REVIEW

on a PhD THESIS entitled:

"Comparative study of the biological effects of natural dietary sources of sulfur on human intestinal cells"

for the award of the Educational and Scientific Degree of "Doctor"
Field of Higher Education: 4. Natural Sciences, Mathematics, and Computer Science
Professional Field: 4.3. Biological Sciences (Biochemistry)

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Reviewer: Prof. Dr. Svetla Dimitrova Petrova
Department of Biochemistry, Faculty of Biology, Sofia University "St. Kliment Ohridski"; Member of the Scientific Jury, appointed by Order No. R-109-320/September 25, 2026, issued by the Rector of the Medical University "Prof. D-r Paraskev Stoyanov" – Varna, Prof. Dr Dimitar Raykov, DSc.

1. General characteristics of the dissertation and the presented materials

Stefka Stoeva's PhD thesis is written following a standard structure: Introduction and Literature Review (30 pages); Aims and Objectives (1 page); Materials and Methods (10 pages); Results (58 pages); Discussion (29 pages); Conclusion (3 pages); Contributions (1 page); Appendices - including scientific contributions, a list of publications (2 publications with JIRanking), and participation in scientific forums (3 international and 3 national), as well as a bibliography citing 298 literary sources. The dissertation spans 154 pages and includes 73 figures, 2 schemes, 1 photograph, and 6 tables, all presented in an excellent format. The abstract accurately reflects the results and content of the dissertation and meets all formal requirements. All presented materials fully meet the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB).

2. Brief biographical data

Stefka Stoeva holds a Master's degree in Biochemistry and Microbiology with a specialization in „Animal and Human Physiology" from the Faculty of Biology at Sofia University "St. Kliment Ohridski," alongside a qualification as a Biology Teacher. Over the following 10 years, she worked as a nurse in the Cardiology Department at the Regional Hospital in Dobrich, the Hematology Clinic in Sofia, and subsequently as a biochemist in the Clinical Laboratory at Diagnostic and Consultative Center 4 (DKC 4) in Varna. Since 2001, Stefka Stoeva has worked as a biochemist at the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, Department of Biochemistry, molecular medicine, and nutrigenomics at the Faculty of Pharmacy. In 2004, she graduated from the Medical College of the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, and since 2021, she has been a full-time PhD student in the Department of Biochemistry, molecular medicine, and nutrigenomics, under the supervision of assoc. prof. Dr. Milka Nashar.

3. Originality and significance of the scientific problem

The topic of this dissertation is devoted to investigating the biological significance of certain natural sources of sulfur whose efficacy and biological role have not been fully studied - sulfur-containing mineral water (a complex natural system), sulforaphane, taurine, and alliin. An experimental design was developed using the specialized intestinal cell line HIEC-6 (not previously used for such studies), which allows an assessment of the effects of the studied biological agents on cytotoxicity and the transcriptional levels of target genes associated with inflammatory, oxidative, and cell adaptive processes, under comparable experimental conditions. The use of this cell line as an experimental cell model is well-explained. The dissertation presents an *extensive new study* that serves as a continuation of in-depth molecular research conducted at the Department of Biochemistry, molecular medicine and nutrigenomics, while opening up potential for the expansion of this scientific field. Stefka Stoeva's research is particularly important and biochemically necessary for determining the individual molecular mechanisms of action of the sulfur sources derived from food and water, but aims to demonstrate their role in the integration and regulation of processes such as inflammation, oxidative stress, cellular adaptation, and biotransformation.

