HABILITATION REPORT OF THE SCIENTIFIC CONTRIBUTIONS

in the publications of Assist. Prof. Dr. Boryana Trusheva

presented for application for the Associate Professor academy position in a professional field 4.2. Chemical Sciences: scientific specialty "Bioorganic Chemistry, Chemistry of Natural and Physiologically Active Substances"

The author's report for the scientific contributions was made on 26 scientific publications (outside the PhD thesis) and one author's certificate for a registered utility model [**6, 7, 9, 11-32, 34, 35**] (attached list of all publications). Seven of the scientific papers published in referenced and indexed journals are equated to habilitation work (group of indicators B) [**9, 13-15, 23, 27, 31**]. At the time of filing the documents, a total of 1229 citations (778 of them on *Scopus/WoS*) were noticed, the **h**-index is **15** (*Scopus*, without the auto-citations).

In general, the scientific work reflected in these publications is in the field of chemistry of natural compounds. The aim of the research is to clarify the chemical composition, biological activity and the possibilities and ways of applying of natural objects traditionally used in folk medicine or their bioactive components in the modern medical practice. In only two of the scientific publications [6, 12] two **wood-decay fungus** species, respectively were investigated. In the other papers, as a continuation of the PhD thesis work, the object of the study is **propolis** (bee glue), produced by honey bees (*Apis mellifera*), with the exception of publication **22**, where geopropolis of stingless bees (*Melipona fasciculata*) is investigated.

I. Scientific contributions

They are mainly related to **obtaining and providing new data and facts and confirming hypotheses for the structural diversity of biologically active natural compounds** and can be grouped as follows:

1. Contributions to propolis research

2. Contributions to the studies of wood-decay fungi (Basidiomycetes)

1. Propolis studies

Propolis (bee glue) is one of the most valuable bee products with healing properties. It is a mixture of resinous plant material, which bees collect from the surface of the buds, young leaves or damaged tree bark, and beeswax. The plant resin is its main ingredient and source of biological activity. Propolis is the bee tool to fight against microorganisms, and humans use it to prevent and treat a number of diseases. Continuous research on propolis is required because of its variable chemical composition, determined by plant biodiversity in different geographical and climatic regions. The study of the chemical

composition and propolis plant origin leads to the formulation of different propolis types. This is important concerning the development of criteria for its standardization and quality control, necessary for propolis use in modern medicine.

The main focus of the study of propolis chemical composition is to identify the compounds that determine its biological activity. As a plant-based product, for this purpose a phytochemical approach is applied: the GC/MS method (after derivatization) and/or isolation of individual components are most commonly used. For the isolation of individual components a combination of chromatographic techniques was used and the structure of the substances was determined using spectral methods such as NMR, MS, UV and IR. For quantitative determination of different groups of phenolic compounds in poplar propolis, validated spectrophotometric methods developed in our laboratory are used. Much of the research is based on the study of the composition of 70% ethanol extract (balsam) of crude propolis, the form which is most commonly used in medicine and apitherapy, and containing the biologically active plant components.

1.1. Isolation of new natural compounds and/or new data for the chemical composition of propolis from different mainly unstudied geographic regions [**14-17, 19, 20, 22, 23, 27, 31].**

- In search of new biologically active molecules, the **chemical composition and biological activity of Iranian propolis from the Isfahan province was studied for the first time**. From 70% ethanol extract five individual components were isolated: the prenylated coumarin suberosin, and four terpene esters: tschimgin (bornyl p-hydroxybenzoate), tschimganin (bornyl vanillate), ferutinin (ferutinol p-hydroxybenzoate), and teferin (ferutinol vanillate). **All of them were found for the first time in propolis**. All isolated terpene esters demonstrated activity against *Staphylococcus aureus* [**14**][1].

- From a biologically active extract of scarcely studied Indonesian propolis from East Java, 11 compounds were isolated and identified: **four alk(en)ylresorcinols** (obtained as an inseparable mixture) **were isolated for the first time from propolis**, along with four prenylflavanones (propolin D, propolin C, propolin F and propolin G) and three cycloartane-type triterpenes (mangiferolic acid, isomangiferolic acid and 27-hydroxyisomangiferolic acid). All prenylflavanones demonstrated significant radical scavenging activity against diphenylpicrylhydrazyl radicals (DPPH). For the first time *Macaranga tanarius* L. and *Mangifera indica* L. are shown as plant sources of Indonesian propolis, which distinguishes it from the so-called "Pacific" or *Macaranga* type propolis (from Taiwan and Okinawa) [**15**].

