological activities in a cellular model, and thus probably also *in vivo* [1]. However, E^0 values cannot be easily determined for mixtures of redox active chemicals such as food products and components containing e.g. antioxidant phytochemicals. Thus, the growing interest in dietary antioxidants requires the fast, cheap, reliable and accessible in every laboratory parameter to assess redox properties of foodstuffs. Stoichiometry value n_{10} determined by DPPH test meets these requirements to some extent. This parameter seems to be a reliable predictor of antioxidant activity being also in line with electrochemical properties, evaluated using potentiometric titration. Its determination is less related to the sample concentration used. Moreover, when the measurements are made after fixed time, also kinetic aspects of redox reaction(s) in which the studied sample is implicated are taken into account. Still, standard redox potential E^0 appears even better parameter, because it directly characterizes the redox active compound. Therefore, we have made an attempt to estimate the latter for natural complex mixtures based on simple measurements performed by DPPH test. Although, theoretically any assay measuring antioxidant activity of samples of interest may be applied, our previous investigations performed for the sets of catechins demonstrated the best correlation between E^0 and n_{10} values determined with the aid of DPPH radical [1].

We propose three-step approach explained in detail in Fig. 1, in which pure redox active compounds are used to provide experimental data (step 1) needed to generate the mathematical formula describing the relationship between E^0 and n_{10} (step 2). In this paper, the linear approximation was applied, however once more data for a larger number of compounds is available, the function $n_{10} = f(E^0)$ may become more complicated. This function equation, when published may be generally used by anybody interested. Finally, in the step 3, the n_{10} value is determined for any sample of interest with the use of strictly defined uniform DPPH test protocol and based on this determination, cumulative reduction potential E_c calculated from the $n_{10} = f(E^0)$ function equation. We named this parameter "cumulative", because it reflects all the redox interactions taking place in natural plant sample containing a mixture of redox active ingredients characterized by divergent electrochemical properties and present in various concentrations.

Author Contributions: conceptualization, B. K. and A. B.; methodology, K. S., B. K. and W. C.; investigation, M. B. and K. S.; writing, M. B. and A. B., funding acquisition, J. N.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract ***Poster 6***

Using Nanoparticles as Gene Carrier (vectors) in Cell†

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† Presented at Natural Products and the Hallmarks of Chronic Diseases – COST Action 16112, Luxemburg 25-27 March 2019

There are many complicated pathogenic diseases caused by cell genes that are incorrectly expressed or not sufficiently active for normal cell functioning. It is clear that the fight against such diseases is extremely difficult. Gene vectors (e.g. plasmids, viruses) are able to move healthy genes into cells and "sew" them into the host DNA. Such designed technologies are hard and expensive, that is why we believe that nanoparticles can be used as gene vectors. The advantage lies in the fact that they are easier to prepare and cheap to produce. In the presented work, two different types of nanoparticles are proposed as gene vectors poly lactic co-glycolic acid (PLGA) and (poly-amidoamine) dendrimers (PAMAM). PLGA and PAMAM nanoparticles were prepared to create complexes with DNA. It should be noted that both types of nanoparticles are able to penetrate cell membranes.

We conducted experiments with complex DNA-PLGA nanoparticles using various physical methods. The purpose of these studies was to determine the structure and stability of the complex of particles in terms of time and temperature. At the outset, tt was important to identify the length of DNA molecule or gene which is placed inside the nanoparticle complex. We used the calf timuse DNA (SERVA) and two different types of PLGA nanoparticles (diameter both particles were d = 150 nm) with different values of surface potentials (negative and positively charged).

Using ZetaSizer (Malvern) method, we established that the PLGA particles have a negative surface potential, while the PLGA nanoparticles coating with chitosan had a positive surface potential. It is clear that the negative charge of DNA in an aqueous solution is due to the phosphoric acid. We mixed the DNA and PLGA with various ratios of DNA:PLGA to obtain an effective correlation of complex. To determine the DNA:PLGA ratio, we used high-speed centrifugal and spectrophotometric methods. The study indicated that there was no possibility to obtain complexes between the negative surface of the PLGA nanoparticles and the negatively charged DNA. Complexes do form between the PLGA with chitosan and DNA. According to the study, we determined the effective DNA:PLGA ratio to be 7:1(W/W). This result can be used to create a complex of PLGA (with chitosan) and DNA, that can be used in practical purposes.

We have a number of recommendations for the preparation of dendrimer solutions for their practical and safe usage as gene delivery systems. DSC calorimeter has also been employed to study the thermodynamic properties of DNA/PAMAM G4 dendrimer complexes. We showed that up to a DNA:dendrimer ratio of 43: 3 (w/w) the solution was homogeneous, with stable aggregates formed at higher PAMAM G4 content. We note that the diameter of PAMAM G4 dendrimers compared to the PLGA (d=150nm) is very small and is 4.5nm. DSC experiments performed with homogeneous solution of dendriplexes revealed the existence of the pH-dependent melting curves that contain several endothermic peaks associated with melting of GC-rich regions. In this study we created a model of the complex of DNA and PAMAM G4 dendrimers, which make it possible to determine the amount of DNA and dendrimer that can be used for practical purposes.

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Abstract ***Poster 7***

Effect of Nigella sativa oil in a rat model of adjuvant-induced arthritis †

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Rheumatoid arthritis is characterized by chronic progressive inflammation and damage at joints leading to disability in about 250 million people between 30 and 60 years of age.

Here we studied the capacity of *Nigella sativa* oil cultivar, produced in the Marche region (Italy), to slow the onset/progression of disease in an adjuvant-induced arthritis animal model [1]. *Nigella sativa* oil was chosen for its anti-inflammatory and antioxidant properties previously measured in a human *in vitro* model of low–grade inflammation [2]. *In vitro* data showed antioxidant and anti-inflammatory properties of this oil; in particular, IL-1 beta and IL6 levels were decreased by *Nigella sativa* oil [2].