4. General characteristics of PhD thesis

Stefka Stoeva's dissertation is written in a highly intelligent and scientifically informative manner, concise and free of unnecessary details. PhD student demonstrates an excellent understanding of the biochemical basis of the research problem, the role of sulfur-containing biologically active compounds, and the differences in their effects on redox homeostasis, cell signaling and survival, the activity of enzymes involved in sulfur metabolism, and their interdependence in maintaining intestinal barrier function. The HIEC-6 intestinal epithelial cell line (proliferating, immature cells) was used as a model. It possesses specific morphological, biochemical, and molecular-biological characteristics, making it advantageous for studying cell viability, cell signaling, the effects of pharmacological agents, cytotoxicity, changes in gene expression, oxidative stress, inflammatory mediators, the cell cycle, and etc.. This model offers significant advantages for investigating cell viability, cellular signaling, the effect of pharmacological agents, cytotoxicity, alterations in gene expression, oxidative stress, inflammatory mediators, the cell cycle, and etc. Although it cannot be directly compared to the effects, regulation, and metabolic integration observed in mature intestinal epithelium, the choice of cell model is appropriate, due to the established ability of HIEC-6 cells to respond to lipopolysaccharide (via the expression of TLR4 and MD-2) as an immunoreactive agent, a factor of importance for the experimental design. At the end of her dissertation, Stefka Stoeva also discusses the methodological limitations of the experimental design - the inability to equate the percentage of SMV in the culture medium to a precisely defined and constant concentration of H_2S/HS^-, which is why the observed biological effects of SMV cannot be compared with the pure substances used in terms of sulfur content by molar concentrations

The *literature review* presents a detailed overview of the study's essense, the necessity of employing a molecular-biological approach to elucidate the role of sulfur in fundamental cellular processes, and the distinct effects exhibited by the sulfur compounds under investigation. The properties, characteristics and biological role of sulfur and all its derivatives as a biogenic element, SMV, sulforaphane (SFN), alliin (ALI) and taurine (TAU) are described in detail, and a comparative analysis of the data published so far is made. The molecular mechanisms and cell signaling pathways, metabolism, and the studied effects (cytoprotection, neuroprotection, antitumor potential, antimicrobial, antifungal and antiviral activity, etc.) are discussed. Special attention is paid to the influence of these compounds on the intestinal epithelium.

The original figures created by Stefka Stoeva (generated using AI) are highly informative, effectively summarizing and directly illustrate the diverse effects of the biologically active sulfur compounds. The advantages and limitations of the HIEC-6 cell line used in the study are discussed, and the overview concludes with a summary and the formulation of a scientific hypothesis: "sulfur-containing mineral waters (SMV) from the Varna basin and selected natural sulfur-containing compounds (SFN, ALI, and TAU) can modulate (in a concentration-dependent manner) cell viability, the inflammatory response, antioxidant and stress-adaptive signaling, as well as the expression of phase II biotransformation enzymes in human intestinal epithelial cells."

Based on this in-depth study and preliminary analyses, the objective of the dissertation has been formulated: "to conduct a comparative analysis of the biological effects of natural dietary sources of sulfur

in the context of LPS-induced inflammation in human intestinal epithelial cells." Four very specific tasks have been defined, requiring extensive experimental and analytical work to compare the effects of various compounds and demonstrate their influence on the expression of target genes associated with cellular responses such as inflammation, oxidative stress, biotransformation, adaptation, and survival.

The *"Materials and Methods"* section is detailed and scientifically informative, covering the range of methods employed: *cell biology techniques* (culturing, morphological characterization, and lipopolysaccharide-induced inflammation in the HIEC-6 cell line); *molecular biology techniques* (RNA isolation, reverse transcription and cDNA amplification, RT-qPCR, and analysis of the transcriptional activity of selected genes); and *statistical analysis* (using Microsoft Excel and GraphPad Prism 10.0 software), all of which demonstrate the *interdisciplinary nature* of the research. The entire experimental design is presented schematically, detailing every step, the combinations of various concentrations of sulfur-containing biological agents, and the experimental conditions (including both controls and conditions where an inflammatory process via stimulation with LPS (*E. coli*, serotype O26:B6 – 1 μg/mL) was induced. This allows for a clear understanding of the research logic and an assessment of the study's scope.

The *"Results"* section determines the primary scientific and educational value of the dissertation. Stefka Stoeva presents her findings logically, following the sequence of the established objectives. They are excellently structured and described in a scientific, direct manner; at the end of each set of results regarding the expression of the target 14 genes, a summary is provided that directs attention specifically to the effects of the 4 biologically active sulfur compounds within the two experimental models (control conditions and LPS-induced inflammation).