[1] The antibacterial tests were conducted by colleagues at the Institute of Microbiology, BAS

- **For the first time, propolis from Malta was investigated**, and it was found to be representative of the “diterpenic” type. Seventeen Maltese propolis samples were studied by GC/MS after silylation. They exhibited the typical Mediterranean chemical profile, rich in diterpene compounds. In 16 samples, terpenyl esters of substituted benzoic acids were also found, **2 of them new propolis constituents**, were isolated and identified as acetylated daucane esters of p-methoxy and p-hydroxybenzoic acids, respectively. Terpenyl esters are characteristic for *Ferula communis* (Apiaceae), a species widely distributed in Malta and designated as a secondary plant source. A statistically significant correlation (Pearson test) between ester content and antifungal activity was established [**16**].

- Next step is to prove the plant species whose resinous secretions, rich in diterpenes, are the main active ingredient of Mediterranean propolis. The chemical composition of resins from cypress (*Cupressus sempervirens*) and pine (*Pinus halepensis*) growing in Malta was analyzed and the resulting GC/MS profiles were compared with that of propolis from Malta and Greece. According to the results, **the main and/or the only plant source of the Mediterranean type propolis is the cypress *C. sempervirens*** (Mediterranean Cypress), and this is the first report of cypress as a plant source of propolis [**17**].

- In recent years, serious problems have arisen concerning bee diseases leading to the extinction of entire bee colonies. Within the 7FP of EU project and in collaboration with Dr Bilikova from the Slovak Academy of Sciences studies on the activity of isolated propolis components against bee pathogens were conducted. From Bulgarian propolis 5 individual compounds were isolated (pinochembrin, pinobanksin-3-O-acetate, benzyl and pentenyl ferulates and **9-oxo-10(E)-12(Z) -octadecadienoic acid, new for propolis**), together with a mixture of caffeic acid esters (pentenyl, isopentenyl and phenethyl caffeate), which were tested *in vitro* against *Paenibacillus larvae* strains. This bacterium, well known as American foulbrood, causes a disease in bees with fatal outcome. As a result, flavonoids and caffeate mixtures show significant and highest activity. **This is the first report of the activity of propolis constituents against *P. larvae*, identifying them as potential antimicrobial agents against bee pathogens**. The use of isolated propolis compounds as a means of combating bee diseases is advantageous as these substances are natural and naturally present in the bee hives [**19**].

- **The composition of propolis from the Perm Region, European Russia, was analyzed for the first time**, and the results led to the formulation of a new mixed type of propolis. Five samples from different regions were analyzed by GC / MS. All the samples were found to belong to a specific, **new type of propolis with triple plant**

origin: *Populus tremula-Betula pendula-Populus nigra*. The quantitative content of the major groups of biologically active components was reported, and samples with a higher total flavonoid content were found to exhibit higher antibacterial activity [**20**].

- In collaboration with colleagues from the State University of Sao Paulo, Brazil, **the chemical composition and pharmacological activity of poorly studied geopropolis from the stingless bee *Melipona fasciculata* were investigated**. In addition to vegetable resins and beeswax, the geopropolis also contains small amounts of soil from which its name originates. The sample was collected from the hives in the Maranhao state, northeastern Brazil. The 70% ethanol extract was analyzed by GC/MS and **the results showed that the major constituents were carbohydrates and their derivatives, triterpenes, anacardic acids, alkylresorcinols, and sugar alcohols.** It was found that geopropolis in combination with chloramphenicol showed synergistic effect against *Staphylococcus aureus* and *Escherichia coli*. **It also displayed anticancer and immunomodulatory activities and these biological properties may be due to the presence of triterpenes**- one of its major chemical constituents [**22**].

