

REVIEW

by Prof. Dr. Svetla Danova, D.Sc.

The Stephan Angeloff" Institute of Microbiology, Bulgarian Academy of Sciences

of the PhD thesis titled:

DEVELOPMENT OF BIOLOGICALLY ACTIVE PRODUCTS FROM NEW NATURAL SOURCES

Presented by **Milena Nikolova Petrova**

for awarding the educational and scientific degree "**Doctor**"
in the field 4 "Natural sciences, mathematics and informatics";

Professional direction **4.3. "Biological Sciences"**

Doctoral program: "**Microbiology**"

With scientific supervisors: **Prof. Dr. Petya Koycheva Hristova**

Prof. Dr. Ganka Todorova Chaneva

ABOUT THE PROCEDURE:

The current procedure for the acquisition of PhD degree "Doctor" is conducted on the basis of Art. 4 of the Law on the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB), in connection with §7 of the Transitional and final provisions of the Regulations on the terms and conditions for acquiring scientific degrees and occupation of academic positions at Sofia University "St. Kliment Ohridski". According to Order (No. RD-38-389/13.07.2022.) of the Rector of Sofia University I was elected as a member of the Scientific Jury for the above-mentioned competition and I was designated as a reviewer at its first meeting. In my capacity as such, I declare that there is no conflict of interest within the meaning of §1, item 2a of the additional provisions of the Law (ZRSARB) between me and the candidate under the procedure for the PhD "Doctor" and I am not subject to the restrictions under Art. 33 of ZRASRB.

The set of documents and materials presented to me on paper and electronic variant meets the requirements of the Law (ZRASRB) and the Regulations on the Terms and Conditions for the Acquisition of Scientific Degrees and for the Occupancy of Academic Positions at BF-SU.

The absence of plagiarism in the candidate's scientific works has been proven according to the law.

RELEVANCE AND SIGNIFICANCE OF THE SUBJECT DEVELOPED

Presented dissertation, submitted for review has the ambitious title: “Development of biologically active products from new natural sources”. The team (the PhD student and supervisors) is trying to contribute to the solution of two serious scientific challenges:

(1) Urgent need to search for new medicinal products in the fight against foodborne pathogens and the growing antibiotic resistance in microbes from different habitats caused by increased and uncontrolled intake of antibiotics;

(2) Plant health and the consequences of pesticide use in terms of disease-causing microorganisms that contribute to the expansion of cross-pathogens and the urgent need to find complex biocompatible solutions limiting this negative for consumer health chain.

In this regard, the thesis looks for opportunities in the potential of hemocyanins from invertebrates and microalgae with their metabolites and cell fractions. Regardless of the fact that these are foundational studies, they are originally combined and will generate a good basis for further development and achieving in the future ready-to-implement products, as the title suggests. All this gives me the reason to rate this work as actual, unconventional and very up-to-date.

SCOPE AND STRUCTURE OF THE DISSERTATION

The dissertation work is well ordered and structured and meets the established requirements. The PhD thesis is laid out on 182 pages and includes: *Introduction* – 3 pages; *Literary review* -47, *Aims and tasks* - 1 page; *Materials and methods* – 20 pages, *Results and discussion* – 60 pages; *Conclusion* – 1 page, *Contributions* -1 page; *References*- 22 pages and *Appendices* - 21 pages. The material is richly illustrated with 22 figures and 44 tables. Thanks to the attached *Detailed list of figures* -2 pages and with the *List of tables* -2 pages, the data summarized in them can be easily found and analyzed. I would only suggest, purely technically, that the sheets with the used abbreviations and those with tables and figures be placed at the beginning and not as the last at the end of the Appendix and the dissertation.

The dissertation is clearly written in good scientific language. A good impression is also made by the logical arrangement in the literature review and in the overall shaping of the results.

LITERARY AWARENESS AND STATEMENT OF GOAL AND OBJECTIVES

The literature review of the dissertation comprehensively and systematically presents the natural sources of antimicrobial substances that are the subject of research - *Microalgae, invertebrates, hemocyanin producers, higher plants and microorganisms*. The targets for the sought-after active products were also examined: *Pathogenic bacteria - human and phytopathogenic*.

In a logical sequence, the PhD student introduced: "*Methods for introducing a natural sample in laboratory conditions*" and "*The possibilities of combined therapy with microalgae extracts*". This structure shows a well-considered scientific framework in the development, the combination of a good knowledge of the theory and an aspiration to evaluate new approaches in solving the outlined challenges related to the need to find new biologically active products.

The knowledge of the novelties in the taxonomic classification of cyanobacteria and the citation of the previous Bulgarian experience make an excellent impression. A lot of scientific literature is summarized. A huge number are cited, even for a DS.c. dissertation, 477 references. Of these, only 19 are in Bulgarian and together with others, they present the Bulgarian experience on the investigated problems. Thus, the building-up research carried out by the doctoral student can be clearly outlined. A properly structured and comprehensive review logically leads to formulating the purpose of the dissertation work. The PhD student has focused her efforts on "research and evaluation of the antioxidant and antimicrobial activity of bioactive substances from new natural sources - products from microalgae and invertebrates (hemocyanin)". For its implementation, 7 clearly defined experimental tasks have been set.