In the *first series of experiments*, the metabolic activity and viability of HIEC-6 cells were assessed following treatment with various concentrations of the sulfur-containing biological compounds, taking into account all requirements for the accurate determination of their concentrations. Based on the MTT assay, a threshold was defined - only concentrations resulting in cell viability exceeding 80% are considered non-toxic. Consequently, the following concentrations were selected for use: for CMV - volume concentrations of 4%, 6%, and 8% in the culture medium (all tested concentrations, ranging from 0 to 16 vol%, were found to be non-toxic); for SFN - concentrations of 0.5, 1, and 2 μg/mL (within a range of 0–10 μg/mL); for TAU - concentrations of 1, 2, and 5 mM (within a range of 0–20 mM); and for ALI - concentrations of 10, 25, and 30 μg/mL (within a range of 0–100 μg/mL). Interestingly, three of the SFN concentrations exert a proliferative effect, increasing cell viability to approximately 109%. Low doses of SFN improve cell survival, whereas high doses exhibit cytotoxicity.

The *second series of experiments* involves determining the gene expression levels of selected genes in cell cultures using a two-step quantitative real-time *PCR (qPCR) assay*. The number of genes analyzed—14 in total *(NF-kB, TNF-α, IL-6, IL-8, COX-2, Casp1, IL-1β, IL1RN, Nrf-2, GPx, Sirt1, FOXO-4, SULT1A1, and SULT1A3)*, is notable. The use of custom-designed primers constitutes a distinct scientific task. An additional experimental series utilizes salicylic acid (3 mM SA) as a reference substance against which the anti-inflammatory properties of the tested biological agents are evaluated. The core of the experiments follows the described experimental design very precisely - untreated cells / treatment with biologically active sulfur compounds / treatment with LPS / (plus an additional control treatment with SA). Given that the whole experiment involves 4 sulfur-containing agents, each at 3 different concentrations, the assessment of expression levels for 14 genes and 2 enzymes, so the analyses are both labor-intensive and large-scale, requiring a high level of biochemical expertise regarding the regulation and integration of metabolism. In this context, the application of correlation analysis is necessary to evaluate the overall impact. The results from this *third series of experiments* are organized according to the genes being monitored and the cellular signaling pathways with which they are associated: *NF-kB, TNF-α, IL-6, IL-8, COX-2, Casp1, IL-1β, IL1RN, Nrf-2, GPx, Sirt1, FOXO-4, SULT1A1 and SULT1A3*.

As a result of the extensive number of analyses conducted, several key aspects emerge:

A. Expression of genes associated with the inflammatory response in human intestinal epithelial cells (HIEC-6)

- ✓ *NF-κB* (a pro-inflammatory transcription factor involved in cytokine synthesis, activated by TNF-α and TLR). In general, the induction of inflammation using bacterial LPS resulted in a statistically significant increase in the relative expression of *NF-κB* compared to untreated cells (1.54 RU), whereas the SA caused a statistically significant decrease in expression to 0.40 RU

(relative to untreated cells). All sulfur sources investigated reduced NF-κB expression. The most pronounced effect on NF-κB expression was observed with SMV (at 6% and 8%), SFN (at all concentrations tested in unstimulated cells and at 2 μg/mL LPS), TAU (at 5 mM in both experimental models, and at 2 mM in LPS-stimulated cells only), and ALI (only at the highest concentration used, 30 μg/mL). NF-κB signaling influences and integrates various cellular processes, such as inflammation, proliferation, and survival, which is why PhD student Stefka Stoeva investigated the expression of genes associated with the NF-κB pathway.