- **The chemical composition of propolis from Fiji islands was determined for the first time**. **Two new prenylated stilbenes with an irregular sesquiterpenyl side chain**, solomonin B and solomonin C, together with four known compounds, glyasperin A (**new for propolis**), kumatakenin, macarangin and mangiferolic acid **were isolated from its ethanol extract**. A GC/MS analysis (after silylation) of the ethanol extract was carried out and **the major components is found to be prenylated flavonoids and stilbenes, alkylresorcinols, anacardic acids and cycloartane triterpenes**. **The sample was found to be of dual plant origin**. While alk(en)ylresorcinols, anacardic acids and cycloartane triterpenes are characteristic for the resinous secretion of the *Mangifera indica* (mango), the source of the prenylated flavonoids and stilbenes is another species most probably *Macaranga*, but different from the one, which is the plant source of Indonesian and "Pacific" type propolis [**23**].

- As a continuation of our work on propolis from unexplored regions, together with colleagues from the Institute of Biophysics and Biomedical Engineering-BAS and the Institute of Microbiology-BAS, **we investigated for the first time propolis from Pitcairn Island**. From the dichloromethane extract of the sample, **four new cycloartane-type triterpenes were isolated**: 3-Oxo-cycloart-24E-en-21,26-diol-21,26-diacetate, 3-Oxo-cycloart-24E-en-21,26-diol, 3-Oxo-cycloart-24E-en-21,26-diol-21-acetate and 3-Oxo-cycloart-24E-en-21,26-diol-26-acetate, together with 17 known compounds (di- and triterpenes). The extract was found to exhibit a potential antitumor activity against triple-negative MDA-MB-231 human breast carcinoma cells, as well as antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and Candida

albicans. The antimicrobial activity of the newly discovered natural compounds was also investigated, finding that three of them were highly active against *Escherichia coli*, and 3-oxo-cycloart-24E-en-21,26-diol-21,26-diacetate also demonstrated strong antifungal activity against *Candida albicans* [**31**].

- By hydrodistillation in a Likens-Nickerson apparatus, essential oils of two distinct types of crude propolis were isolated: Brazilian red (originating from *Dalbergia ecastaphyllum*) and Taiwanese green propolis (originating from *Macaranga tanarius*). The volatile fractions obtained were analyzed by GC/MS and it was found that **the main volatile components of Brazilian red propolis**, that have been poorly studied, **were the phenylpropanoids**: elemicin, methyl eugenol, transmethyl isoeugenol, isoelemicin and trans-anethole. While **the major constituents of Taiwanese green propolis essential oil, which has not been studied so far, were: β-eudesmol, 6-methyl-3,5-heptadiene-2-one, γ-eudesmol, geranial and 6-methyl-5-heptene-2-one** [**27**].

1. 2. Summarized and comparative data for propolis

Summarized and comparative data for propolis are presented in review papers published in scientific journals and books (by invitation), concerning:

- **Propolis plant sources from different geographical and climatic regions**, data published in the period 2000-2006. Particular attention was paid to plant sources determined on the basis of a comparison of the composition of propolis and plant material, as well as direct observation of the bee behavior [**7**].

- **Chemical composition, biological activity and plant sources of propolis**. Particular attention is paid to the newly discovered natural and propolis components [**11, 26**].

- **Different propolis types, based on plant sources and their respective chemical profiles**, data published after 2000. The composition-activity relationship for the different propolis types is discussed, and the compounds responsible for a given biological activity are described in details [**18**].

- **For the first time data on the volatiles content of propolis from different geographical regions were collected and summarized**. Contrary to the statement (based on pre-1948 literature) that bee glue contains up to 10% of essential oils, it is concluded that, regardless of geographical origin, propolis typically contains no more than 1% of essential oil, in rare cases up to 2-3%. For the various propolis samples the main components of the essential oil fractions are indicated. The contribution of volatiles and their constituents to the biological activities of propolis is considered [**21**].

- **For the first time, data on the new emerging fields of application of propolis**, other than those related to improving and protecting human health, have been summarized [**25**].

- **The most recent developments concerning propolis plant sources, bees' preferences to particular plants, the application of metabolomic approaches and chemometrics to propolis research and the problems regarding its standardization** are summarized, data published in the period 2007-2017 [**32**].