The *in vivo* indicated that 25 days of treatment with *Nigella sativa* oil can reduce the edema of inoculated and contralateral paws in the animal model of arthritis. Anti-hyperalgesic and anti-allodinic actions of *Nigella sativa* were observed during the anti-inflammatory process. Arthritic scoring was improved only in the positive control treated with indomethacin.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract ***Poster 8***

Oxidative stress applied in *Diabetes Mellitus*-A new paradigm†

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Abstract: Although obesity and a sedentary lifestyle are well-known risk factors for type 2 diabetes, at molecular level, the oxidative stress is regarded as the primary contributor to the pathogenic process. Our work intends to evidence how the thinking models influence the way that medical practitioners understand the pathogenic mechanisms. Some research groups focused lately on the system's dynamics and Complex Systems Theory. The living organisms as a complex system could be analyzed applying the network concept. Alternative methods for characterizing biological processes or phenomena based on fractal methods and algebraic fractals, from mathematical theory and Euclidian space, have been developed [1].

Keywords: oxidative stress, antioxidants, complexity theory, algebraic fractals, diabetes mellitus, public health.

1. Introduction

During last decade, many research groups proved that the oxygen species generate adverse effects [2, 3] due to the imbalance between the production of ROS (reactive oxygen species) and the body's biological capacity to neutralize them (i.e. enzymes) [4, 5]. In the meantime, the evolution of some significant diabetes complications, such as cardiovascular or renal disease, is related to the oxygen species as the primary pathogenic mechanism.

2. Discussion

Almost all of the chronic diseases have been associated with a high quantity of free radicals, or modified signal transductions. In dedicated medical research, two approaches could apply from nutritional interventions point of view: the situation when it is used to prevent a particular disease or, when it is able to alleviate progression, symptoms or complications of the disease. In addition, the considered specific diseases are divided as follows: the group that involves the so-called mitochondrial oxidative stress conditions (cancer and diabetes) and the group that involves the inflammatory or oxidative conditions (atherosclerosis, chronic inflammation, ischemia and reperfusion injury) [6].

Hyperglycemia in diabetes mellitus is one of the main factors leading to specific structural changes, as

protein and lipid oxidation, which are the most common (Figure 1). For instance, the free radicals induce damage to sulfhydryl groups. As a consequence, the proteins are not recognized anymore, resulting in cross-reactions and finally triggering the autoimmune diseases.

In the meantime, abnormal LDL produced by the peroxidation of plasmatic lipids is not identified by liver's LDL receptors and subsequently, macrophage scavenger receptors take modified LDLs, forming engorged lipid macrophages (LEM), and infiltrate under blood vessel endothelium. It should be also considered that the lipid peroxidation mechanism is governed by the loss of membrane functionality and integrity [7].

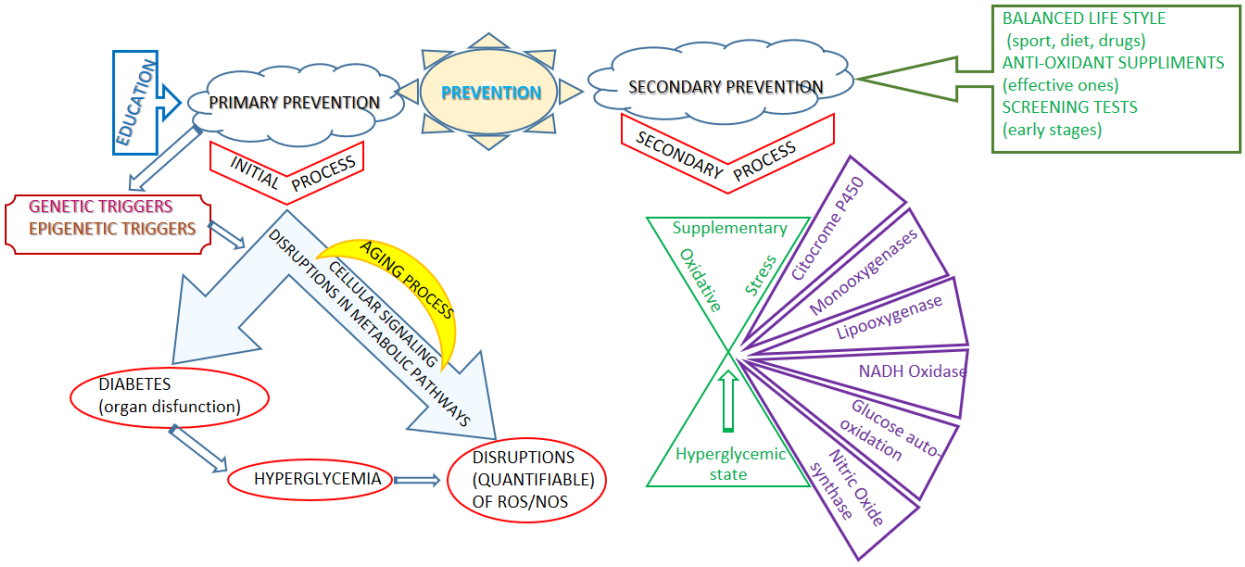

Figure 1. Presentation of main primary and secondary prevention approaches

The membrane lipids are influenced by the chain reaction between fatty acids polyunsaturated and ROS. Such chemical processes have as consequence increased cellular membrane permeability as well as an increased calcium influx. The subsequent effect of all these chemical transformations at the membrane level leads to mitochondrial damage. Another aspect to be considered is related to the effects of certain antioxidants whose molecules might change their first functionality *in vivo*. For instance, melatonin is a proven antioxidant *in vitro* [8] generates circadian rhythm through protein-coupled receptors [9].

3. Conclusions

In our work, we proved that the approach of the biological processes by help of complex mathematical models allows a correct understanding of pathogenic processes, without applying the simplifying hypothesis or artificial extension of general models.

It has been shown that it is possible to better interpret the oxidative stress using fractals, which present same patterns at lower scale – as cells. The studies that we started could bring a significant difference on approaching the primary and secondary prevention and on other public health issues as well. Such dedicated research could be important for explaining the signaling pathways, which generate chronic diseases (i.e. diabetes).

Author Contributions: The contribution of the authors: concept, A.P.S. and G.M.; methodology, F.C., C.S.; investigation, A.P.S., V.M.; resources, D.M., E.M.C.; data curation, E.E.T.; writing—original draft preparation, A.P.S., E.M.C., E.E.T.; writing—review and editing, E.E.T.

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Abstract ***Poster 9***

Determination of antioxidant activity of vitamin C by voltammetric methods†

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Abstract: Voltammetric methods – cyclic (CV) and differential pulse voltammetry (DPV) are considered the most appropriate way to evaluate antioxidant activity of redox active compounds. They provide information about both mechanism and kinetics of electrochemical oxidation of antioxidants as well as their physical and chemical properties such as the redox potential or the number of electrons transferred. These methods are helpful for understanding the mechanisms of oxidation or reduction processes of antioxidant compounds. This work presents the *electrochemical properties* of vitamin C obtained by both CV and DPV methods.