- ✓ *TNF-α* (pro-inflammatory cytokine): all biological agents used *reduced* TNF-α expression under various concentrations and conditions: SMV – at all tested concentrations in LPS-stimulated cells, and at an 8% concentration in unstimulated cells; SFN – in LPS-stimulated cells at concentrations of 1 and 2 μg/mL; TAU – in both experimental models; ALI – at a concentration of 10 μg/mL in unstimulated cells, and at 25 μg/mL in LPS-stimulated cells.
- ✓ *IL-6* (a cytokine that regulates the immune system): SMV and TAU exhibit an inhibitory effect on IL-6 expression, which is particularly pronounced under conditions of induced inflammation; ALI shows a limited effect, and only in LPS-stimulated cells; SFN does not induce statistically significant changes.
- ✓ *IL-8* (pro-inflammatory chemokine): SMV, SFN, and ALI do not show statistically significant changes in *IL-8* expression compared to the respective controls, in both unstimulated and LPS-stimulated cells. The biological effect of TAU is limited, suppressing *IL-8* expression only at the highest concentration tested (5 mM) and only in LPS-stimulated cells.
- ✓ *COX-2* (the inducible form of cyclooxygenase, which expression is regulated by NF-κB-dependent signaling pathways). Stefka Stoeva demonstrated that in LPS-stimulated cells, COX-2 expression is 1.8 times higher than in untreated cells, whereas SC reduces it to 0.51 RU—a statistically significant decrease. The results regarding the effect of TAU are also noteworthy: it reduces COX-2 expression across all three concentrations and experimental conditions tested.
- ✓ *Caspase 1/IL-1β* and the endogenous inhibitor, the product of the *IL1RN* gene. Based on the statistically significant increase in *CASP1* expression (up to 2.34 RU) in the presence of LPS, and the marked decrease in the presence of SA (down to 0.42 RU), Stefka Stoeva determined that all four sulfur sources investigated reduce *CASP1* expression, under specific concentrations and experimental conditions, in LPS-stimulated cells. A statistically significant reduction was observed with SMV (at all concentrations tested). The effects of SFN, TAU, and ALI were limited.
- ✓ *IL-1β* (a pro-inflammatory cytokine associated with NF-κB-dependent signaling). TAU and ALI show the most pronounced decrease in *IL-1β* expression, whereas no statistically significant changes were observed for SMV and SFN.
- ✓ SMV, SFN, and ALI *increase IL1RN* expression in unstimulated cells, though the concentration-dependent profiles differ. The effect of ALI is the most pronounced, showing a statistically significant increase. In LPS-stimulated cells, none of the four sulfur sources investigated induce a statistically significant change in *IL1RN* expression.

B. Expression of genes related to the antioxidant response – **Nrf2, GPx**

All investigated sulfur sources increase the expression of *Nrf2 and GPx* under specific experimental conditions: SFN shows a statistically significant increase in expression in both unstimulated and LPS-stimulated cells (at all tested concentrations); the effect of TAU is evident in both unstimulated and LPS-induced cells at the two higher concentrations; ALI increases expression in unstimulated cells, and in LPS-stimulated cells only at the highest concentration; SMV has no significant effect.

C. Expression of genes related to cellular adaptation – *Sirt1 and FOXO-4* (regulators of various cellular processes associated with metabolism, redox homeostasis, and the response to cellular stress).

Treatment of cells with LPS induces a statistically significant decrease in *SIRT1* expression to 0.70 RU compared to untreated cells, whereas SA induces a statistically significant (2-fold) increase in the expression of both *Sirt1 and FOXO4*. LPS induction has no statistically significant effect on *FOXO4*. SFN demonstrates a statistically significant increase in the expression of *SIRT1 and FOXO4* at all tested concentrations in unstimulated cells. The other biologically active compounds used do not significantly affect the expression of *SIRT1 and FOXO4*. Under conditions of LPS-induced inflammation, *none of the four sulfur sources*

investigated leads to a statistically significant increase in SIRT1 expression at any of the applied concentrations.

D. Expression of genes associated with Phase II of biotransformation – *SULT1A1 and SULT1A3* (isoforms of the enzymes sulfotransferase and catecholamine-sulfotransferase).

SMV *demonstrates the most pronounced effect on the expression* of *SULT1A1*- an increase in unstimulated cells and a decrease in LPS-stimulated cells at specific concentrations, whereas no statistically significant change was observed in the expression of *SULT1A3*. SFN and TAU induce an increase in expression only at the highest concentrations applied, and exclusively in unstimulated cells, regarding both *SULT1A1 and SULT1A3*. ALI did not induce any statistically significant changes.

