Review

by Assoc. Prof. Dr. Nikolina Mihaylova Mihaylova, Laboratory of Immunohistochemistry and Immunopathology, Department of Immunology, Institute of Microbiology "Stefan Angelov," Bulgarian Academy of Sciences

Selected as a member of the Scientific Jury by Order No. I-81/30.06.2023 of the Director of the Institute of Microbiology and as a reviewer by the Scientific Jury with Protocol No. 1/09.08.2023.

on a PhD dissertation for the award of an educational and scientific degree "Doctor"
Author: Kristiana Miroslavova Amirova
Topic: Isolation of natural plant-derived molecules modulating the function of the transcription factor Nrf2
Academic Supervisors: Prof. Milen Georgiev and Assoc. Prof. Petia Dimitrova
Field of Higher Education: 5. Technical Sciences, **Specialty**: 5.11 "Biotechnology," **Doctoral Program**: "Technology of Biologically Active Substances"

1. General description of the submitted materials

The author of the dissertation is Kristiana Miroslavova Amirova, a regular doctoral student in the Laboratory of Metabolomics at the Department of Biotechnology, Institute of Microbiology, Bulgarian Academy of Sciences, under the scientific supervision of Prof. Milen Georgiev and Assoc. Prof. Petia Dimitrova.

The set of materials presented for review, both in printed and electronic form, complies with the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulation for the Implementation of LDASRB, and the Regulation of the Bulgarian Academy of Sciences (BAS) for the implementation of LDASRB. It also meets the criteria of the Regulation on the conditions and procedure for acquiring academic degrees and academic positions at the Institute of Microbiology "Stefan Angelov" at BAS for obtaining the educational and scientific degree of "Doctor."

2. Brief biographical data

The doctoral student Kristiana Miroslavova Amirova was born on May 5, 1993, in Plovdiv, Bulgaria. In 2017, Kristiana graduated from “Paisii Hilendarski” Plovdiv University with a

master's degree in Molecular Biology and Biotechnology. In the same year, she was appointed as a Biologist at the Center for Plant and System Biology and Biotechnology (CPSBB) in Plovdiv. In 2018, she enrolled as a regular PhD student in the field of Biotechnology, specialty 5.11, at the Institute of Microbiology, BAS, in the Laboratory of Metabolomics.

3. Relevance of the topic

For centuries, plants have been used for medicinal purposes and have been proven to be a source of natural products with numerous health benefits for humans. Previously based mainly on empirical knowledge without in-depth research into their pharmacological activity and chemical composition, plants are now being used as a basis for the development of new therapeutic drugs. The search for and validation of the biological activity of natural molecules isolated from plants is a trendy topic in recent years. The development of proteomics, metabolomics, and bioinformatics enables the study of such natural raw materials as potential molecules with anti-inflammatory, antibacterial, antifungal, and antitumor activity, and their therapeutic potential.

Due to the side effects associated with conventional therapies, the search for new and safe sources of natural products with proven therapeutic qualities is becoming increasingly urgent. The dissertation provided for my review addresses a topic that is relevant from both a scientific and a scientific-applicative perspective. The interest of the doctoral student and her supervisors is focused on the search for plant-derived substances that have a modulating effect on the pathogenesis of degenerative and inflammatory joint diseases, specifically the transcription factor Nrf2.

4. Appropriateness of Objectives and Tasks

The aim of this dissertation is to identify and characterize low-molecular-weight compounds from plant extracts of *Ballota nigra L., Clinopodium vulgare L., Leonurus cardiaca L.*, and *Haberlea rhodopensis Friv.*, with the potential to influence the transcription factor Nrf2 in a targeted population of neutrophils and to demonstrate their therapeutic action on granulocyte function in models of joint inflammatory and degenerative diseases. The 8 experimental tasks systematically and logically describe the steps to achieve the stated goal.