Keywords: L-Ascorbic acid, vitamin C, antioxidant activity, cyclic voltammetry, differential pulse voltammetry, electrooxidation

1. Introduction

Redox reactions, describing oxidation-reduction via electron transfer, occurs commonly in cells of living organisms in order to maintain metabolism and to generate energy. Reactive oxygen (ROS) and nitrogen (RNS) species are produced during normal metabolism and take part in redox signalling that governs cell fate [1]. However, an excessive level of ROS causes oxidative stress and various harmful effects. Thus, the maintenance of cellular redox homeostasis is crucial for healthy living. Subsequently, natural antioxidants derived from foods such as fruits or spices have received a great deal of attention in supporting endogenous antioxidant system of cells [1, 2]. One of the food ingredients that plays a key role in supporting antioxidant barrier of the body is L-Ascorbic acid (AA). The reduced form of vitamin C is capable of reacting with strong oxidants (compounds with high value of E^0) which can be evaluated by voltammetric methods – cyclic (CV) and differential pulse (DPV) voltammetry. Voltammetric techniques are based on the measurement of the current arising from oxidation or reduction on the electrode surface, following the application of variable potential. CV and DPV are characterized by good sensitivity, thus they are very suitable methods for a wide range of applications and the most effective electro-analytical technique for the study of electroactive species [3].

The objective of the work is to present electrochemical parameters that can be obtained by carrying out CV and DPV measurements of L-Ascorbic acid in aqueous solution (pH = 7.4).

2. Materials and Methods

2.1. Materials

For the electrochemical studies L-(+)-Ascorbic acid (AA) from Sigma Aldrich (USA) was used. Sodium phosphate buffer prepared using $Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ (Sigma Aldrich, USA) was applied. To clean working electrode and electrochemical cell, H_2SO_4 (POCH, Poland) and potassium permanganate (Sigma Aldrich, USA) were used. Water, purified using a QPLUS185 system from Millipore (USA), was applied.

2.2. Methods

Voltammetric measurements were carried out using a Gamry Reference 600 potentiostat (Gamry Instruments, Inc., USA) and were performed in a three-electrodes system consisting of a glassy carbon electrode (GC, 1.6 mm in diameter), platinum wire and the 3 M KCl Ag|AgCl (type R-10/S, Hydromet S.C., Poland), which were the working (WE), the counter (CE) and the reference (RE) electrode, respectively. Different concentrations of L-Ascorbic acid (2, 3, 4, 5, 6 mM) were prepared by dissolving it in 1 M sodium phosphate buffer as the supporting electrolyte (pH = 7.4 ± 0.1). Prior to the measurements, the solution was deoxidize by 3 min percolation with high purity argon and the surface of WE was carefully polished using alumina suspension (MicroPolish Alumina, 0.05 µm particles, Buehler, USA) on microcloth pads (MF-1040, BASi, USA) and then rinsed with distilled water and methanol. The RE was stored in 3 M KCl and rinsed with water prior to use. All cyclic (CV) and differential pulse (DPV) voltammograms were performed at positive potentials, in the range - 0.3 to + 0.8 V. The number of cycles was set at 4 in CV. To assess the impact of scan rate on the oxidation process of tested samples, the following rates were selected: 10, 20, 30, 50 and 100 mV/s. In case of DPV, the settings were: pulse size 50 mV, pulse time 0.1 s and sample period 0.5 s. All experiments were performed at 25 °C. Temperature was controlled using Ultra Thermostat AD 07R-20-A12E model (PolyScience, USA). Three independent repetitions of each sample were performed. Voltammograms were analyzed by SigmaPlot 13.0 software (Systat Software Inc., USA).

3. Results

Electrochemical oxidation of L-ascorbic acid at the GC electrode was studied by cyclic and differential pulse voltammetry. Selected CV and DPV voltammograms for the electrooxidation of vitamin C and the supporting electrolyte are shown in Fig. 1a.

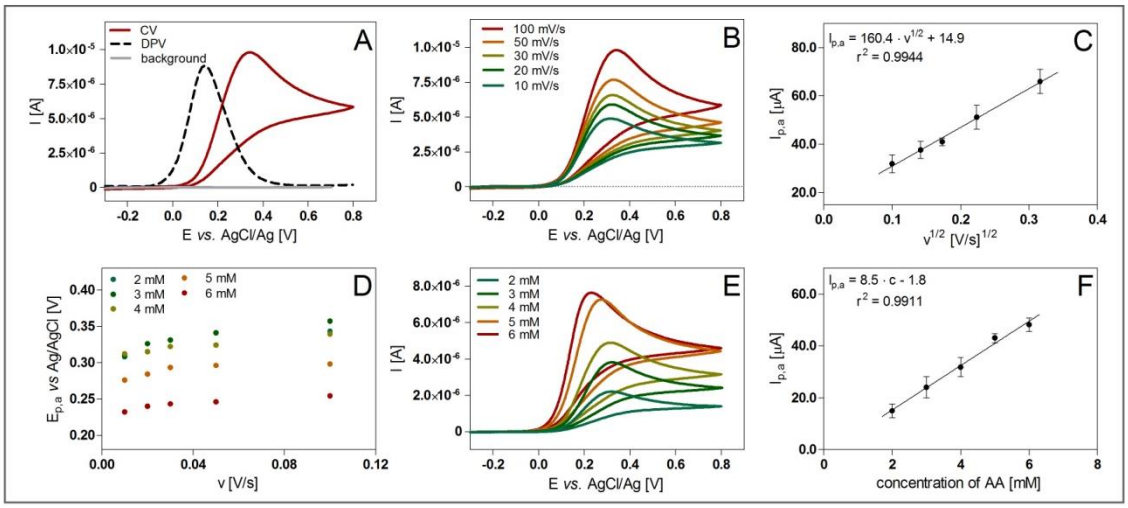

Figure 1. (a) CV – cyclic voltammogram DPV – differential pulse voltammogram; c = 4 mM, potential scan rate (v) 100 mV/s. (b) CVs, c = 4 mM at various v; 100 mV/s, 50 mV/s, 30 mV/s, 20 mV/s, 10 mV/s of the supporting electrolyte. (c) The dependence of the anodic peak current ($I_{p,a}$) on the square root of v, c = 4 mM. (d) The dependence of the anodic peak potential ($E_{p,a}$) on the v for various concentrations: 2 mM, 3 mM, 4 mM, 5 mM, 6 mM. (e) CVs for various concentriatons, v = 10 mV/s. (f) The dependence of $I_{p,a}$ for various concentrations, v = 10 mV/s; electrooxidation of L-ascorbic acid at GC electrode, solutions prepared in 1 M sodium phosphate buffer.