A particularly valuable part of Stefka Stoeva's scientific research consists of the *correlation analyses* she conducted, which examine the expression patterns of all genes following treatment of HIEC-6 cells with the biologically active sulfur-containing compounds used in both experimental models — untreated cells and LPS-stimulated cells. The presented relationships demonstrate the integration of various processes influenced by the action of the compounds used and highlight the interdependencies that exist among them. A comparative analysis was performed between the experimental models; in LPS-induced inflammation, the structure of the network of expression interdependencies reorganizes, and the pro-inflammatory module becomes more active (there is a strong correlation between *NF-κB, TNF-α, IL-8, COX-2, and Caspase-1*. On the other hand, a distinct correlation is demonstrated between *Nrf2, GPx, SIRT1, and FOXO4*.

It has been established that, under conditions of LPS-induced inflammation, the effect of SMV and SFN leads to a marked negative correlation between the genes of the pro-inflammatory and antioxidant *Nrf2/GPx/SIRT1/FOXO4*, which is logical from the perspective of the cellular response. The *Nrf2/GPx* axis maintains an exceptionally strong positive correlation regardless of the inflammatory context, which establishes the coordination between *Nrf2 and GPx* as one of the most stable characteristics of the transcriptional response to SFN and SMV, i.e., the inflammatory stimulus more strongly reorganizes the interactions of the antioxidant module with the rest of the expression network. *IL1RN and SULT1A1 partially lose the connections* characteristic of unstimulated cells with these two main correlation blocks.

The action of TAU in unstimulated cells exhibits a distinct *IL1RN/SIRT1/FOXO4/SULT*-dependent pattern, in contrast to *NF-κB/TNF-α*-mediated expression. Of particular interest is the change in *IL1RN* in control cells, the expression of this gene is strongly opposed to *NF-κB and TNF-α* and positively correlated with antioxidant and stress-adaptive genes *Nrf2, GPx, SIRT1, and FOXO4* while under inflammatory conditions, it correlates positively with *NF-κB, TNF-α, IL-6, and Caspase-1*, i.e., a concentration-dependent compensatory anti-inflammatory response.

Upon treatment with ALI, a heterogeneous profile is observed in unstimulated cells, along with a polarized structure (a more pronounced distinction between pro-inflammatory and antioxidant-adaptive expression patterns). Particularly indicative is the change in *the NF-κB/Nrf2* expression relationship, whose negative correlation ($r = -0.70$) in unstimulated cells increases to $r = -0.92$ upon application of LPS. The *Nrf2/FOXO4* correlation nearly doubles (from $r = 0.42$ to $r = 0.81$), and the *Nrf2/SULT1A3* expression relationship increases from $r = 0.74$ to $r = 0.96$. At the same time, *SULT1A3* exhibits stronger negative correlations with some pro-inflammatory markers, including Caspase-1 (from $r = -0.48$ in unstimulated cells to $r = -0.97$ upon induced inflammation) and *IL-1β* ($r = -0.32$ and $r = -0.87$).

Stefka Stoeva compares the eight correlation matrices and reveals a recurring pattern in the expression profiles across two functionally distinct pathways: 1. *NF-κB, TNF-α, IL-6, IL-8, COX-2, Caspase-1*, and *IL-1β*, which is defined as an inflammatory expression module; and 2. *Nrf2 and GPx*, to which depending on the specific treatment, *SIRT1, FOXO4, IL1RN, SULT1A1, and/or SULT1A3 are added*. In SMV, SFN, ALI, and TAU, LPS stimulation manifests through the amplification of positive interactions between *NF-κB, TNF-α, COX-2, and Caspase-1*. As a result of all the experiments, two consistent expression patterns were identified: the Nrf2/GPx relationship, which is positive under all conditions, and the negative NF-κB/Nrf2 expression relationship, which stands out as the most consistent example of opposing changes in the inflammatory and antioxidant expression profiles.

Thus, in *this first scientific study*, SMV stands out as a "*complex natural agent*" that primarily influences the expression of target inflammatory markers and, to a more limited extent, antioxidant and stress-adaptive mechanisms. SFN exhibits a pronounced antioxidant-adaptive transcriptional profile, influencing the expression of *Nrf2, GPx, SIRT1, and FOXO4*, which is considered to be involved in the cellular response to

redox and metabolic stress. Its anti-inflammatory effect is more selective depending on the specific experimental conditions. TAU demonstrates a stronger *anti-inflammatory effect* in LPS-induced inflammation. Its profile is characterized by a pronounced effect on the expression of *IL-1β and IL-8* and is concentration-dependent with regard to the expression of *SIRT1 and FOXO4*. ALI also proves to be more effective under inflammatory conditions, but compared to SFN, its involvement in antioxidant and stress-adaptive regulation is more limited; in LPS induced inflammation, a clearer distinction emerges between the inflammatory and the protective-adaptive expression profiles.

