

## REVIEW

by **Prof. Dr. Velizar Kostadinov Gotchev,**  
**Head of the Department of Biochemistry and Microbiology,**  
**Faculty of Biology, "Paisii Hilendarski" University of Plovdiv**

of a thesis for the award of the educational and scientific degree “Doctor”(PhD)  
in the field of higher education 5. Technical sciences, professional field 5.11 Biotechnologies,  
doctoral program Technology of bioactive substances

**Candidate:** *Kristiana Miroslavova Amirova*

**Title:** “Isolation of natural substances from plant origin, modulating the function of transcription factor Nrf2”

**Scientific supervisors:** *Assoc. Prof. Petia A. Dimitrova, PhD and Prof. Milen I. Georgiev, PhD*

### **1. General presentation of the procedure and the candidate**

By Order № I-81 dated 30.06.2023 of the Head of The “Stefan Angeloff” Institute of Microbiology at Bulgarian Academy of Sciences (IM-BAS) I was appointed as a member of the scientific jury of a thesis entitled “*Isolation of natural substances from plant origin, modulating the function of transcription factor Nrf2*” for the award of the educational and scientific degree “Doctor”(PhD) in the field of higher education 5. Technical sciences, professional field 5.11 Biotechnologies, doctoral program Technology of bioactive substances by Kristiana Miroslavova Amirova – PhD student at department “Biotechnology” at IM-BAS, scientific supervisors Assoc. Prof. Petia A. Dimitrova, PhD and Prof. Milen I. Georgiev, PhD.

The set of digital materials submitted by Kristiana Amirova complies with Academic Staff Development Act in the Republic of Bulgaria (ASDARB) and Academic staff development requirements and procedures act at IM-BAS (ASDRPA-IM-BAS) and includes the following documents: application form to the Head of IM-BAS; CV in European format; Master’s degree of higher education; orders; examination protocols and certificates; reference for the fulfilment of the minimum national requirements stipulated in ASDARB; protocol by National seminar of “Applied microbiology and biotechnologies”, PhD-thesis, summary of the thesis in Bulgarian and English; list of scientific publications and citations; declaration of originality and absence of plagiarism.

## **2. Brief Biographical Information About the Candidate**

In 2016, Kristiana Amirova obtained a Bachelor's degree in Molecular biology from the Faculty of Biology at “Paisii Hilendarski” University of Plovdiv, qualification Bachelor in Molecular biology. In 2017, she obtained Master's degree in Molecular biology and biotechnology, qualification Master in Molecular biology and biotechnology by the same faculty. In the period 2017-2022, she was a specialist – biologist at Centre for Plant systematic biology and biotechnology in Plovdiv and in parallel, she was full time PhD student at IM-BAS.

## **3. Actuality of the subject, aim and research tasks**

The search for new medicinal substances to combat various infectious, oncological, autoimmune, etc. diseases is essential for the implementation of efficient therapeutic approaches for their use. Natural sources of bioactive substances (BAS) characterized with pharmacological activity are plants and microorganisms. Plants are well known from centuries and are widely used as natural remedies. Plants are the basis of modern pharmaceutical science. On the other hand Bulgarian flora is rich in medicinal and aroma plants, some of which are endemic and/or poorly studied, with unique metabolic characteristics, which makes them particularly suitable for the search of new BAS. Degenerative and inflammatory joint diseases are debilitating and affect a significant part of the population, which makes the search for new and effective natural substances for their treatment particularly important. All this allows me to define the topic of the present PhD thesis as current.

