Expanded habilitation reference for scientific contributions of assoc. prof. Petko Denev, PhD

After acquiring the academic position "Associate professor" in 2014, I continued my scientific work on topics on which the Laboratory of Biologically Active Substances (LBAS), Plovdiv, headed by me, has built traditions. The scientific contributions of my research work have both fundamental and applied character and are directed mainly to studying the chemical composition of medicinal and economically important plants and their biological activity. I sincerely believe that my scientific work contributes to the enhancement of LBAS's accountability and recognition as a leading research center in the field of functional foods, and a sought partner for joint scientific and applied projects, among business and scientific community. The expanded habilitation reference for scientific contributions covers research papers №№ 1-31 of the attached list of scientific publications, patents, utility models, and monograph chapters. The main scientific and applied contributions from these publications can be summarized in the following directions:

1. Investigation of chemical composition and biological activity of medicinal and economically important plants:
   1.1. Investigation of chemical composition and biological activity of fruit and functional foods of aronia (*Aronia melanocarpa*);
   1.2. Investigation of chemical composition and biological activity of other medicinal and economically important plants;
2. Utilization of waste from the essential oil industry to obtain new products;
3. Investigation of chemical composition and biological activity of pectic polysaccharides.

All these directions are priorities in several national strategic documents, such as the National Strategy for Development of Scientific Research in the Republic of Bulgaria 2017-2030, the Innovation Strategy for Smart Specialization and others.

1. Investigation of chemical composition and biological activity of medicinal and economically important plants.

1.1. Investigation of chemical composition and biological activity of fruit and functional foods of aronia (*Aronia melanocarpa*);

Central to the research of LBAS, over the last 15 years, are the fruits of aronia (*Aronia melanocarpa*). Black chokeberry (*Aronia melanocarpa*) fruits are among the richest sources of polyphenols and anthocyanins in plant kingdom, and suitable raw material for production of

functional foods. The popularity of chokeberries is not due only to their nutritional value, but also to the constantly emerging evidence for their health-promoting effects. In the period 2014-2019, I deepened the studies on the fruits of aronia and in 2016, under my leadership, the National Science Fund funded a project № DN09/20 entitled "Integrated approach to improve the quality, organoleptic properties and biological activity of functional foods from black chokeberry (*Aronia melanocarpa*) by co-pigmentation and synergism in antioxidant activity". In the frames of the project, we performed a comprehensive study on the chemical composition of 23 aronia fruit samples from two harvest years – 2016 and 2017. We demonstrated that chokeberry samples differ significantly in both the content and the composition of organic acids, sugars and phenolic compounds. In several cases, these differences were more than 100%. The total polyphenol content of aronia fruits varied between 1022 mg/100 g fresh weight (FW) and 1795 mg/100 g FW, and ORAC antioxidant activity from 109 μmol TE/g to 191 μmol TE/g FW. The differences in the chemical composition of the fruits resulted in functional foods that differ significantly in their chemical composition and antioxidant activity. Additionally, we demonstrated that temperature of juice pressing and nectar extraction has a profound effect on the polyphenol content and composition of these products. This is very important, since differences in chemical composition of raw chokeberries and variation of technological parameters during processing could result in functional foods with different chemical composition, rendering different biological activity (**Publication №19**). In another set of experiments, we investigated the antioxidant, antimicrobial and neutrophil-modulating activities of different polyphenolic preparations from black chokeberry fruits. Quercetin and epicatechin were the strongest antioxidants among aronia polyphenols, but the high content of proanthocyanidins in fresh aronia fruits makes them the major contributor to their antioxidant activity. Besides, we revealed that black chokeberry proanthocyanidins are the most potent antimicrobial agents in the fruit. Studied polyphenols and preparations had no effect on the spontaneous chemiluminescence (CL). Greater effects were observed on OZP-activated CL) for quercetin and rutin (**Publication №24**). Black chokeberry fruits are particularly rich in anthocyanins, which are very instable during processing and storage of aronia products. Co-pigmentation is one of the main mechanisms for natural stabilization of anthocyanins and it was interesting to investigate the impact of different phenolic co-pigments and herbal extracts on color intensity of aronia anthocyanins. Tested compounds provoked different co-pigmentation effect, manifested by hyperchromic and batochromic shifts. The co-pigmentation was accompanied by a magnification of color intensity and decrease of color hue, both related to a more pleasant color. The hyperchromic effect was the most significant for rosmarinic acid