The supporting electrolyte (1 M sodium phosphate buffer) shows no characteristic peaks, thus the observed peaks are the results of oxidation of the AA. The values of the anodic peak potential and current are well determined for electrode reaction. The received voltammograms (Fig. 1a) show that L-Ascorbic acid is irreversibly oxidized in one step, where two electrons are changing. The potential anodic peak ($E_{p,a}$), as determined by CV is 0.34 V (*vs* RE). This is somewhat variable with concentration, however (Fig. 1d), thus suggesting that potential found in this way may be hardly correlated with antioxidant activity measured by biochemical and biological tests. The influence of potential polarisation rate on the electrooxidation of AA was studied by CV with scan rates of 10 – 100 mV/s (Fig. 1b). The analysis of linear dependence between $I_{p,a}$ on $v^{1/2}$ or $\ln(I_{p,a})$ on ln(v) allows to define whether a reaction is controlled by adsorption or diffusion processes.

The influence of AA concentration on the reaction on the GC electrode was investigated in the range from 2 mM to 6 mM. CV and a dependence of $I_{p,a}$ on the concentration is presented in Fig. 1e and Fig. 1f. This dependence is described by linear regression in all concentration range of AA. The linear dependence of $I_{p,a}$ on concentration of the depolarizer (Fig. 1f) is maintained also at other scan rates.

4. Discussion

Reactions on the surface of the electrode are characterized by the dependence of the current on the electrode potential. Potential of the DPV anodic peak is shifted relative to CV (Fig. 1a). This shift results from the difference in measured current in both techniques. DPV as a pulsed technique is highly sensitive, because the charging current (non faradaic process) is minimized. Thus, the height of the peak current in DPV is directly proportional to the concentration of redox active species in solution, while in CV both faradaic and non-faradaic currents are measured.

Considering studied potential scan rate ranging from 0.01 to 0.1 V/s, the anodic peak current depends linearly on the square root of the scan rate. It is described by the following equation (1)

(4 mM AA, Fig. 1c):

$$I_{p,a} = 160.4\ (v)^{1/2} + 14.9 \quad r^2 = 0.9944 \quad (1)$$

This equation may suggest whether the electrode reaction is diffusion or adsorption-controlled**.** Moreover, the dependence of $\ln(I_{p,a})$ on ln(v) is characterized by linear regression (equation 2):

$$\ln(I_{p,a}) = 0.3198\ln(v) + 4.9 \quad r^2 = 0.9803 \quad (2)$$

The slope of the fit reaches 0.3198, which suggests that the process is controlled by diffusion. Diffusion-controlled electrode processes are characterized by value of the slope close to 0.5. However, processes, which are controlled by adsorption are described by a slope close to 1.0 [5, 6, 7, 8]. The same results are observed for other investigated concentrations of vitamin C. For a reversible reaction, the peak potential is independent of v. Therefore, it may be considered that the heterogeneous electron transfer at peak is irreversible, thus determination of values of the electron transfer coefficient ($\beta_{n\beta}$) for the reaction is possible [5]. The following equation (3) is valid for a totally irreversible diffusion-controlled process.

$$\boldsymbol{E_{p,a} = \left(\frac{RT}{2\beta_{n\beta}}\right) lnv + const} \quad (3)$$

where: $E_{p,a}$ – anodic peak potential (V), R – gas constant (8.314 $JK^{-1}mol^{-1}$), F – Faraday constant (96 487 $Cmol^{-1}$), T – temperature (K), $\beta_{n\beta}$- anodic transfer coefficient, v – scan rate (Vs^{-1})

Based on the dependence of $E_{p,a}$ on ln(v), described by following equation (4), $\beta_{n\beta}$ is equal to 0.65 for electrooxidation of 3 mM AA.

$$E_{p,a} = 0.0199 \cdot \ln(v) + 0.4019 \quad r^2 = 0.9938 \quad (4)$$

Analysis of the CVs received allows to calculate the *Tafel slope* (b) as information on the rate-determining step. The following equation (5) describes a totally irreversible diffusion-controlled process [8, 9].

$$E_{p,a} = b/2\ln(v) + \text{const} \quad (5)$$

The slope above dependence is equal 19.9 mV, thus *Tafel slope* was found to be 39.8 mV in this work.

5. Conclusions

L-Ascorbic acid is considered as a compound with strong antioxidant activity. In this study, the electrochemical behavior of AA at GC electrode was investigated. It was observed, that vitamin C is irreversibly oxidized in one electrochemical step with the exchange of two electrons. Based on cyclic voltammetry technique, electrochemical parameters (the anodic transfer coefficient, the Tafel slope) were calculated. These values help understand the kinetics of underlying reactions of redox active compounds. Voltammetric studies may provide information on electrochemical oxidation mechanisms of other ascorbate compounds in aqueous media.

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Abstract ***Poster 10***

Effects of an exercise test on inflammation and oxidative stress biomarkers in patients with metabolic syndrome †

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Abstract: Metabolic syndrome is characterized by an increase in oxidative stress and chronic low-grade inflammation. The effects of an exercise test at 60-70% of the maximum capacity was evaluated on inflammatory and antioxidant response in elderly people suffering from metabolic syndrome. Exercise induced significant increases in plasma levels of inflammatory cytokines and malondialdehyde. The expression of these cytokines and antioxidant enzymes were also increased in peripheral blood mononuclear cells. In addition, plasma levels of tumour necrosis factor alpha were unchanged. In conclusion, the exercise test induces a situation of oxidative stress that promotes the activation of a proinflammatory cytokine cascade.

Keywords: aging; antioxidants; exercise; inflammation; metabolic syndrome; oxidative stress.

1. Introduction

Metabolic syndrome is a cluster of common factors: hypertension, hyperglycaemia, excess of visceral fat, altered cholesterol and/or triglyceride levels that occur together, increasing cardiovascular risk and diabetes [1]. This pathology is characterized by an increase in the degree of oxidative stress and is associated with an imbalance in adipocytokine production by adipose tissue promoting chronic inflammatory status and vascular endothelial dysfunction [2]. This situation is exacerbated by the high degree of sedentary lifestyle of the population in general.