1. *Isolation, identification and laboratory cultivation of promising strains of microalgae;*
2. *Preparation of extracts and exopolysaccharides from selected microalgae strains;*
3. *Determination of the antioxidant activity of microalgae products;*
4. *Determination of the antimicrobial activity of microalgae extracts and exopolysaccharides against human and plant pathogens;*
5. *Investigation of the antimicrobial activity of a combination of microalgae extracts;*
6. *Determination of the antimicrobial activity of natural antibiotics in relation to phytopathogens;*
7. *Determination of the antimicrobial activity of hemocyanin against human pathogens.*

EVALUATION OF USED MATERIALS AND METHODS

Solving the set tasks is based on a panel of different research approaches and the combination of different methods in full compliance with the interdisciplinary nature of the dissertation. The PhD student used 3 types of microalgae and selected a large group of microorganisms as follows: 6 isolates from food products: *E. coli*, *Bacillus* sp., *Listeria* sp., *Enterococcus* sp., *Staphylococcus* sp.; 1 strain isolated from polluted waters: *P. aeruginosa* and strains of 4 types of phytopathogenic bacteria: *Xanthomonas gardneri* 62t, *X. gardneri* 64t, *X. euvesicatoria* 105t, *X. euvesicatoria* 269p, *X. vesicatoria* 68t, *X. vesicatoria* 60t, *P. syringae* pv. *tomato* 32f. The number of tests is impressive - the cultures of human pathogens (9 Gram-positive and 10 Gram-negative bacteria and 4 species of yeast representatives of the genus *Cryptococcus* and genus *Candida*) as well as phytopathogenic bacteria (9 Gram-negative - and 1

species of Gram-positive: *Clavibacter michiganensis*) against which an inhibitory effect was sought. This selection is well justified by the high pathogenic potential of the species.

Methodical approaches to the assessment of biological activity are correct. The modifications of the classic microbiological methods for activity assessment by the diffusion in agar method are described, in view of the specifics in the characterization of the different microalgae fractions. Minimum inhibitory and minimum bactericidal concentrations were correctly estimated. The selection of nutrient media for microbial test cultures and experiments is correct. I especially want to highlight the created algorithm with a mathematical model for estimating inhibition zones from photographic material. I appreciate it as a very good author's solution and it shows the developed skills of a microbiologist who successfully solves problems, in search of new solutions.

Both classic microbiological techniques for purification, cultivation and taxonomic determination of microalgae strains, as well as modern molecular methods - PCR and sequencing analysis of genes recognized as the gold standard in taxonomic research - have been mastered. The noted analyzes within the framework of a master's thesis and with the help of specialists from the Institute of Plant Physiology - BAS make a good impression. which have allowed the doctoral student to acquire new knowledge in the field of microalgae.

It is correctly stated that hemocyanin was provided by the "Structural Organic Analysis" laboratory at the "*Institute of Organic Chemistry with Phytochemistry Center*", BAS.

The PhD student also applies various biochemical methods: Obtaining extracts from microalgae biomass, isolation of endopolysaccharide; determination of absolute dry matter, Beltuck according to Lowry; on the amount of total lipids, the content of photosynthetic pigments in algal extracts. The PhD student successfully combined 2 different methods for evaluating antioxidant activity - DPPH-radical-trapping activity and total antioxidant activity (TAA).

EVALUATION OF THE RESULTS OBTAINED

We can conditionally consider the results of the dissertation in 3 sections

(1) selection, isolation and characterization of microalgae - producers of active substances, in which 3 strains of microalgae were studied, two of which were isolated from the environment and were taxonomically defined and 1 isolated by the doctoral student;

(2) obtaining a total of 16 extracts, for the purposes of the dissertation

(3) laboratory evaluation of antimicrobial and antioxidant activity of the different extracts.

The exposition de facto merges the first two tentatively outlined sections of the experimental part. An interesting case was solved regarding the identification of microalgae isolate HPV-A1. Of undoubted interest are the results with the identification of this isolate. I highly appreciate the polyphasic approach combining classical morpho-physiological with genetic identification and

the inclusion of sequence analysis not only of the two taxonomically significant *ITS* regions but also of the *rbcL* gene.

The tasks of obtaining extracts and exopolysaccharides from the 3 microalgae strains and the tasks of determining the antioxidant activity of the obtained extracts were successfully solved. Milena Petrova's assessment of which of the extracts have promising antioxidant activity makes a good impression. These are new data because the antioxidant properties of microalgae are still poorly studied. Objectively, the alcoholic extracts obtained from the biomass of the investigated microalgae were evaluated as the best antioxidants with maximum amounts of phenolic and flavonoid compounds.