(51.02%), syringic acid (43.24%) and catechin (39.73%). However, it was observed at the highest pigment/co-pigment ratio (1:50), not achievable in plant matter. Targeting the potential practical application of co-pigmentation, we tested eight herbal extracts for their co-pigmentation ability with aronia anthocyanins. The use of herbal extracts led to a significant hyperchromic effect at much lower co-pigment/pigment ratios, compared to pure compounds. The use of selected herbal extracts as co-pigments opens realistic prospects for development of aronia functional foods with improved sensory properties and biological effects, due to enhanced color and anthocyanin stability (**Publication №21**). Further technological attempts were made to synergistically increase the antioxidant activity of aronia beverages by adding an extract obtained by homogenization of rosehip fruit. A utility model protected these results with a practical application (**Utility model №28**).

An important stage in the production of functional foods is the study of their biological activity *in vitro* and *in vivo*. Within the framework of the above-mentioned project, in a collaboration with teams from the Medical University of Plovdiv, various experiments with functional drinks from aronia were carried out. In these collaborative studies, I participated in the preparation and analysis of aronia functional drinks, the design of the research itself and the interpretation of the results. Age-related diseases are a social problem of a global significance and their prevention by natural products is a research area of particular interest. Therefore, we investigated the possibility to counteract the risk factors for atherosclerosis arising in the aging process by supplementation of chokeberry juice (**Publication №4**). It employed a model of healthy adult rats monitored for a number of somathometrical, serum lipidogram and histopathological parameters, and their response to supplementation with antioxidant-rich chokeberry juice. Treatment with aronia juice significantly lowered the proatherogenic low-density lipoprotein fraction of the animals and led to a 16.5% decrease in their total cholesterol. Atherogenic indices in aronia-supplemented animals clearly showed lower atherogenic risk and cardioprotective indices indicated protection of the cardiovascular system. Besides that, chokeberry juice retarded the age-related changes in the aortic wall and can be recommended as a prophylactic tool for healthy aging. Using the same animal model, we revealed that aronia-supplemented animals were characterized with reduced atherosclerotic lesions and a lower level of restructuring of aortic walls. These data confirm that *Aronia melanocarpa* juice successfully retards age-related vascular aging **(Publication №6)**. In a recent study, we investigated the effect of aronia juice on learning ability, memory and brain morphology of aged rats. Activity cage test showed that aronia supplemented rats increased the number of vertical movements compared with old controls. Aronia supplementation did not affect the mean neuronal number

in the dentate gyrus but significantly increased the density of nerve fibers in the perforant path of the hippocampus, which is an evidence for a neuroprotective effect. Aronia juice also increased acetylcholinesterase activity in hippocampus, which is a marker of improved functional activity of the cholinergic neurons. These results indicate that aronia juice induced ameliorating changes in the ability of old rats to learn tasks and improved their locomotor functions (**Publication №27**).

1.2. Investigation of phytochemical composition and biological activity of other medicinal and economically important plants.

In the period 2015 – 2019, we continued our research on the phytochemical composition and biological activity of Bulgarian medicinal plants. In a review article, we collected our results for ORAC antioxidant activity of nearly 80 Bulgarian fruit, vegetables and herbs and extended them with unpublished data for 11 mushrooms, 4 vegetables and 2 herbs (**Publication №10**). In addition, we have systematized the available information on the health effects of flavonoids and their role in healthy nutrition, and presented our own results for flavonoid content (flavonoids, flavonols and flavones) of selected fruits and vegetables, searching for a correlation with their ORAC antioxidant activity. This work was published in the prestigious edition of "Handbook of Food Bioengineering" by Elsevier Publishing House (**Monograph chapter №30**). Based on the large volume of accumulated results for antioxidant activity of Bulgarian plant materials, we have deepened the experiments on the application of medicinal plant, rich in antioxidants for production of nutraceuticals and functional foods. Blackberry (*Rubus fruticosus*) leaves, chokeberry (*Aronia melanocarpa*) leaves, hawthorn (*Crataegus monogyna*) leaves, lady's mantle (*Alchemilla glabra*) aerial parts, meadowsweet (*Filipendula ulmaria*) aerial parts and raspberry (*Rubus idaeus*) leaves revealed particularly high antioxidant activity by several assays (ORAC, TRAP, HORAC and inhibition of lipid peroxidation). All extracts blocked almost completely the opsonized zymosan particle-activated ROS production by neutrophils from human whole blood, indicating that their polyphenols (apart from their antioxidative activity) interfere with the signaling cascade of phagocyte activation upstream of the protein kinase C activation. Meadowsweet and blackberry leaves extracts revealed very high antimicrobial effect (**Publication №1**). In a subsequent study, we determined the antioxidant activity of extracts from *Valeriana officinalis, Melissa officinalis, Crataegus monogyna, Hypericum perforatum, Serratula coronata* and their commercially available combinations Antistress 1 and Antistress 2, which are used as food supplements and recommended for chronic fatigue, anxiety and stress (**Publication №11**). The most pronounced was the antioxidant

