REVIEW

by

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Subject: Dissertation thesis titled "Comparative Study of the Biological Effects of Natural Dietary Sources of Sulfur on Human Intestinal Cells," prepared and submitted by: Stefka Marinova Stoeva, a full-time PhD student in the doctoral program "Biochemistry," professional field 4.3. "Biological Sciences," Faculty of Pharmacy, Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, for the acquisition of the educational and scientific degree "Doctor" (PhD).

1. GENERAL OVERVIEW OF THE PROCEDURE AND THE DOCTORAL CANDIDATE

By Order No. R-109-320 dated September 25, 2026, issued by the Rector of the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna (MU-Varna), I was appointed as a member of the scientific jury for the procedure for the acquisition of the Educational and Scientific Degree "Doctor" (PhD) in the specialty of "Biochemistry" (Professional Field 4.3: "Biological Sciences") and was tasked with preparing a review.

The author of the dissertation is Stefka Stoeva, a full-time doctoral student in the "Biochemistry" program at the Department of Biochemistry, Molecular Medicine, and Nutrigenomics, Faculty of Pharmacy, Medical University "Prof. Dr. Paraskev Stoyanov" – Varna. Her academic supervisor is Assoc. Prof. Milka Asparuhova Nashar, PhD, from the same department.

For the purpose of preparing this review, I received the materials required by the „Regulations on the Conditions and Procedures for Acquiring Scientific Degrees and Occupying Academic Positions at MU-Varna", namely:

- ✓ An application to the Rector to initiate the defense procedure;
- ✓ A curriculum vitae (CV);
- ✓ Records of passed doctoral examinations ("minima") in Biochemistry and a foreign language;
- ✓ A protocol from the Department Council regarding the completion of the doctoral program;
- ✓ Orders for enrollment and for completion of the doctoral program;

- ✓ An abstract (summary) of the dissertation and the dissertation itself;
- ✓ Declarations attesting to the originality of the work and the authenticity of the submitted documents. Report on similarity and verification of an existing current profile;
- ✓ List and copies of publications related to the topic of the dissertation;
- ✓ Lists of participation in scientific forums, training sessions, and seminars, as well as participation in projects;
- ✓ Certificates.

My assessment is based on the requirements of the „Act on the Development of the Academic Staff in the Republic of Bulgaria", the „Regulations for its Implementation", and the „Regulations for the Development of the Academic Staff at the Medical University of Varna". The submitted documents meet the requirements of the aforementioned regulatory acts.

The doctoral candidate, Stefka Marinova Stoeva, was born in the town of General Toshevo (Dobrich Municipality), where she completed her secondary education. In 1984, she completed specialized secondary education as a nurse at the "Dr. N. Nikolaev" Institute for Paramedical Personnel in Varna. In 1994, she graduated from Sofia University "St. Kliment Ohridski" with a Master's degree in Biochemistry and Microbiology, specializing in Animal and Human Physiology. In 2004, she also obtained a Bachelor's degree in Nursing from the Medical College of the "Prof. Dr. Paraskev Stoyanov" Medical University of Varna.

In the period 1986-1989 and in 1995, she worked successively as a nurse in the ORKB Dobrich and in the DIBA-KVB, Hematology Clinic in the city of Sofia, and from 1995 to 2000 - as a biochemist in the IV Diagnostic-Consultative Center, Clinical Laboratory, Varna. From 2001 to the present, Stefka Stoeva holds the position of biochemist in the Department of Biochemistry, Molecular Medicine and Nutrigenomics of the Faculty of Pharmacy at the Medical University "Prof. Dr. P. Stoyanov" - Varna. By order No. R-109-282 of 07/08/2021, she was enrolled as a full-time doctoral student at the same department. She was expelled from doctoral studies with the right to defend by order P-109-249/20.07.2026 after successfully passing an internal defense.

2. GENERAL CHARACTERISTICS OF THE DISSERTATION

2.1. Relevance of the Topic

This study focuses on a comparative assessment of the biological effects of various natural sources of sulfur-containing biologically active compounds—specifically, diverse food products and mineral waters with a distinct composition that includes sulfur compounds, such as the mineral water from the Varna Basin. The research was conducted in vitro using human intestinal epithelial cells.

The diversity of bioactive molecules of plant origin underpins the traditional use of plants across various cultures for the purposes of prevention and therapy. Certain plant species—such as those belonging to the genera *Brassica* and *Allium*—synthesize unique sulfur-containing compounds (secondary metabolites) that activate the body's endogenous defense systems against oxidative stress and inflammation. By regulating key signaling pathways, these plant-derived phytochemicals facilitate cellular adaptation and the maintenance of tissue homeostasis.