Reactive oxygen species (ROS) are a double-edged sword because they act as redox signal molecules in physiological processes but they also can induce oxidative damage and play a role in pathological processes [3]. In this sense, low-moderate levels of ROS exert stimulating effect (signalling, receptor and enzymatic stimulation), whereas a massive level of ROS inhibits enzyme activity and induces apoptosis or necrosis. During exercise, contraction processes cause an increase in ROS production and a transient situation of oxidative stress. The aim of this study is to evaluate the effects of 60-70% exercise test on inflammatory and antioxidant response in elderly people suffering from metabolic syndrome.

2. Materials and Methods

9 men over 55 years of age with a body mass index ≥27 and <40 kg/m2 fulfilling at least three of the metabolic syndrome parameters were tested. Physical activity consisted of a treadmill session for 30 minutes at an initial speed of 4 km/h that was increased progressively from minute 2 until reaching 60-70% of participant's maximum ability. Blood samples were collected in heparinized tubes before and 1 hour after the exercise test.

Antioxidant and inflammatory parameters were measured in plasma and mononuclear immune cells (PBMCs). The plasma levels of intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6) and tumour necrosis factor (TNF-α) were determined by ELISA according to manufacturer's instructions. Malondialdehyde

(MDA) was analysed in plasma by colorimetric assay, based on the reaction of MDA with a chromogenic reagent that produces a stable chromophore with maximal absorbance at 586 nm. Gene expressions of TNF-α, IL-6, catalase and glutathione peroxidase were measured in PBMCs by real-time polymerase chain reaction (PCR) with 18S ribosomal as the reference gene. The PCR was performed using a LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I.

Statistical analysis of data was carried out with the SPSS statistical program (Statistical Package for Social Sciences Inc., v.25 for Windows) applying the Student's t-test for paired data.

3. Results

The results evidenced a significant increase in plasma levels of ICAM-1 (+88%), IL-6 (194%), and MDA (0,3 ± 0,10 vs 0,8 ± 0,18 pg/ml, p<0,001) after the exercise test, whereas the levels of TNF-α remained unchanged.

The gene expression of the antioxidant enzymes catalase (1 ± 0.37 vs 1.71 ± 0.08%, p<0.001) and glutathione peroxidase (1 ± 0.19 vs 2.5 ± 0.39%, p=0.001) and IL-6 (1 ± 0.16 vs 3.61 ± 0.67%, p=0.003) were significantly induced after the exercise test in PBMCs, while no changes were found in TNF-α.

4. Discussion

Aging is an inevitable process that involves a progressive loss of homeostasis and therefore, the function of the different structures of the organism over time, especially in regulatory systems such as the immune, the endocrine and the nervous systems [4]. In aging, there is an accumulation of ROS in the mitochondria that promotes a chronic oxidative stress situation contributing to the presence of oxidative damage in DNA, lipids and proteins and promoting tissues' degeneration [4].

The patients in the present study were advanced in age and symtomatic of metabolic syndrome, both of which are characterized by an increase in oxidative stress and inflammatory status. Physical activity promotes an increase in respiratory flow that leads to an increase in ROS production [5]. Accordingly, the present study has evidenced how an exercise test at 60-70% of the maximum capacity in elderly individuals with metabolic syndrome induces a situation of oxidative stress and the instauration of a pro-inflammatory environment. Exercise increases the concentration of circulating IL-6 and ICAM-1 and an induction of the expression of IL-6 and antioxidant enzymes PBMCs in response to the stress generated [6,7]. The increase in IL-6 and ICAM-1 would be indicative of the existence of an inflammatory response aimed at repairing muscle tissue damaged by exercise as evidenced by the high levels of MDA. Unlike the activation of the pro-inflammatory cytokine cascade due to an infection, the levels of TNF-α and IL-1 are not modified [8,9]. Moreover, it has been evidenced that subjects with metabolic syndrome present a higher pro-inflammatory response to exercise than that observed in healthy people of the same age [10-13]. Regular exercise practice would allow continued stimulation to maintain high antioxidant defences and contribute to the adaptive response of the body to future situations of acute stress associated with exercise. These results show how physical activity practice for healthy aging is a key point in the management and prevention of chronic diseases and therefore it would be highly recommended to promote it.

Author Contributions: conceptualization, A.S., A.P. and J.P.; methodology, A.P., A.S. and X.C; investigation, X.C., M.M., L.G., C.M.M., E.A. and P.A.B.; data curation, X.C. and M.M.; writing—original draft preparation, A.S.; writing—review and editing, all authors; funding acquisition, A.P. and J.T.

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Abstract **Poster 11**

Regulation of cellular redox homeostasis by (-)-epicatechin and cocoa extracts – a pilot study.

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Abstract: Cocoa polyphenols play an important role in protection against diseases in which oxidative stress is implicated as a causal or contributing factor. The main aim of this study was to elucidate the influence of different cocoa extracts and main cocoa bioactive compound (-)-epicatechin on cell growth and support of antioxidant cellular barrier in colon adenocarcinoma cell line model (HT29). Results show that the tested cocoa bioactivity results from concerted interactions between epicatechin and other components. Hence, cocoa is a very good example which supports the food synergy concept that is attracting growing interest.

Keywords: cellular antioxidant activity, cocoa extract, (-)-epicatechin, food synergy

1. Introduction

Living in an oxygenated environment has forced the evolution of effective cellular strategies to take advantage of endogenous sources of reactive oxygen species (ROS), which are generated as a consequence of metabolic activities. ROS regulate several signalling pathways affecting a variety of cellular processes. However, many environmental stimuli generate high levels of ROS that can perturb the normal redox balance and increase the risk of chronic diseases. Therefore, the enhancement of an endogenous antioxidant defence system through dietary supplementation with antioxidants seems to present a reasonable approach to reduce undesirable oxidative stress. However, epidemiological studies suggest that regular consumption of products rich in antioxidants is more effective in the decreasing risk of chronic diseases than isolated antioxidants [1]. The reasonable explanation to these observations may be the food synergy concept, which is defined as additive influence of different food ingredients on modulation of biological processes being involved in redox homeostasis. To fully understand this concept, it is necessary to investigate the influence of both isolated antioxidants and whole food products on the redox biology of cells.

