

# Opinion

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on a PhD Thesis for awarding the educational and scientific degree “doctor” in the field of higher education 5. Technical science, Professional filed 5.11. Biotechnology, PhD program: Technology of biologically active compounds

**Author:** Kristiana Miroslavova Amirova

**Title:** Isolation of natural molecules of plant origin modulating the function of transcription factor Nrf2

**Supervisors:** Assoc. Prof. Petya A. Dimitrova, PhD (Stephan Angeloff Institute of Microbiology-BAS; IMicB-BAS) and Prof. Milen I. Georgiev, PhD (IMicB-BAS).

## 1. General presentation of the procedure and the PhD student

According to order № I-81/30.06.2023 of the Director of IMicB-BAS, I have been appointed as a member of the scientific jury in the procedure for the defense of a dissertation for awarding the educational and scientific degree “doctor”.

The set of materials presented by Kristiana Amirova for the defense of a PhD thesis for awarding the educational and scientific degree "doctor" is in accordance with the Regulations for the Development of the Academic Staff of the IMicB-BAS, and meets the criteria of the IMicB-BAS for the awarding of the educational and scientific degree "doctor".

The PhD candidate has presented 4 publications on the topic of the dissertation and the corresponding supporting material, a list of citations of the papers included in the dissertation, a list for participation in research projects and scientific conferences, and the awards received. *The overall scientific production of Kristiana Amirova is 11 publications in international scientific journals with 152 citations; her h-index is 6, which is amazing.*

Kristiana Amirova completed her MSc in the field of Molecular Biology and Biotechnology, Department of Plant Physiology and Molecular Biology, Paisii Hilendarski University of Plovdiv in 2017. In the period 2017-2022, she worked at the Center for Plant and System Biology and Biotechnology, Plovdiv as a biologist. She became a full-time PhD student at the Department "Applied Microbiology" at the IMikB-BAS on 01.10.2018.

## 2. Relevance of the topic

The topic of the PhD Thesis concerns an important and up-to-date area of research related to the search and evaluation of plant species and their metabolites as potential "chemical agents" for the therapy of chronic diseases and their accompanying pathologies. The focus of the dissertation is on joint inflammatory and degenerative diseases and, accordingly, on the transcription factor Nrf2, playing a role in the regulation of metabolism, differentiation and function of immune and bone cells involved in the pathogenesis of these diseases, and the modulation of its function by applying substances of plant origin. The goal is clearly formulated, and the specific scientific tasks for its achievement are well and logically defined.

## 3. Knowing the problem

The dissertation includes a total of 217 references. The literature review is written clearly and concisely. The number of the references and the fact that a large number of them are from recent years indicate that the PhD student has an excellent knowledge of the current state of research in the various fields and the issues, considered and studied in the dissertation, and that she is able to summarize the available literature.

#### 4. Research methodology, characterization and evaluation of the dissertation work

The dissertation is written on 175 pages and is constructed in a classic style with a good balance among the sections. The results are illustrated with 36 figures, 7 tables and 4 illustrations. Information about the publications on the topic, citations, participation in research projects and scientific conferences, and awards received is also included.

The results of the own research are presented in seven sub-sections, according to the sequence of the research, and data on the phytochemical and pharmacological research carried out using a wide range of methods are presented in detail. The discussion section is divided in two sub-sections, logically reflecting the direction of the work, including selection of plant species and their metabolites, monitoring and analyzing the effects, *in vitro* and *in vivo*, of the application of obtained extracts, fractions and individual compounds (incl. isolated in the course of work) on the transcription factor Nrf2 in the selected target population of neutrophils in models of joint inflammatory and degenerative diseases, as well as the mechanism of action of substances on Nrf2 in a model of joint chronic inflammation. All studies were conducted using a systematic, well-planned and consistent approach, based on ethnopharmacological data and a precise literature review of plants and their metabolites with anti-inflammatory activity.

The metabolic profiling of the obtained plant extracts and the identification of the isolated compounds was carried out by NMR spectroscopy (1D and 2D experiments), and for the purposes of quantification of the components in extracts and fractions - HPLC. For *in vitro* and *in vivo* assessment of biological activity and therapeutic potential flow cytometry, immunofluorescence, ELISA, RT-qPCR, etc. were used. The instrumental methods used are up-to-date and modern.

My overall impression is that the dissertation is serious and thorough research that has been conducted at a high scientific level. The high competence of the scientific supervisors Assoc. Prof. Dr. Petya Dimitrova and Prof. Dr. Milen Georgiev also has an indisputable contribution to the choice of highly relevant topic of the PhD Thesis and its high scientific quality.

The research presented in the dissertation and the obtained original results on the effect of pharmacological modulation of Nrf2 expression in neutrophils in degenerative and inflammatory joint diseases are of a scientific and scientific-applied nature.