The results of determining the antimicrobial activity are of practical importance. The established panel of 44 strains of test microorganisms, of which 28 are pathogenic for humans and 16 are phytopathogens causing diseases on fruit and agricultural crops, outlines a very high inhibitory potential. In an effort to objectively evaluate the antimicrobial potential of the new extracts, the doctoral student searches for and creates a new method for determining the zone of inhibition based on photographic material, together with mathematicians. The method has been validated and the results have been reported in a scientific forum. A valuable algorithm for standardizing the results of the agar-diffusion method has been developed and I recommend that it be made available to the wider scientific community. The results are proof. The PhD student evaluates 6 products from each of the three types of microalgae - four are from the biomass (high-temperature extract, low-temperature extract, endopolysaccharide and alcoholic extract), the cell-free supernatant and exopolysaccharide. An algorithm has been created for the evaluation, which includes:

(1) . Determination of microbial sensitivity to extracts in two variants - Antibacterial activity against phytopathogenic bacteria and against human pathogens evaluated against control - copper sulfate for phytopathogens and antibiotic gentamycin for human pathogens

2) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of microalgae extracts

The summary made for each of the three microalgae developed in the dissertation makes a very good impression. The obtained results complement the information on the biological activity of *Nostoc commune* Vaucher and *Arthonema africanum*. The doctoral student has preemptively included phytopathogens new to our country, which makes the obtained results very valuable. Moreover, a synergistic effect of the microalgae extracts selected as the most active against phytopathogenic bacteria was sought, combining them in different concentrations. The analyzes were performed against three strains of phytopathogenic bacteria: *X. euvesicatoria* 269p and *P. syringae* pv. tomato 32f and *E. amylovora* NBPMKK 8492.

I consider as an original element in the part with antimicrobial activity, the inclusion of hemocyanin fractions obtained from marine crabs *Eriphia verrucosa*, which were collected in the region of Kamchia, Black Sea. Thus, the group of newly characterized biologically active substances, which the doctoral student defines as potential products, has been expanded.

CONTRIBUTIONS AND SIGNIFICANCE OF THE DEVELOPMENT FOR SCIENCE AND PRACTICE

The 9 conclusions and 5 contributions made are a logical consequence of the entire development and provide objective information about the value of the conducted research. They are clearly and precisely formulated. The obtained results can serve as a basis for the development of plant protection products, which Milena Petrova seems to have set as a long-term goal, coded under the title of her dissertation. At the stage of the presented work, the tested extracts cannot yet be summarized as a finished product.

CRITICAL NOTES, QUESTIONS AND RECOMMENDATIONS

The dissertation work is up-to-date and unquestionably represents an original interdisciplinary development in accordance with the requirements of ZRASRB and its Regulations. Without diminishing the merits, I would make a few recommendations and critical notes for the future development of the subject and the presentation of the undeniably good results:

The PhD student is very sparing in her discussion of the results. E.g. in the section on the identification and study of the biochemical composition of microalgae, it would be good to compare the data obtained with those of other microalgae. The paper has useful information on the composition of the microalgae, which would also be good to evaluate in relation to the extracts obtained and the likely nature of the biologically active metabolites/extracts. Nowhere is an important product indicator noted - the yield of biomass, especially with this long growth cycle of a microscopic strain, and the yield of the desired biologically active extracts that lead to the desired new products. Thus, a significant volume of the work remains as if aside from the purpose and title of the dissertation work.

Only in the contributions is it noted which results were obtained for the first time. And there are undoubtedly such, and it is good to outline them in the discussion. In this regard, I would ask the doctoral student if it is possible that the HPV - A1 isolate is a new species from the *Chlorellaceae* family and closely related to those of the *Muriella* genus. I would recommend bolder presentation of the original/authored elements of the experimental protocols. Based on the comparative evaluation of microalgae extracts and hemocyanins, which has greater application potential? What is the reason that the data obtained on the evaluation of the antioxidant activity, set as the aim of the dissertation, do not find a place in the conclusions drawn?

CONCLUSION

The dissertation contains scientific and scientific-applied results, which 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 (ZRASRB), the Regulations for the Implementation of ZRASRB and the relevant Regulations of BF-SU and SU.

The dissertation unequivocally shows that PhD student Milena Petrova possesses the theoretical knowledge and professional skills of a microbiologist and molecular biologist, demonstrating qualities and skills for independent conduct of scientific research, incl. and solve scientific challenges.

Based on the above, I give my positive assessment of the dissertation work and propose to the honorable scientific jury to award the educational and scientific degree "Doctor" to Milena Petrova in field 4 "*Natural sciences, mathematics and informatics; professional direction 4.3. "Biological Sciences"* (Microbiology).

09/09/2022

Reviewer:.....

(Prof. S. Danova, PhD)