activity of the extracts from *M. officinalis* and *H. perforatum*. These extracts contributed to the greatest extent for the activity of Antistress 1 and Antistress 2 formulations. The conducted study for acute toxicity *in vivo* indicated non-toxicity both of individual extracts and their combinations. In another study, the ORAC and HORAC antioxidant activities of various flavonoid glycosides isolated from dwarf elder (*Sambucus ebulu*s) were determined (**Publication №2**). The data on antioxidant activity of Bulgarian natural products were enriched with results for ORAC antioxidant activity of *Salvia tomentosa* essential oil, measured by the lipophilic modification of the assay (**Publication № 5**). To this set of studies, we could add also a recent study on the kinetic parameters of co-pigmentation between strawberry anthocyanins and caffeic acids, indicating that co-pigmentation process is irreversible (**Publication №18**).

Chaenomeles maulei is little known plant in Bulgaria and few people are acquainted that this mountain crop has been extensively studied at the Institute for high-mountain crops in Trojan, in attempt to introduce it in Bulgaria. For the first time, we investigated the chemical composition and antioxidant activity of the *Chaenomeles maulei* fruit juice (CMFJ) from fruits harvested in Bulgaria. The results showed that CMFJ was extremely rich in polyphenolic substances. The sour taste of the juice and its low pH were due to the high content of organic acids (malic, quinic, citric, shikimic, ascorbic, oxalic). The carbohydrates were presented by glucose, fructose, galactose, xylose, rhamnose and arabinose. The antioxidant activity measured by ORAC and HORAC assays was very high and correlated well with its high polyphenolic content. Based on these results, it could be concluded that fruits of *C. maulei* could be used in food industry as natural acidifying agent, which in the same time will increase the functionality of foodstuffs, adding substantial amounts of phenolics with antioxidant activity (**Publication №23**).

Pepper (*Capsicum annuum* L.) is the most widely cultivated and economically important vegetable crop worldwide. The rich agro-biodiversity of peppers on the Balkan region is a valuable source of new alleles for improvement the quality and nutritional value of the fruits. In Bulgaria, there is a lack of a complex evaluation of the pepper genetic diversity in regards to substances with antioxidant properties in the fruits. In a recent study, we used the rich biodiversity of peppers in the Balkan region and characterized 63 pepper accessions from five cultivar types (Pungent; Sweet; Pumpkin; Kapia; For powder), in view of their phytochemical composition and antioxidant activity (**Publication №26**). The obtained results showed high variation in the compositional profiles between studied cultivar groups, as well within individual groups. On the basis of fresh weight (FW), the most abundant sugar in the studied accessions was glucose (0.36-3.79%), followed by fructose (0.16-2.98%). The accessions from