2. Research on the wood-decay fungi (Basidiomycetes)

These studies were carried out in the framework of a project funded by the National Science Fund. The interest in these objects is provoked by the scarce data or the complete lack of such data in the scientific literature, and at the same time the availability of sufficient evidences for the powerful healing properties of other closely related species of wood-decay fungi.

- The macromycete *Hygrophorus agathosmus* was found to be toxic to *Artemia salina* nauplii and the active chloroform fraction of the total extract was chemically studied. Several hydrocarbons, including paraffins, prystan, phytan, and squalene were identified. **The sterol composition of this mushroom was analyzed for the first time** by GC/MS and the main sterol being ergosterol (22E-ergosta-5, 7, 22-triene-3β-ol) (82%), typical fungal sterol, accompanied by two other ergostane derivatives: (22E)-ergosta-5,7,9(11),22-tetraene-3β-ol (11.5%) and ergosta-7-ene-3β-ol (6.5%). 5α, 8α-Epidioxi-24(ξ)-methylcholesta-6,22-diene-3β-ol was isolated for the first time from *H. agathosmus* but its presence might be regarded as an artifact obtained by oxidation of the corresponding Δ 5,7 sterol [**6**].

- **The secondary metabolites of the wood-decay fungus *Fomitopsis rosea* were investigated for the first time.** This fungus is now Red-Listed in many European countries, including Bulgaria. Two new lanostane triterpenic acids along with three known lanostane triterpenes and an epidioxysterol derivative were isolated and identified. The isolated compounds exhibit activity against *Staphylococcus aureus*. All triterpenes are characterized by the presence of an additional carbon atom at C-24, which is characteristic of the fungus *Daedalea quercina* and confirms the close relationship between *Fomitopsis* and *Daedalea* [**12**].

II. Scientific and applied contributions [**9, 13, 24, 28-30, 35**]

- Within the framework of a project funded by the National Science Fund, **a study was conducted for the first time on the feasibility of applying the modern techniques for the extraction of biologically active substances from propolis** and evaluating their effectiveness. For the extraction of Bulgarian propolis (poplar type) with 70% ethanol, the following methods have been applied: the used so far classical extraction by maceration (at room temperature), ultrasonic (UE) and microwave-assisted extraction (MAE), under varying conditions (extraction time, raw propolis/solvent ratio, etc.). The quantities of extracted phenolics and flavonoids were determined spectrophotometrically, on the basis of which **the efficiency of the methods applied was evaluated**. Compared to the maceration extraction, MAE and UE methods provided high extraction yield, requiring short timeframes and less labour. UE was shown to be the most efficient method based on yield, extraction time and selectivity [**9**].

- Under the FP7 EU project and in collaboration with colleagues from the University of Chemical Technology and Metallurgy and the University Rovira i Virgili, in Spain, **dead-end nanofiltration has been successfully implemented for the first time as a method for concentration of biologically active compounds derived from poplar propolis**. Two membranes were used: Starmem^{TM} 122 (polyimide) and Duramem^{TM} 200 (modified polyimide), resistant to organic solvents and with molecular weight cut-off 220 Da and 200 Da, respectively. As a stock solution, a chemically well-characterized ethanol-water extract of poplar propolis was used. To evaluate the efficiency of the method, the content of flavones, flavonols, flavanones, dihydroflavonols as well as total phenolics was determined spectrophotometrically in the feeds and permeates of dead-end nanofiltration experiments. **Rejection of more than 95% of all analytes and concentration of propolis extract, more than three times, was achieved** with the Duramem^{TM} 200 membrane. The established conditions for extract concentration could facilitate cross-flow nanofiltration experiments regarding a technology for medicine or nutritional additive production [**13**].

- **A novel micellar form of poplar propolis based on a biocompatible** poly (ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) block **copolymer ($PEO_{26}PPO_{40}PEO_{26}$) is developed** in a collaboration with colleagues at the Institute of Polymers-BAS. **The composition of this water-soluble form has been registered as a utility model.** The rapid and easy preparation of the colloidal aqueous solution, its stability over a time at a relatively low copolymer to propolis weight ratio, and the solubilization of all biologically active lipophilic constituents of propolis in the absence of ethanol, makes it possible to be used in medicine, pharmacy, food and beverage industry, cosmetics, etc. The micellar form of the propolis exhibits high *in vitro*

cytotoxicity at relatively low concentrations, which in combination with the nanometer sized particles and the sustained release of propolis molecules, **makes it a promising candidate for cancer treatment applications** [**24, 35**].