A product characterized by very high content of antioxidants is cocoa powder, thus it seems to be an important contributor to the total dietary intake of antioxidants. Cocoa has been shown to protect against diseases in which oxidative stress is implicated as a contributing factor [2]. Over 10 % of the weight of cocoa powder consists flavan-3-ols, that makes it one of the most concentrated source of (-)-epicatechin, (+)-catechin, and procyanidins. Among all bioactive compounds of cocoa, (-)-epicatechin has been pointed out as the most active ingredient [3]. It has been revealed, however, that cocoa extract exhibits stronger redox-related activities in cell culture than the equivalent concentration of the main cocoa antioxidant, (-)-epicatechin. Cocoa extract appears to be more efficient in modulating redox dependent processes, such as anti-inflammatory and neuroprotective processes, in comparison to equivalent concentrations of (-)-epicatechin [4].

Once the synergy of cocoa bioactives was revealed, the need of further investigation of its impact on regulation of cellular redox status arose. The objective of this study was to elucidate the impact of main cocoa antioxidant, namely (-)-epicatechin, and cocoa extract son cell growth and support of cellular antioxidant barrier in colon adenocarcinoma cell line (HT29) as a cellular model of alimentary tract.

2. Materials and methods

2.1. Chemicals and reagents

In MTT test, thiazolyl blue tetrazolium bromide (MTT) from Sigma-Aldrich (USA) was applied. The OxiSelect™ Cellular Antioxidant Assay Kit was purchased from Cell Biolabs, Inc. (USA). All reagents for cell culture were purchased from Sigma-Aldrich (USA). Water was purified with a QPLUS185 system from Millipore (USA).

2.2. Preparation of cocoa extract

This investigation was conducted on two natural cocoa powders (CE1 – product code PNGH11; CE2 - PARN12) obtained from leading Serbian chocolate manufactures and one commercial standardized cocoa extract purchased from Oryza oil & fat chemical co., ltd, Japan.

To obtain cocoa extract, 100 mg of cocoa powder was suspended in 1 mL of 70% ethanol (v/v). The suspension was vortexed for 1 min and centrifuged (13 000 rpm, 5 min, 25°C). The supernatants were used in MTT and CAA tests.

2.3. Cell culture

HT29 cell line was purchased from the ATCC culture collection. The cell culture was maintained in McCoy's medium supplemented with L-glutamine (2 mol/L), sodium pyruvate (200 g/L), fetal bovine serum (100 mL/L) and antibiotics (100 U/mL penicillin, 100 g/L streptomycin). Cells were maintained at 37°C under humidified atmosphere with 5% CO_2 in the Smart cell incubator (Heal Force).

2.4. MTT test

To determine the impact on growth of HT29 cells MTT test was used as described previously [Baranowska et al., 2018]. Briefly, exponentially growing cells were seeded in 96-well plates ($5x10^3$ cells / 0.18 mL of medium) and allowed to settle for 24 h at 37°C. Then the cells were treated for 24 with 0.02 mL of different concentrations of cocoa extracts. 70% ethanol extract was diluted to 30% ethanol extract. Final concentrations of (+)-catachin and (-)-epicatechin in cell culture ranged from 10 nM to 50 μM. Control cells were treated with the corresponding solvent only.

Following incubation, the medium was removed from the wells and replaced with 0.2 mL of fresh medium. The cells were incubated at 37°C until 24 h. After this time, 0.05 mL solution of MTT (4 g/L) was added to well and. The plate was left for another 4 h at 37°C. Then, medium was removed from wells and 0.05 mL of DMSO per well was added. The absorption of solutions was determined at 540 nm with TECAN Infinite M200 plate reader (Tecan Group Ltd., Switzerland). Treatments were performed in four technical replications. Three independent experiments for each treatment were performed. The impact on cell growth was expressed as growth inhibition of cells exposed on tested antioxidants compared to control cells treated with solvent only whose growth was regarded as 100%.

2.5. CAA test

The cellular antioxidant activity of EC and extracts in HT29 cells was studied using a CAA assay (The OxiSelect™ Cellular Antioxidant Assay Kit, Cell Biolabs, Inc., USA). The cells were seeded in black 96-well tissue culture plates with a transparent base ($3x10^4$ cells / 0.2 mL of medium). The cells were allowed to settle for 24 h at 37°C, then were treated with 0.05 mL of different concentrations of samples for 1 h. Final concentrations of investigated samples ranged from 1 μM to 100 μM. Subsequent steps were performed strictly according to the manufacturer's procedure available from the website: https://www.cellbiolabs.com. Treatments were performed in four technical replications. Emission of fluorescence at 538 nm in cell cultures was measured every 5 min for 1 h after excitation at 485 nm using TECAN Infinite M200 plate reader. Three

independent experiments for each treatment were performed. The calculation of cellular antioxidant activity expressed as CAA value has been described previously [5]

3. Results

This study examined the impact of cocoa extracts (CE 1, CE 2 and CE 3) and main cocoa bioactive compound – (-)-epicatechin (EC) on regulation of redox status of cell. In the initial stage, the impact on HT29 cell growth was assessed (Fig. 1). Our earlier study showed that physiological concentrations of (-)-epicatechin (0.01–1 μM) caused significant growth stimulation of HT29 cells, while concentration of 10 μM maintained cell growth at the control level. The higher doses of main antioxidant of cocoa inhibited cell growth [5]. In the case of cocoa extracts, equivalents of physiological EC concentrations (0.01 – 1 μM) did not cause cell growth stimulation. It turned out, that cocoa extracts maintained cell growth at control level at the broad range of investigated concentrations, both physiological and those relevant only for intestinal epithelium (Fig. 1).