Sulfur-containing (sulfide and sulfate) mineral waters exert their physiological effects through a combination of chemical, thermal, and mechanical factors. The primary bioactive agent within them is dissolved free hydrogen sulfide (H2S), as well as hydrosulfide (HS^-) and sulfate (SO_4^{2-}) ions. H2S is the third known endogenous gasotransmitter (alongside NO and CO) and possesses exceptional lipophilicity, allowing it to rapidly permeate biological membranes (including the skin barrier and mucous membranes). The significance of this research is particularly great for Varna, as it focuses on a natural resource that is readily accessible and traditionally utilized by the city's residents and visitors. The accumulated scientific data provide a foundation for further research and for the evidence-based promotion of mineral waters as a component of the city's natural and health-related potential. This creates a basis for future interdisciplinary studies in the fields of disease prevention, balneology, and health tourism.

Based on the above, it can be concluded that the subject of this dissertation is timely, significant, and holds high potential for practical application.

2.2. Aim and Objectives

The aim of the dissertation is clearly formulated based on an analysis of existing knowledge, as well as the ambiguities and gaps in the current understanding of the problem. The research work aimed at achieving this goal—"to conduct a comparative analysis of the biological effects of natural dietary sources of sulfur under basal conditions and under conditions of LPS-induced inflammation in human intestinal epithelial cells"—is logically structured into three main research objectives. These objectives facilitate the collection of data and a comparative analysis of the anti-inflammatory, antioxidant, and adaptogenic profiles of the sulfur sources under study, as well as their influence on detoxification processes.

2.3. Scope and Structure

The dissertation spans a total of 154 pages and is organized into the sections standardly recommended for a PhD thesis: introduction, literature review, aims and objectives, materials and methods, results, discussion, conclusion, final takeaways, scientific contributions, and references. The text is written in a rigorous scientific style that is both clear and readable. It is well-supported and illustrated by 73 figures, 2 diagrams, 1 photograph, and 6 tables. The

bibliography comprises 298 entries, 2 of which are in Cyrillic, while the remaining 296 are in the Latin script.

2.4. Funding

The experimental work for this dissertation was funded by the "Science" Fund of the Medical University of Varna, under Project No. 22003: "A Comparative Study of the Biological Effects of Natural Dietary Sources of Sulfur on Human Intestinal Cells."

3. CHARACTERIZATION AND ASSESSMENT OF THE DISSERTATION AND ITS CONTRIBUTIONS

3.1. Literature Review

The literature review is based on contemporary sources. In it, the doctoral candidate provides a comprehensive and in-depth overview of existing knowledge regarding sulfur as a biogenic element. She characterizes sulfur-containing mineral waters (referred to as "sulfur waters" throughout the remainder of the text) and discusses the origin and biological effects of sulforaphane, alliin, and taurine, thereby identifying the scientific problem and the need for further comparative research. The review also includes a section that examines, in detail, the potential for using the adherent cell line of human intestinal epithelial cells (HIEC-6) as a cellular model for screening the biological effects of foods and waters.

In my view, the literature review establishes the necessary foundation and context for understanding the aim, objectives, and hypotheses of the study. The comparative design allows for the assessment of similarities and differences among the agents under investigation, without assuming that their mechanisms of action are identical. The doctoral candidate demonstrates an excellent grasp of the relevant scientific literature, as well as the ability to select, systematize, and analyze scientific information.

3.2. Research Methodology

The methodology is characterized by a detailed and competent presentation of the research process. The doctoral candidate employed research methods that are both reliable and sufficient for achieving the stated objective. Furthermore, she utilized a model of LPS-induced inflammation in the HIEC-6 cell line—a model previously established within the Laboratory of Nutrigenomics—using cells obtained from the American Type Culture Collection (ATCC® CRL3266™). Following cytotoxicity testing, the assessment of gene expression levels for 14 specific genes addressed the research objectives of the dissertation; this was achieved through a comparative analysis of the anti-inflammatory, antioxidant, and adaptogenic profiles of the sulfur sources under investigation.

The statistical methods were appropriately selected to provide the level of information required for the study. Changes in gene expression were analyzed using the 2^−ΔΔCt method, with results expressed in relative units (RU) of mRNA. The relative expression of the target genes was determined by comparison with untreated cells (control, C), for which the gene expression level was arbitrarily set to 1. The genes for β-actin (ACTB) and RPLP0 were used as endogenous controls.

All samples were analyzed in three independent technical replicates, and mean values were used for statistical analysis. Data are presented as mean ± standard error of the mean (Mean ± SEM) for expression levels, or as mean ± standard deviation (Mean ± SD) for the MTT assay. Correlation analyses were performed using GraphPad Prism 10.0 via Pearson correlation matrices, visualized as heatmaps. These analyses were used to assess similarities in gene expression profiles across different experimental conditions. Differences between groups were considered statistically significant at a significance level of $p \leq 0.05$.

3.3. Results and Analysis

The results are presented clearly, in detail, and in a logical sequence. Their analysis is contextualized within existing scientific literature, and the doctoral candidate demonstrates analytical and critical thinking throughout the discussion. The discussion is concise, avoiding excessive or unsubstantiated interpretation. The tables are well-organized. The figures and diagrams effectively illustrate the results and further facilitate their understanding.