“For powder” group were the richest source of uronic acids – 0.77%, followed by accessions from “Pungent” group – 0.64%. In four of the five groups, citric acid was the predominant organic acid, whereas only in the “Sweet” group, malic acid was the major representative of organic acid. The highest amount of ascorbic acid (Vitamin C) was found in a genotype CAPS-7, which is in the group of pungent accessions. The polyphenol content varied in a broad range from 42 mg/100 g FW to 266 mg/100 g FW. The differences in the flavonoid content were even more significant reaching 60-fold difference – from 1.0 mg/100 g FW to 64.3 mg/100 g FW. The accessions from the “For powder” group are leaders in regards to their total polyphenol, total flavonoid contents, as well as for their antioxidant activity, measured by ORAC and HORAC assays. This variation in fruit composition might be manipulated via plant breeding to create new pepper varieties with improved nutritional value of their fruits. This study was performed in the frames of a project DN06/4 – 2017 entitled “Phenotyping and genotyping of pepper (*Capsicum annuum* L.) specimens with Balkan origin for creation of core-collection”, funded by the National Science Fund of Bulgaria, whose coordinator for IOCCP is me. In another study, we investigated the main parameters that affect extraction process of polyphenolic compounds from tomato fruits. The most suitable extragent was acetone-water mixture 80:20 (v:v). The optimal extraction time, temperature and solid to solvent ratio were 45 min, 60ºC and 1:40, respectively. Using these extraction parameters, the yield of total polyphenols in tomato variety “Desperado” was 27.80 GAE/100g fresh weight. The kinetics of extraction process was investigated and theoretical model describing extraction process was proposed (**Publication №2**).

Stevia (*Stevia rebaudiana*) is a perennial shrub, growing naturally in Paraguay and Brazil. Dried stevia leaves are used industrially as a source of steviol glycosides, which are used in food industry as a food sweetener. Being 300 times sweeter than sucrose, steviol glycosides are considered as one of the strongest natural sweeteners. The Agricultural institute in Shumen, Bulgaria performs field trials directed towards the selection of Bulgarian stevia cultivars. However, under the climatic conditions of Bulgaria stevia can be grown only as an annual crop. In a collaborative study, we determined the content of steviol-glycosides in 24 genotypes of Stevia (*Stevia Rebaudiana*), cultivated in Bulgaria. The HPLC analysis showed that the content of stevioside in the separate groups reached 6.80 g/100g dry mass and that of rebaudioside A – 8.27 g/100g dry weight. Such a comparative study is the initial step in the development of Stevia cultivar with high content of steviol-glycosides. These results allow us to make a conclusion that trough the path of individual selection, it is possible to develop stevia genotypes with high content of steviol-glycosides, under the climatic conditions of Bulgaria (**Publication №9**).

Quinoa (*Chenopodium quinoa*) is a plant that recently have been successfully grown in Egypt, providing seeds rich in nutrients and bioactive compounds. In the frames of an inter-academical agreement between Bulgarian Academy of Sciences and Benha University in Egypt, we investigated the chemical composition, nutritional value, amino acid and fatty acid profiles of selected quinoa accessions (Shams17-2, Shams16 and Shams14) cultivated in Egypt (**Publication №13**). Based on the results, it could be concluded that quinoa seeds from the new accessions, cultivated in Egypt are a good source of essential nutrients such as minerals, essential amino acids and essential fatty acids.

2. Utilization of waste from the essential oil industry to obtain new products.

Studies in this direction were mainly carried out in the frames of project DN 17/22-2017 entitled "Utilization and application of waste materials from the essential oil industry for "green synthesis of metal nanoparticles", whose coordinator for IOHCP-BAS is me. My contribution to these studies consists mainly in the chemical characterization of extracts derived from waste biomass from essential oil industry. Thus, in a series of studies (**Publications №№ 8, 12, 17, 22, 25**), a variety of polyphenol and polysaccharide extracts from *Rosa damascena, Calendula officinallis, Lavandula angustifolia* and *Melissa officinalis* were obtained and characterized. The effect of lavender (*Lavandula angustifolia*) and melissa (*Melissa Officinalis*) waste on preparation, characteristics and shelf life of bread was investigated **(Publication №17**). It was found that lavender and melissa waste, were rich on polyphenols (especially rosmarinic acid) and aroma compounds, and exhibited high antioxidant and antimicrobial activity. The total dietary fiber increased three times and the polyphenols and flavonoids increased more than four times for breads with added 5% lavender and melissa waste, compared to control sample. The breads with 2.5% and 5% added lavender waste had increased shelf life (up to 96 h) compared to the control. Especially interesting was the study on the synthesis of nanoparticles mediated by *Rosa damascena* waste extracts and their application for an electrochemical sensing of hydrogen peroxide and vanillin (**Publication №25**). Silver nanoparticles (AgNPs) were synthesized by reduction of Ag^+ with four extracts of *Rosa damascena* waste post-distillation water (PW), water extract (WE), 30% (30 EE) and 70% (70 EE) ethanol extracts. The ethanolic extracts were rich in polyphenols and particularly catechin and epicatechin, while the water extracts were rich in reducing sugars, proteins and pectic substances. Biosynthesized AgNPs were deposited onto a spectroscopic graphite electrode and stabilized using chitosan to build modified electrodes-catalysts. The developed electrodes exhibited a sensitive and reproducible response for quantitative determination of H_2O_2 and vanillin.