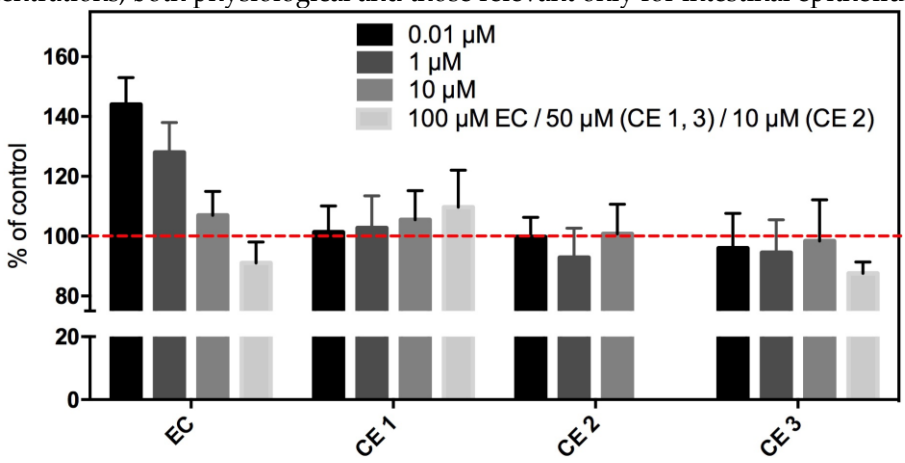

Figure 1. Biological activity of cocoa extracts and equivalent concentrations of (-)-epicatechin in terms of cytotoxicity evaluated in HT29.

In cellular antioxidant activity (CAA) test, cocoa extracts exhibited similar as EC antioxidant protection at low physiological concentrations up to 10 μM. Interestingly, cocoa extracts were the most efficient in cell protection against ROS, in particular at the highest concentration (100 μM) (Fig. 2).

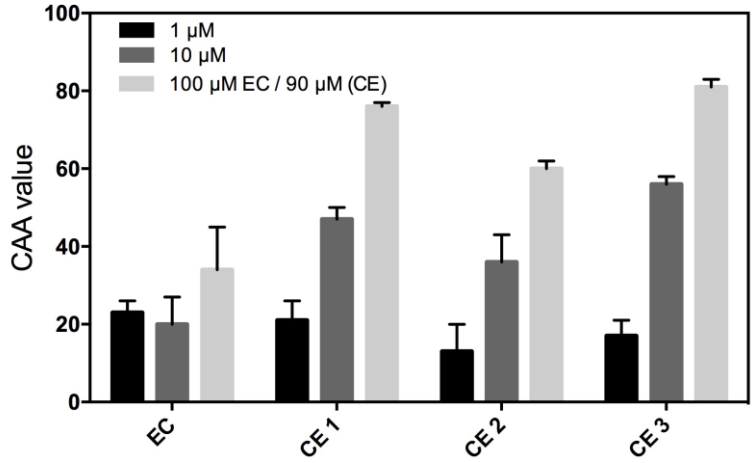

Figure 1. Biological activity of cocoa extracts and equivalent concentrations of (-)-epicatechin in terms of cellular antioxidant activity evaluated in HT29.

4. Discussion

The objective of this study was to elucidate whether the impact on redox homeostasis observed for pure (-)-epicatechin, the main antioxidant and attributed active component of cocoa, will be comparable to the biological activity of cocoa extracts containing equivalent concentration of this compound. It appeared that the natural mixture of bioactive compounds, even if containing equivalent concentration of EC, exhibited different impact on cancer cell growth, then pure compound. In comparison to isolated EC, cocoa extracts did not stimulate cell growth at physiological concentrations. Because experiments were performed in colon adenocarcinoma cell line, this observation can have a crucial meaning for anticarcenogenic efficacy of catechin based prevention. Interestingly, at physiological concentrations, the support of cellular antioxidant barrier is similar for both the pure compound and cellular extracts. Higher doses of natural mixtures of bioactive compounds offered much better protection against oxidants than the pure compound. It seems that fraction of oligomeric catechins may play an important role in cellular antioxidant activity at concentrations relevant for intestine epithelia.

From the results of this study, it can be concluded that cocoa bioactivity in term of chemoprevention is a result of interactions between various components. Therefore, impact of (-)-epicatechin on cellular antioxidant activity is enhanced by other cocoa compounds and cocoa as a full matrix represent a good candidate, in aiding the understanding of the food synergy concept.

Author Contributions: conceptualization, Agnieszka Bartoszek and Sladjana Sobajic; methodology, Monika Baranowska; investigation, Monika Baranowska and Vanja Todorovic; writing—original draft preparation, Monika Baranowska and Vanja Todorovic; writing—review and editing, Agnieszka Bartoszek and Sladjana Sobajic.

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Conflicts of Interest: The authors declare no conflict of interest.

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Abstract **Poster 12**

The Effect of High Pressure Processing on Antioxidant Activity of Irish Potato Cultivars†

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Abstract: The effect of High Pressure Processing (HPP) on Irish potato cultivars' antioxidant activity (AOA) was examined. High Pressure Processing at 600 MPa for 3 min was applied to two coloured (Rooster and Kerr's Pink) and two white (Saxon and Gemson) Irish potato varieties. Antioxidant activity was assayed spectrophotometrically by ferric reducing antioxidant power and diphenyl-1-picrylhydrazyl methods. No statistically significant ($p \geq 0.05$) change in antioxidant activity was observed in both the AOA methods irrespective of the HPP treatments, although a slight increase in the activity was noted in the majority of the HPP treated samples. This implies that HPP treatment has little role in improving the functional qualities, and can be tailored to improve the quality and safety of the commonly consumed potatoes.

Keywords: high pressure processing; potatoes; polyphenols; antioxidant

1. Introduction

Potatoes are amongst the most important crops grown for human consumption [1]. Potatoes constitute a staple nutritional diet worldwide, and are also considered as one of the richest sources of antioxidants in human diet [2]. Some of the antioxidants reported in the potatoes are polyphenols, ascorbic acid, carotenoids, tocopherols, alpha lipoic acid, and selenium [3].

In Ireland, there has been an increased sale of ready-to-eat processed potatoes as per the recent reports of the Irish Farmers' Association. As conventional food processing may have damaging effects on certain physical and sensory characteristics of food, and considering the high demand by the consumers for "fresh-like" products, the food industry is constantly seeking for new methods of processing and preservation that have less negative effects not only on the organoleptic properties but also on the nutritional and functional values of food and food products [4].

High pressure processing (HPP) is an emerging non-thermal technology and its applications in food industry, especially for the production of minimally processed food, are increasing each year [5]. Therefore, the aim of this work is to examine the effect of HPP on the functional value (i.e. antioxidant activity) of commonly consumed Irish potato cultivars' (Saxon, Gemson, Rooster and Kerr's Pink).

2. Materials and Methods

2.1 Samples

Freshly harvested potatoes (*Solanum tuberosum* L.) of Rooster cultivar were provided by Country Crest Ltd., Lusk, Co. Dublin. Saxon, Gemson and Kerr's Pink potatoes were purchased from a local market in Dublin, Ireland.