In the experimental model employed, sulfur-rich mineral water, sulforaphane, alliin, and taurine preserve cell viability within the tested concentration ranges, thereby enabling further analysis of their biological effects. The data obtained regarding the viability of the human intestinal epithelial cells (HIEC-6) provide an experimental basis for interpreting subsequent changes in gene expression—changes that may be linked to the effects of consuming sulfur-containing mineral waters and foods.

It has been established that the sulfur sources investigated differ in their ability to modulate the inflammatory response in human intestinal epithelial cells. Sulfur-rich mineral water exerts a broad range of effects on the expression of key genes encoding pro-inflammatory markers, highlighting its potential to influence molecular mechanisms associated with the cellular inflammatory response. As expected, sulforaphane has the most significant impact on the expression of genes involved in antioxidant defense and cellular adaptation, whereas the effects of taurine and alliin on these signaling pathways are more limited and highly dependent on the concentration applied. In addition to its influence on pro-inflammatory mediators, sulfur-rich mineral water also positively modulates the expression of genes associated with the antioxidant response. The sulfur sources studied exhibit varying selectivity regarding the expression of sulfotransferases: sulfur-rich mineral water primarily affects the expression of SULT1A1, while

sulforaphane and alliin have a more pronounced effect on SULT1A3. The most consistent expression relationship observed is the Nrf2/GPx axis; this correlation is positive, with both components changing in the same direction across all treatments—both in unstimulated cells and under conditions of LPS-induced inflammation. Conversely, NF-κB and Nrf2 show a consistent negative correlation across all matrices. These results support the existence of coordinated expression profiles, the manifestation of which depends on the nature of the agent and the cellular state.

3.4. Evaluation of contributions

I accept the doctoral candidate's self-assessment regarding the contributions of the dissertation work—specifically, two contributions of an original nature, one of an applied nature, and one of a confirmatory nature. It should be highlighted that this research provides the first in vitro experimental data on the effects of sulfur-containing mineral water from the Varna basin on the expression of genes associated with inflammatory and redox-related cellular responses, as well as biotransformation, in HIEC-6 intestinal cells—including in a model of LPS-stimulated immune response. Furthermore, knowledge regarding the molecular effects of the under-researched organosulfide alliin has been expanded, and its potential to modulate the expression of genes linked to inflammatory and redox-related cellular responses and biotransformation has been established. A comparative cellular approach was developed and applied to preliminarily assess the molecular effects of natural sulfur sources in HIEC-6 cells under both basal and LPS-stimulated conditions. The results obtained for sulforaphane and taurine complement and support existing literature regarding their influence on the expression of genes associated with inflammatory and redox-related cellular responses.

4. ASSESSMENT OF PUBLICATIONS RELATED TO THE DISSERTATION

The results of the dissertation are presented in two scientific publications and six scientific communications, demonstrating the successful dissemination of the scientific and applied scientific contributions of the work in reputable journals. One publication appears in the *Journal of IMAB* (Web of Science, IF 0.2), and another—in which the PhD candidate is the first author—appears in *Pharmacia* (Web of Science, IF 1.5). Three of the abstracts correspond to scientific communications presented at European and global congresses, and the PhD candidate is the first author of four of the published scientific communications.

5. CRITICAL REMARKS AND RECOMMENDATIONS

I recommend expanding the scope of the study to include the detection of the investigated cytokines and transcription factors—factors that regulate DNA transcription as part of inflammatory processes and the immune response, contribute to the maintenance of cellular redox balance, and play a role in biotransformations. I expected the literature review to provide a rationale for the genes under study, as well as to establish the connection between the regulation of redox homeostasis and detoxification processes, and to explain how these elements fit into the LPS-induced inflammatory response model employed.

6. CONCLUSION

The presented dissertation thesis constitutes a complete, independent, and methodologically sound scientific study of distinct scientific value and indisputable contributions. It advances the understanding of the biological significance of sulfur-containing foods and waters, as well as their potential to influence signaling pathways related to the regulation of redox status, inflammation, and stress adaptation, along with enzymes involved in detoxification processes.

The PhD candidate has demonstrated the necessary scientific knowledge and skills to conduct independent research—ranging from assessing the significance of a scientific problem and formulating a research concept, to designing, planning, and executing studies, and critically analyzing the obtained results. The recommendations I have provided in no way diminish the merits of the dissertation. The dissertation meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Implementation, and the Regulations for the Development of the Academic Staff at the Medical University of Varna. In light of the above, I provide a positive assessment of the dissertation and the candidate's qualifications, and I vote in favor of awarding the educational and scientific degree of "Doctor" (PhD) in the specialty of "Biochemistry"—within the professional field 4.3 "Biological Sciences"—to Stefka Marinova Stoeva.

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Заличено на основание чл. 5, §1, б. „В“ от Регламент (ЕС) 2016/679

21.10.2026