**The main contributions** of the dissertation can be summarized as follows:

✓ The chemical composition of four plant species *Ballota nigra*, *Clinopodium vulgare*, *Leonurus cardiaca* and *Haberlea rhodopensis* was revealed by NMR profiling, and main biologically active components were isolated from *H. rhodopensis*;

✓ Therapeutic effect of the oleanane triterpene CDDO-Me as an activator of Nrf2 in a model of degenerative joint disease (collagenase-induced osteoarthritis in mice) at topical application was confirmed, and for the first time was proven that the activation reduces the sensitivity of mouse neutrophils to senescence;

✓ Osteoarthritis progression was found to be accompanied by the acquisition of a senescent neutrophil phenotype;

✓ *C. vulgare* extract and its metabolites caffeic and chlorogenic acids were found to suppress COX-2 cyclooxygenase expression;

✓ For a fraction of *H. rhodopensis* extract containing myconoside and calceolarioside E (1:0.6) as well as for the individual components, it was found: influencing the expression of the *NFE2L2* gene and the percentage of Nrf2 positive neutrophils; the mechanism of action of the two pure substances on Nrf2; improvement of symptoms and pathological changes in the arthritis model. *The results lead to the conclusion that a combination of the active components is applicable for effective pharmacological modification of Nrf2;*

✓ Biotechnological approach for cultivation of *H. rhodopensis* Friv was developed, registered as a utility model, in which the bioproduction of the pharmacologically active phenylethanoid glycoside myconoside exceeds that of the wild species by several times.

## 5. Assessment of the publications and personal contribution of the PhD student

The research included in the PhD thesis is summarized in 4 publications in Q1 quartile and high impact factor scientific journals: *International Journal of Molecular Sciences* (IF<sub>2021-2022</sub> 6.208), *Cellular and Molecular Life Sciences* (IF<sub>2021</sub> 9.234) and *Food and Chemical Toxicology* (IF<sub>2019</sub> 4.679). This, as well as the 25 citations noted, is sufficiently indicative of the relevance, quality and significance of the conducted research. The presentation of the results at international conferences and the distinction of the works with a total of 3 awards is also indicative. The PhD student is first author in three of the scientific papers and third in one, which undoubtedly confirms her personal contribution to the research.

## 6. Abstract

The presented abstract is prepared according to the requirements and reflects the structure of the dissertation.

## 7. Critical remarks

I have no critical comments on the research conducted. Regarding the presented dissertation, apart from noticing inaccuracies in the statements, I have remarks related to terminological and factual inaccuracies:

- incorrect description: "core structure" (**it is correct basic structure**); in Table 1 - "chemical shift constant" (*chemical shift*), "pairing constant" (multiplicity, *coupling constant*); "triterpenoid CDDO-Me that contains  $\beta$ -unsaturated carbonyl groups in rings A and C" (*triterpenoid CDDO-Me that contains an  $\alpha,\beta$ -unsaturated carbonyl fragment in rings A and C*);

- incorrect classification: "phenolic acids (caffeic, chlorogenic, catechin)" - catechin is a flavonoid, it should be *rosmarinic acid*; "phenylethanoid glycosides (myconoside, rosmarinic acid, forsythoside B)" - rosmarinic acid is not a glycoside; "diterpenes (ballotetroside, ballonigrin, ballotinol)" - ballotetroside is a phenylpropanoid glycoside; probably ballotenol or ballotinone, not ballotinol;

- in item V.2. no data for identification of forcytoside B, "ballotinol" and ballotetroside are provided as stated on pp. 133, 134, 143 and 145. Conclusion VII.2., and contribution 1 accordingly, is incorrect and incomplete with respect to all identified metabolites;

- for the results of the NMR profiling of the extracts, it is referred Table 7 and Table 1 in the dissertation and the abstract, respectively. However, no data are presented for the compounds ballonigrin, verbascoside, leonurine, stachydrine, myconoside and calciolarioside E. Apart from the missing data in the Table(s), NMR data for all these compounds are described/presented/cited in the dissertation, but not in the abstract, which adds to the ambiguity;

- it is necessary to present the chemical structures of the corresponding compounds in Figures 5b, 6 and 7, as the NMR data for three of the compounds are discussed in detail in the text; the arrows in Figure 6 do not indicate the cross peaks corresponding to the interactions described in the text; Figures 7b and 7c are identical, and the range (up to 4.5 ppm and 85 ppm) is indicative only of the presence of stachydrine;

- for *H. rhodopensis* (pages 83 и 132) - it is stated that paucifloside is identified, correctly it is paucifloside, but, as it is actually correct, structure and data for calceolarioside E are presented and discussed;

- on page 134 a link between *L. cardiaca* and labdane diterpenes is stated, one of them is "*H. rhodopensis* extract contains structurally different compounds, phenylethanoid glycosides (myconoside, calceolarioside E) of *L. cardiaca* where we identify diterpenes (ballotetroside,

ballonigrin, ballotinol)" - ballotetroside is a phenylethanoid glycoside, and the combination of ballotetroside, ballonigrin and ballotenol/ballotinone is characteristic for *B. nigra*. Moreover, according to item V.2 in *L. cardiaca* alkaloids are identified and the focus is on them. Therefore, it is not clear which extract was tested.

The above-mentioned remarks do not detract from the results achieved by the PhD student and do not affect the essence of the work, and the overall excellent impression and merits of the dissertation.

### CONCLUSION

The PhD Thesis contains original scientific and scientific-applied contributions and developments, which cover and exceed the requirements of the Law for the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for its Implementation and the Regulations for its Implementation of the Bulgarian Academy of Sciences. The presented materials and dissertation results fully comply with the specific requirements of the Regulations of the IMikB-BAS for the application of LDASRB.

Due to the above, I confidently give my **positive assessment** of the research conducted, the results achieved and the contributions, and **I propose to the Honorable Scientific Jury to award the educational and scientific degree "doctor"** to Kristiana Miroslavova Amirova in the field of higher education 5. Technical science, Professional filed 5.11. Biotechnology, PhD program: Technology of biologically active compounds.

Sofia, 25 September 2023

Reviewer: .....  
/Prof. Dr. Milena Popova/