The main scientific aim of the PhD study is to identify and characterize low molecular BAS, isolated from plant extracts, obtained from *Ballota nigra* L., *Clinopodium vulgare* L., *Leonurus cardiaca* L. and *Haberlea rhodopensis* Friv., and to evaluate their potential to modulate the activity Nrf2 transcription factor in neutrophils. The aim is clearly formulated and for reaching the main goal 8 experimental tasks are pointed. I believe that the second research task, II.2.2, should definitely be the first, because it is focused on the systematic literature review on the issue of low-molecular BAS from plant origin, regulating Nrf2, and it is logical to be the first step in the research process. It would be better if the *in vitro* cultivation of *H.rhodopensis* Friv. and the production of secondary metabolites was singled out in a separate tasks, in order to emphasize the biotechnological element of the PhD thesis, which is presented for the defense under PhD program Technology of BAS. The fulfillment of the set tasks is a prerequisite for the successful fulfillment of the purpose of the PhD thesis.

#### **4. Familiarity of the problem**

The literature review is presented on 45 pages and is based on 217 up-to-date literary sources. Conditionally, it can be considered as composed of two main parts - the first, focused on the plant BAS and phytochemical characterization of the studied species and the second, dedicated to the structure, function and approaches to modulating the activity of the Nrf2 factor. The first part of the review lists various plant-based BASs that are the subject of clinical trials and approved for therapeutic use. Methods for determining the qualitative and quantitative composition of plant extracts, techniques for *in vitro* cultivation and transformation of plant cultures, as well as methods for studying the biological effect of BAS isolated from plants (*in silico* analysis, *ex vivo* cell-free filtrate assay, *in vitro* cell assay, *in vitro* phenotype assay, *in vivo* experimental models, etc.). I positively appreciate the fact that the PhD student presented the information analytically, highlighting the advantages and disadvantages of the various methods and the possibilities of their application for the purposes of the specific research. The second part of the review is extremely detailed and presents the various regulatory mechanisms and natural BASs acting as activators of the Nrf2 factor. Both parts of the review end with brief conclusions that motivate the need to develop the present dissertation and define the purpose of the research. To some extent, the information in the overview is unnecessarily fragmented into numerous points and sub-points. In places there are discrepancies between section titles and the content of the text in them, for example in section II.33 “*Isolation of BAS from plants*”, techniques are described for *in vitro* cultivation and transformation of plant cultures and no method for the isolation of BAS. The scope and the way of organization of the literature review allows me to conclude that Kristiana Amirova is very well acquainted with worldwide achievements on the research problem in details.

#### **5. Research methodology**

To reach the major scientific goal of the study and to evaluate the research hypothesis wide range experimental scheme is realized. For the realization of the research tasks, a wide range of modern spectrophotometric, immunological, molecular, cytological, histological, instrumental, biotechnological and microscopic methods were used, described in detail, in a way that guarantees accuracy and allows reproduction of the obtained results. The way the methods are explained ensure accuracy and reproducibility of the results. The PhD student has mastered and applied an impressive array of methods, which shows that the educational component of the PhD degree has been definitely fulfilled.