One of the most consistent expression correlations in the entire dataset is *Nrf2/GPx*. It is positive under all eight conditions. The second consistent correlation is the *negative expression relationship between NF-κB and Nrf2*, which is maintained across all eight matrices, with or without LPS, respectively: SMV -0.79/-0.99; SFN -0.96/-0.71; TAU -0.58/-0.57; and ALI -0.70/-0.92. This is the most consistent example of an opposite change in the inflammatory and antioxidant expression panels.

I consider the discussion section of the dissertation as a very necessary part for evaluating, comparing, and integrating all experimental variants in order to give biological meaning to the entire study, despite its large volume and the repetition of the main results. The main contributions of Stefka Stoeva's dissertation are summarized as follows: "new experimental data on the effects of sulfur-containing mineral water from the Varna Basin on the expression of specific genes; and expanded knowledge of the molecular effects of the poorly studied organosulfide alliin." In fact, the experimental approach and design of the comparative cellular study, along with the development of a method for the preliminary assessment of the molecular effects of natural sulfur sources in HIEC-6 cells under control and LPS-stimulated conditions, represents a very significant methodological contribution that can also be applied to other cellular models."

5. Publications

Stefka Stoeva has presented her findings in two publications in specialized journals (1 in the Journal of IMAB (International Medical Association Bulgaria), JIF 0.2 (ESCI & Web of Science); SJR 0.3; Q3, and 1 in Pharmacia, JIF 1.1 (Web of Science), SJR 0.334; Q2. The results have been presented at 6 scientific conferences (3 international). In the publications cited, the PhD student is the third author in one and the first author in the other, which attests to her personal contribution. Stefka Stoeva's dissertation is included in four research projects. The scientific works meet the minimum national requirements (under Article 2b, para. 2 and 3 of the LDASRB for the award of the educational and scientific degree of "Doctor."

6. Fulfillment of the minimum state requirements and the individual plan

Ph.D. student Stefka Stoeva has successfully completed her individual PhD study plan and has passed all the exams specified therein. The report on compliance with the minimum requirements for the "Doctor" academic degree in professional field 4.3, Biological Sciences, shows that out of the required 80 points, Stefka Stoeva has earned 85 points. This includes: a dissertation that successfully passed the preliminary review 50 points; 1 article in a Q2 journal - 20 points; and 1 article with a Q3 ranking - 15 points.

7. Conclusion

Stefka Stoeva demonstrates an excellent understanding of the scientific problem and, through her work, has developed the ability to interpret the obtained results, propose hypotheses, and test them. This large-scale study sheds light on the under-researched issue of the unity and specificity of the molecular-biological effects of natural sulfur sources with different chemical natures. The use of a single cellular model and comparable experimental conditions as an experimental approach, along with the study design for tracking the expression of so many genes and the correlations between them, provides new comparative information on the action of dietary sulfur sources and their biological effects on vital processes that ultimately determine the cells' capacity for metabolic biotransformation, regulation, and adaptation.

Having reviewed the dissertation and the accompanying scientific papers submitted as part of this procedure, and based on an analysis of their significance and the scientific and applied scientific contributions they contain, I confirm that the submitted dissertation and the scientific publications associated with it, as well as the quality and originality of the results and achievements presented therein, fully meet and satisfy the minimum national requirements of the LRASRB and its implementing regulations for the award of the

educational and scientific degree of "Doctor" in scientific field 4. Natural Sciences, Mathematics, and Computer Science, professional field 4.3. Biological Sciences (Biochemistry).

Based on the foregoing, I recommend that the Scientific Jury award Stefka Stoeva the educational and scientific degree of "Doctor" in professional field 4.3. Biological Sciences (Biochemistry).

05.11. 2026
Sofia

Member of the Scientific J

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

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