5. Understanding of the Problem

The impression from the presented literature review is that Kristiana is very well acquainted with the current state of the issues she is investigating. The literature review is divided into six

subsections, each addressing different aspects of the research. Five tables summarizing information based on published sources are included. The PhD student begins with a historical overview of phytotherapy and presents currently known plant-derived bioactive substances with pharmacological potential. Data on plant-derived substances approved for therapeutic use and those included in clinical trials are presented in tabular form. An overview of methods for collecting plant samples and characterizing active substances from them, as well as methods for isolating bioactive substances, is provided. Once again, in tabular form, the PhD student has summarized the most common methods for analyzing the biological activity of secondary metabolites, presenting their advantages and disadvantages. This creates a strong impression and demonstrates the doctoral candidate's familiarity with the methodology.

A concise description of the plants that are the subject of the present dissertation is provided. A separate section provides an overview of the structure of the transcription factor Nrf2, the mechanisms of gene and protein regulation, approaches to activation and expression of Nrf2, and its significance in the pathogenesis of degenerative and inflammatory joint diseases. A table of known plant activators of Nrf2 is included, summarizing information about their mechanisms of action and the model systems in which they have been studied.

From the literature review, the doctoral candidate draws three important conclusions that further confirm the relevance of the dissertation topic and suggest the innovative potential of the research.

6. Research Methodology

The methodology used in the development of the dissertation is impressive. Various biotechnological methods have been employed, including obtaining plant fractions, phytochemical analysis using high-performance liquid chromatography and nuclear magnetic resonance; work with cell cultures, the establishment of primary cell cultures; various molecular biology and immunological methods - determining cell viability, programmed cell death, and necrosis; flow cytometric analysis of cell phenotype, intracellular levels of cytokines; signaling; immunofluorescence, real-time polymerase chain reaction (RT-qPCR).

The PhD candidate has also included three of the most commonly used in vivo models of inflammatory and degenerative joint diseases, namely the Model of Zymosan-Induced Inflammation, Collagenase-Induced Osteoarthritis Model, and Anti-Collagen Antibody-Induced Rheumatoid Arthritis Model. Additionally, histological analysis of experimental animals has been performed. A wide range of statistical tests has been applied to analyze the obtained results.

7. Characterization and Evaluation of the Dissertation

The dissertation follows the structure outlined in the Regulations for the Development of the Academic Staff at the Institute of Microbiology, BAS, including the following chapters:

- Title
- Table of Contents
- List of Abbreviations and a list of the used pure substances with their Latin names
- Introduction - 1 page
- Literature Review - 45 pages including 4 illustrative images, 3 figures, and 5 tables and divided into 6 subsections: Historical Overview of Phytotherapy; Biologically Active Substances of Plant Origin with Pharmacological Potential; Methods for Obtaining, Characterizing, and Isolating Bioactive Substances; Methods for Studying the Biological Effects of Metabolites; Ethnopharmacological Approach to Plant Selection as Sources of Bioactive Substances; Structure and Function of Nrf2.
- Objectives and Tasks - 1 page, with 8 defined tasks
- Materials and Methods - 19 pages, with a detailed description of chemicals and reagents used, pure substances, antibodies, and kits categorized by manufacturer with catalog numbers; description of commonly used solutions and media with their full composition; a list of used media and related chemicals with manufacturer information and catalog numbers; description of the cell line used; description of the laboratory animals used; in the section on methods, there are detailed descriptions of the Systematic Literature Analysis Method, Methods for Preparing Plant Extract, Fractionation and Isolation of Pure Substances, Phytochemical Analysis - High-Performance Liquid Chromatography Analysis and Nuclear Magnetic Resonance Analysis, Methods for Isolation, Cultivation, and Differentiation of Cells, Treatment of Differentiated Granulocytes or Mouse Neutrophils with Low-Molecular-Weight Compounds, Methods for Determining Cell Viability and Apoptosis and Necrosis, Flow Cytometric Analysis for Phenotyping, Expression of Cell Receptors, Intracellular Cytokine Production, Nrf2, Phosphorylated/Unphosphorylated p38, and β-Galactosidase, In Vitro Chemotaxis Towards SDF-1, Immunoblot for Determining NRF2 in Neutrophils or Granulocytes, Immunofluorescence for Determining β-Galactosidase and CXCR4, Determination of β-Galactosidase Activity, Real-Time Polymerase Chain Reaction (RT-qPCR) of mRNA. A table with precise descriptions of the primers used in the dissertation is also included; In vivo experiments - Zymosan-Induced Inflammation Model, Collagenase-Induced Osteoarthritis

Model, Anti-Collagen Antibody-Induced Arthritis; Histological methods for analysis; Statistical analysis methods.