#### **6. Characteristics and evaluation of the dissertation**

The PhD thesis is structured in the accepted way, including the sections Introduction (1 p.), Literature review (45 p.), Aim and tasks (1 p.), Materials and methods (20 p.), Results (54 p.), Discussion (15 pages), Conclusions (2 pages), Contributions (2 pages) and References (2 pages). The ratios between the individual parts of the PhD thesis are optimal. The introduction categorically states the relevance of the development and in this sense fully fulfills its function. As I have already noted, the Literature Review is comprehensive, demonstrates knowledge of the state of the research problem, and allows motivated determination of the purpose of the dissertation work. The fulfillment of the set tasks is a prerequisite for the successful fulfillment of the goal. The applied experimental scheme and the description of applied methods are correct. The experimental work began with the preparation of extracts from wild *B.nigra* L., *C.vulgare*, L., *L.cardica* L. and *H.rhodopensis* Friv., and extracts from in vitro cultivated plants were obtained from *H.rhodopensis* Friv. Two extraction regimes were applied with 50% methanol/d H<sub>2</sub>O in a ratio of 1:20 and 1:30 combined with ultrasound. The obtained extracts of *H.rhodopensis* Friv. were fractionated chromatographically and divided into 10 fractions (from A to J). Pure myconoside was obtained from fraction B, calceolaroside E from fraction E, and hispidulin from fraction H. Chemical composition of all the obtained plant extracts was determined by NMR and HPLC and the presence of amino acids, carbohydrates, organic acids, flavonoids, phenylpropanoids, etc. was established. By *in vitro* cultivation a 12-fold increase in myconoside content and a 30-fold increase in calceolaroside E content in the extracts was reached. Unfortunately, neither the Results section nor the Discussion section comment on the cultivation conditions, media composition, composition optimization, and cultivation conditions to somehow explain the 12- and 30-fold increase in the content of myconoside and calceolaroside E, respectively. Differentiated granulocytes and mouse neutrophils were treated with the obtained extracts, separate fractions and pure substances. The expression of the NFE2L2 gene was determined in differentiated granulocytes treated with the plant extracts, pure substances CDDO-Me, myconoside, verbascoside, forsythoside, etc. The influence of *H.rhodopensis* Friv. extracts, separate fractions and pure substances on the expression of Nrf2 at the protein and transcription level in human granulocytes and at the protein level in mouse neutrophils was investigated. The obtained results are correctly discussed parallel to the background of the researches achievements reached by other authors. Twelve conclusions are formulated, which are based on the obtained results. I accept the formulated confirmatory and original contributions, and some of them definitely need editing, for example, confirmatory contribution 1. I accept scientific-applied contribution 1, which is very important, with certain reservations, because the PhD thesis lacks results of such investigations.

## **7. Evaluation of the publications and the personal contribution of the candidat**

The results of the PhD thesis are presented in 4 scientific papers, which are published in scientific journals indexed in Scopus and Web of Science, belonging to Q<sub>1</sub>. Three of the publications, with the leading author PhD student Kristiana Amirova, are related to the results, published in the PhD thesis, but one of the articles, with the leading author Assoc.Prof. Marchev PhD, published in *Cellular and Molecular Science Life Science*, is a scientific review and does not no published any results from the present PhD research. In less than two years, two of the three publications have been cited 9 times, which is an indisputable certificate of the high quality and originality of the obtained results.

## **8. Evaluation of the Personal Contributions of the Candidate**

I believe that the personal contribution of Kristiana Amirova to the realisation of the experimental work, the discussion and publications submitted is relevant to the level of her competence. I would like to emphasize that in each of the listed elements, the teamwork in a large team, including various specialists with a high degree of competence in areas such as immunology, organic instrumental analysis, etc., is clearly visible.

## **9. Summary of the dissertation**

The summary of the dissertation fulfills of the formal and adequately reflects its essence and achievements.

## **10. Recommendations for future use of dissertation contributions and results**

Notes and recommendations for individual parts of the PhD thesis are indicated in sections 3, 4 and 7 of the review. Overall, I would venture to recommend the PhD student and team to expand the relative share of biotechnological experiments and their results, because they have an undisputed expertise in this regard, with the aim of finding a better balance between biotechnology and immunology, especially in a PhD presented for the acquisition of the ESD "Doctor" in Technology of BAS.

I would like to ask how the PhD student would explain the dramatic increase in the production of myconoside and calciolaroside E by *in vitro* cultivated *H.rhodopensis* Friv., compared to wild plants

## CONCLUSION

The evaluated PhD thesis contains scientific and applied results, which represent an original contribution to science and meet the requirements of Academic Staff Development Act in the Republic of Bulgaria and Regulations of the “S. Angeloff” Institute of Microbiology BAS on the Implementation of the Academic Staff Development. The PhD thesis shows that Nikolina Atanasova Atanasova has theoretical knowledge and professional skills in the scientific specialty Technology of BAS. Due to the above, I give my positive assessment of the research pre-sented in the PhD thesis, summary, results and contributions, and I propose to the esteemed jury to award the educational and scientific degree “Doctor” (PhD) to Kristiana Miroslavova Amirova 5. Technical sciences, Professional field 5.11 Biotechnology, scientific specialty Technology of bioactive substances.

20.09.2023

Reviewer:.....

(Prof. Dr. V. Gochev)