- As a continuation of our work **three amphiphilic poly(ethylene oxide)-*block*-poly(n-butyl acrylate)** (PEO-b-PnBA, n= 53, 99 and 157 monomer units) **diblock copolymers for the first time were successfully applied for preparing spherical propolis-loaded polymeric micelles in aqueous media**. PEO-b-PnBA copolymer micelles were selected for their small size, low cytotoxicity and excellent resistance upon severe dilution. **Stable, slightly opalescent colloid solutions were obtained** at a copolymer/propolis extract mass ratio ≥2 **for the three copolymer compositions studied**. The very similar hydrodynamic diameters of blank and propolis-loaded micelles and the absence of any precipitate in solutions **indicated for a complete loading of all biologically active lipophilic propolis constituents into the micellar cores**. Propolis-loaded micelles exhibited a superior structural stability upon rigorous dilution and maintained their morphology at a concentration as low as 0.01 g/L. Based on the results obtained, **PEO-b-PnBA micelles**, due to numerous favorable characteristics like nanoscopic size, good colloid stability, high loading capacity and resistance to fast dissociation at very low concentrations, **have great potential for application as long-circulating carriers of propolis in biomedicine via parenteral administration** [**29**].

- In the search for new drug-delivery systems of propolis to overcome the barriers associated with its low solubility, **it has been demonstrated for the first time that silver-modified MCM-41 and SBA-15 mesoporous materials are suitable carriers of poplar propolis**. The study was conducted jointly with the Hungarian Academy of Sciences and the Institute of Microbiology - BAS. The mesoporous channels of the silicate carrier have been found to be suitable for the storage of relatively high amounts, 15-17 wt. %, of propolis extract. **Loading of propolis** from ethanolic solution **on silver containing silica carriers resulted in significantly improved water solubility, as well as to enhanced antimicrobial activity against some widespread human pathogens** probably due to synergistic effect of silver and propolis. The results of this study indicate that **silver-modified MCM-41 and SBA-15 mesoporous materials are promising carriers for the preparation of poplar propolis loaded dermal formulations** [**30**].

- **The quantitative characteristics of Bulgarian propolis were studied for the first time**. Bulgarian propolis belongs to the poplar type, since its plant source is the resin of the black poplar (*Populus nigra*). The components responsible for its biological activity are flavonoid aglycones (flavones, flavonols, flavanones and dihydroflavonols),

substituted cinnamic acids and their esters. Twenty two samples of Bulgarian propolis collected from different regions of the country were examined. In each of the samples the balsam and three main groups of biologically active substances (total phenolics, total flavones/flavonols and total flavanones/dihydroflavonols) were quantified. Based on the results, **we characterized raw poplar propolis in terms of minimum content of its bioactive components (antimicrobial and antioxidant) as follows: 46% balsam, 24% total phenolics, 7% total flavones/flavonols and 5.4% total flavanones/dihydroflavonols**. These values can be applied as a basis for standardization and quality control of Bulgarian propolis [**28**].

III. A methodological contribution to propolis research [**34**]

- **An overview of the available methods for studying propolis in different aspects is presented for the first time (including based on our experience**): propolis in the bee colony, chemical composition and plant sources of propolis, biological activity of propolis with respect to bees and humans, and approaches for standardization and quality control for the purposes of industrial application. The understanding of the chemical diversity of propolis is very important in propolis research [**34**].

Guidelines for future research:

Over the next few years, I intend to:

1. Continue the research on propolis, and in particular propolis from the stingless bees.

2. Perform studies related to the use of natural deep eutectic solvents as green solvents for extraction of biologically active compounds from natural sources, including propolis. This will be implemented in the framework of an ongoing project in the field of Green Chemistry funded by the National Science Fund.

3. Continue the studies on the synthesis of caffeic acid phenethyl ester, a very important biologically active component of poplar propolis.