- Results - 46 pages, 1 table, and 32 complex figures with good quality.
- Discussion - consisting of 15 pages, synthesizing and summarizing information from the obtained results in the dissertation.
- Conclusions - 1 page, 11 in total, all of which correspond to the obtained results and are in line with the defined objectives and tasks.
- Contributions - 1 page

8. Contributions and Significance of the Research for Science and Practice

Contributions with Confirmatory Character:

1. Metabolomic analysis of samples from plant extracts prepared from B. nigra, C. vulgare, L. cardiaca, and H. rhodopensis confirmed the literature data on the presence of secondary metabolites - phenolic acids (caffeic, chlorogenic, catechin), phenylethanoid glycosides (myconoside, calceolarin E), and diterpenes (balotezid, balonigrin, balotinol).
2. Local application of the Nrf2 activator CDDO-Me has a therapeutic effect in a model of degenerative joint disease.
3. Human granulocytes and mouse neutrophils show increased expression of Nrf2 at the transcriptional and protein levels when Nrf2 is activated by CDDO-Me.

Contributions with Scientific-Fundamental Character:

1. Evidence is presented for the first time that pharmacological activation of Nrf2 with CDDO-Me significantly reduces sensitivity to aging (senescence) in mouse neutrophils.
2. It is established that the progression of osteoarthritis is accompanied by the acquisition of an aging phenotype in neutrophils.
3. The extract of C. vulgare and the active metabolites caffeine and chlorogenic acid suppress the expression of cyclooxygenase COX-2.
4. Fraction D, myconoside, and calceolarin E affect the expression of the NFE2L2 gene and the percentage of Nrf2-positive neutrophils.
5. Different mechanisms of action of the two pure substances on Nrf2 are established, which are due to the characteristics of their structure, their selective action on Nrf2 signaling, and the expression of PI3K.

6. The application of Fraction D, myconoside, and calceolarin E improves the symptoms and pathological changes in an arthritis model by modifying the expression of Nrf2 and Nrf2 target genes (SOD-1 and PRX1) in synovial extracts and by reducing the number of TNF-α+ neutrophils in circulation.

Scientific Applied Contributions:

1. An advantageous model for cultivating Haberlea rhodopensis Friv in vitro has been introduced, in which the level of myconoside in the extract of Haberlea rhodopensis Friv surpasses that of the wild species several times.
2. The plant extract of Haberlea rhodopensis Friv can be used to obtain fractions with different ratios of the metabolites myconoside and calceolarin E.
3. Myconoside and calceolarin E can be applied in combination for effective pharmacological modification of Nrf2.

9. Evaluation of the Publications Related to the Dissertation

The dissertation's topic has resulted in four published articles in refereed international scientific journals (all in Q1), with an impressive total impact factor of 27.222, and three of which Kristiana is the first author. In Scopus, there are 26 citations, excluding self-citations. During her dissertation, Kristiana Amirova participated in two projects, received three awards, and presented five posters at international scientific events.

10. Summary of the dissertation

The summary of the Kristiana Amirova's dissertation work reflects the main results achieved in the dissertation and complies with all the generally accepted requirements in the Regulations for the Application of the Law on the Development of the Academic Staff in the Republic of Bulgaria (RASASRB) for its preparation.

CONCLUSION

The dissertation work contains scientific and scientifically applied results that represent an original contribution to science and meet all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (RASASRB), the Regulations for the Application of RASASRB, and the Regulations for the Application of RASASRB at BAS. The

presented dissertation results fully correspond to the specific requirements of the Regulations of IMicB for the application of RASASRB.

The dissertation work demonstrates that Kristiana Amirova possesses in-depth theoretical knowledge and professional skills and shows qualities and skills for independent scientific research. Based on the above, I confidently give my positive assessment of the conducted research and recommend to the esteemed jury to grant Kristiana Amirova the educational and academic degree "Doctor" in the field of 5.11 Biotechnology.

September 25, 2023

Reviewer:

[Assoc. Prof. Dr. Nikolina Mihaylova]