Chemicals for antioxidant assays: namely 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-striazine, Iron(III) chloride hexahydrate, sodium acetate anhydrous, acetic acid, sodium

hydroxide, hydrochloric acid, methanol, and 2,2-Diphenyl-1-picrylhydrazyl were purchased from Merck, Wicklow, Ireland.

2.3 HPP treatment

Potatoes of the different cultivars were packaged in polyethylene/polyamide pouches and then vacuum sealed. HPP treatment was performed in HPP Tolling, St Margarets, Co. Dublin. A commercial-scale high pressure press was used (Hiperbaric 55HT, Hiperbaric U.S.A.) at 600 MPa (6000 bar) for 3 min at 10. 6°C (max. temperature reached).

2.2 Extraction of antioxidants

Phenolic compounds were extracted according to Wang *et al.*, 2015 [6] with minor modifications.

2.3 Determination of antioxidant activity (AOA)

Ferric Ion Reducing Antioxidant Power (FRAP) assay was performed according to Stratil, Klejdus and Kubáň, 2006 [7] and Ou *et al.*, 2002 [8], while radical scavenging activity (2,2-Diphenyl-1-picrylhydrazyl - DPPH) assay was employed according to Goupy *et al.*, 1999 [9] to evaluate the AOA of potato samples.

2.4 Statistical analysis

Results are expressed as means of three replicates ± standard deviation (SD). All experimental data were analysed using paired samples t-test by SPSS Statistics 23. The values were considered significantly different when $p<0.05$.

3. Results and Discussion

3.1. Effects of HPP on antioxidant activity (AOA) of potatos

The AOA of potatoes as evaluated by FRAP analysis is shown on Figure 1. AOA was increased ~12%, and 10% ($p<0.05$) in HPP treated Saxon and Rooster potato samples, respectively compared to those untreated. However, there were no statistically significant changes in AOA of HPP treated Gemson and Kerr's Pink potato samples as compared to those untreated.

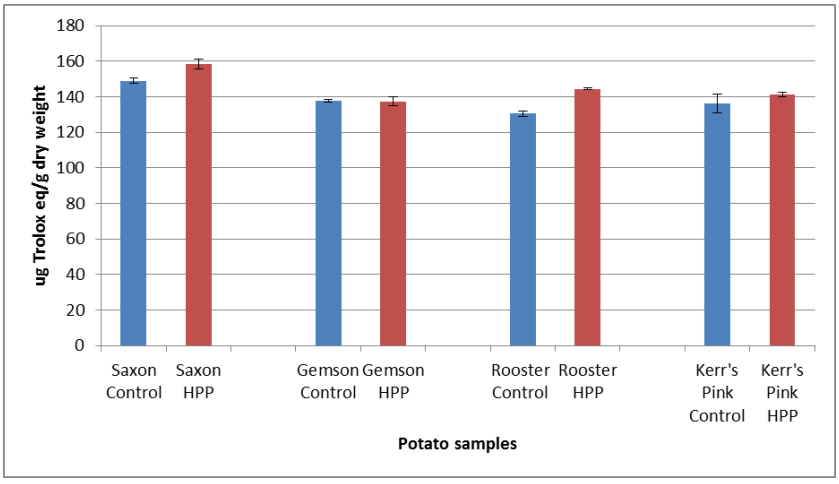

Figure 1. Effect of HPP treatment on antioxidant activity of potatoes as measured by FRAP assay and expressed as µg of Trolox equivalents per g of potato dry weight. Values presented are the average ± SD.

Figure 2 shows the effect of HPP treatment on the AOA of potatoes as measured by DPPH analysis. It can been seen that there were no statistically significant changes in AOA of HPP treated potato samples as compared to those untreated, in all cultivars.

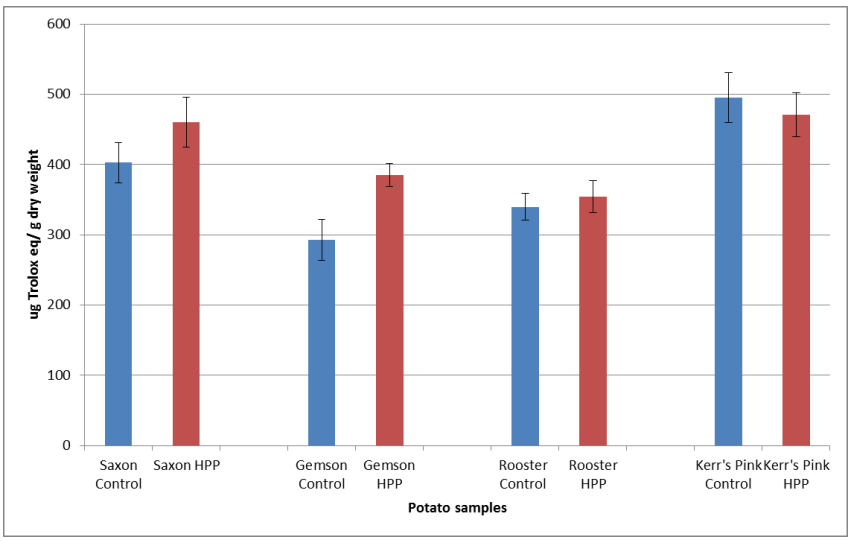

Figure 2. Effect of HPP treatment on antioxidant activity of potatoes as evaluated by DPPH assay expressed as μg of Trolox equivalents per g of potato dry weight. Values presented are the average ± SD.

A possible explanation of the contradicting results between FRAP and DPPH analysis could be that different antioxidant mechanisms are invovled. For instance, FRAP is based on the ability of compounds to reduce ferric ion to ferrous ion whilst DPPH is a free-radical scavenging power. In the later scenario, depending on the radical-generating system, certain compounds might exert pro-oxidant activity [10]. In addition, antioxidant activity may be due to a combined effect of different compounds, acting either synergistically or antagonistically. A number of factors that influence the antioxidant activity such as oxidation system, degree of glycosylation, partition coefficient and concentration, which are not determined here have been reported [11].

4. Conclusion

High pressure processing overall did not have a statistically significant impact on the antioxidant activity of potatoes. However, HPP treated potatoes have shown improvement in quality and safety of the potatoes, which will be reported elsewhere. This study provides a scientific and technological basis to further develop HPP coupled chemistries for enhancing nutritional and functional qualities of potato cultivars.

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