3. Investigation of chemical composition and biological activity of pectic polysaccharides.

In the frame of this direction, for the first time, we characterized the polysaccharide composition in 11 European and in particular Bulgarian medicinal plants (**Publications №№14, 15, 16, 20, monograph chapter №29**). Long-term hot water extraction has been found to be a reliable way to produce pectin-rich polysaccharide complexes with high *in vitro* complement-fixation activity and high *ex vivo* intestinal immunomodulatory effect. A unique pectin polysaccharide, with an anti-inflammatory potential, that is unusually acetylated in Rha and highly galacturonated on GalA (almost 1:1) main chain of rhamnogalactorunan type I was isolated for the first time from lime flowers. The structure and immunomodulatory activity of the water extracted pectic polysaccharides from lavender showing *ex vivo* immunostimulatory action on phagocytic leukocytes and intestinal immunomodulatory activity were studied for the first time. It has been discovered that lavender pectins activate the inborn and acquired immune response, through the blood payer's patches, neutrophils, macrophages and immunocompetent cells in the intestinal payer's patches. In addition, the structure and immunomodulatory activity of water-extractable pectin polysaccharides from nettle leaf has been studied for the first time. From the large-scale study carried out, it can be concluded that that pectin-type acid heteropolysaccharides in Bulgarian medicinal plants show a pronounced immunomodulating activity with immunostimulating and anti-inflammatory potential. The results obtained are of practical application and can serve as a basis for the development of value-added by-products from cultivated Bulgarian herbs. These studies confirm the leading role of LBAS in the studies on chemical composition and biological activity of pectic polysaccharides in our country - a scientific field that should be preserved and developed in our laboratory.

Beyond the four directions in my work, I would add a chapter in the **collective monograph №31,** where upon an invitation from Elsevier publishing house, with a wide range of specialists, we reviewed and analyzed current and emerging technologies for cold pasteurization of food products. In **publication №7,** different assays were applied for analyzing the antioxidant activity of extracts from biomass of Antarctic yeast strain *Sporobolomyces salmonicolor* AL1.

Prospects for research over the next 5 years

I anticipate that my future work will be focused mainly on studying the chemical composition and biological activity of medicinal and economically important plants and the waste from their processing, for the development of nutraceuticals and functional foods. In a near term (in the next 2 years), my priority will be the successful implementation of the project, leaded by me and the other 3 projects of the laboratory, funded by the National Science Fund:

1. Project DN 09/20 "Integrated approach for improving the quality, organoleptic properties and biological activity of new functional foods from chokeberry (*Aronia melanocarpa*) by copigmentation and synergy in antioxidant activity".
2. Project DN 06/4 “Phenotyping and genotyping of pepper (*Capsicum annuum* L.) specimens with Balkan origin for creation of core-collection”.
3. Project DN 17/22 / 12.12.2017 "Utilization and application of essential oil industry wastes for “green synthesis” of metal nano-particles".
4. Project DN 19/14 NSF "Complex approach for evaluation of changes in biologically active substances and the antioxidant potential of irradiated plant foods and herbs. New protectors against gamma irradiation ".

Meanwhile, I will look for opportunities for project funding for one of my ideas related to the development of functional foods naturally enriched with gamma-aminobutyric acid (GABA) and to study the impact of GABA and polyphenol components in foods on the expression of certain genes associated with aging (jointly with teams from MU - Plovdiv and MU-Varna).

I would like to deepen the relationship with the business and the industry in solving real problems that would have a pronounced economic effect. One such project is due to be officially signed in September 2019.

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