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

THESIS SUMMARY

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The dissertation has been discussed, accepted, and referred for defence before a Scientific Jury by the Department Council of the Department "Biochemistry, Molecular Medicine, and Nutrigenomics" at the Medical University - Varna

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The dissertation comprises 154 pages and includes 73 figures, 2 diagrams, 1 photograph, and 6 tables. The reference list contains 298 titles.

The dissertation defence will take place on November 25, 2026 (Wednesday) at 13:30 in the Meeting Hall of the Faculty of Pharmacy and in a virtual room on the Webex platform at the Medical University of Varna.

***Note**: The numbering of figures and tables in the Thesis Summary does not correspond to that in the dissertation.*

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1. INTRODUCTION

Natural sources of sulfur include plant- and animal-based foods, as well as mineral waters with specific ionic and gaseous compositions. Of particular interest, because of their potential benefits for human health, are cruciferous vegetables, plants of the genus *Allium*, taurine-rich foods, and sulfur-containing mineral waters. Their biological significance is determined by the ability of their active components to influence signalling pathways related to redox homeostasis, inflammation, cellular adaptation to stress, and detoxification processes.

Sulfur-containing mineral waters are widespread, including in regions lacking volcanic activity, such as Bulgaria. Their properties and applications depend on the chemical form of the sulfur. Waters in which sulfur is present primarily as the sulfate ion (SO_4^{2-}) are classified as sulfate waters, while natural waters containing hydrogen sulfide (H_2S) are classified as sulfurous mineral waters. In an aqueous environment, H_2S exists in equilibrium with hydrosulfide (HS^-) and sulfide (S^{2-}) anions. Undissociated H_2S is lipophilic, can cross cell membranes, and functions as a gaseous signalling mediator. Mineral waters from the Varna Basin are classified as sulfurous due to their H_2S content.

Key dietary sulfur-containing compounds include sulforaphane, alliin, and taurine. Sulforaphane is an isothiocyanate characteristic of plants in the *Brassica* genus, such as broccoli, cauliflower, and kale. It is one of the most studied compounds in this group because of its established antioxidant, anti-inflammatory, and anti-tumour properties. Furthermore, consuming cruciferous vegetables is associated with a reduced risk of metabolic disorders characterised by chronic low-grade inflammation.

Alliin is a major organosulfide in garlic (*Allium sativum*) and a precursor to allicin. The plant's antioxidant, anti-inflammatory, immunomodulatory, cardioprotective, antitumor, hepatoprotective, and antimicrobial effects are attributed to the organosulfides found in garlic.

Taurine is a conditionally essential sulfonated β-amino acid synthesised in the human body from cysteine. Although it is not involved in protein synthesis, it is widely distributed in the body and found in high concentrations in the brain, retina, muscle tissue, and

phagocytic cells. Taurine has antioxidant and cytoprotective properties and helps maintain cellular homeostasis during acute and chronic inflammation.

Despite accumulated data on various natural sulfur sources, comparative studies of their biological effects on intestinal epithelial cells remain limited. The gastrointestinal tract is the primary site where active compounds, ingested via food and water, interact with the body; consequently, intestinal cell lines are a suitable model for investigating their mechanisms of action under both normal and inflammatory conditions.

In this context, the present study comparatively assesses the biological effects of sulfur-containing biologically active compounds from dietary sources and sulfur-rich mineral water from the Varna Basin on human intestinal epithelial cells.

2. AIM AND OBJECTIVES

2.1. AIM

This study aimed to compare the biological effects of natural dietary sulfur sources in human intestinal epithelial cells under basal conditions and during LPS-induced inflammation.

2.2. OBJECTIVES

1. Determination of cell viability in the HIEC-6 cell line after treatment with:
    - sulfur-containing mineral water
    - sulforaphane
    - taurine
    - alliin

2. Assessment of the potential of the studied sulfur sources to influence the expression of target genes associated with:
    - the regulation and mediation of the inflammatory response
    - the antioxidant response
    - cellular adaptation and survival
    - biotransformation
3. Integration of the obtained data and comparative analysis of the anti-inflammatory, antioxidant, and adaptogenic profile of the investigated sulfur sources
4. Analysis of the potential of the investigated sulfur sources to influence detoxification processes

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Cell line

In this study, we used an adherent human intestinal epithelial cell line (HIEC-6) (Image 1), purchased from the American Type Culture Collection. (ATCC® CRL3266™)

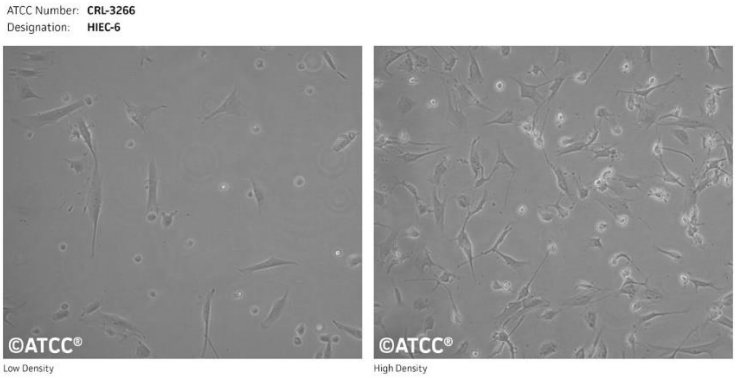


Image 1. HIEC6 cell line (ATCC® CRL3266™) used in the study

3.2. METHODS

3.2.1. Sampling of Varna Mineral Water

The cells were treated with sulfurous mineral water (SMW) collected from the "Aquarium" public fountain in the city of Varna. The samples were stored at 4°C for 24 hours, then equilibrated to room temperature for two hours before the experiment. Immediately before each experiment, the SMW samples were passed through a sterile filter and incubated at 37°C.

3.2.2. Subculturing of cell culture

Opti-MEM™ I Reduced Serum Medium, modified with L-glutamine and phenol red, was used to culture this cell line. The cells were expanded in 75 cm^2 flasks and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. We used Opti-MEM™ I medium without phenol red to prepare treatment concentrations for the experiments.

3.2.3. Cytotoxicity test (MTT)

The method for determining the cytotoxicity of various agents is based on the ability of the enzymatic systems of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is yellow in colour, into a purple-violet formazan. Cell viability is expressed as a percentage of the untreated control (100%). Based on the results of the MTT assay, three concentrations for each treatment were selected for subsequent experiments, as follows: SMW % (4, 6, 8), and sulfur-containing biologically active compounds (SBAC): SFN μg/mL (0.5, 1, 2); ALI μg/mL (10, 25, 30); TAU mM (1, 2, 5).

3.2.4. Induction of inflammation with lipopolysaccharides (LPS)

Serotype 026:B6 LPS from *Escherichia coli* was used to induce an inflammatory response. After seeding for an experiment, cells were pre-treated with the selected concentrations of sulfur-containing source for 24 h, followed by treatment with 1 μg/mL LPS.

3.2.5. Determination of the level of gene expression

A two-step quantitative Real-Time PCR (polymerase chain reaction) is used to determine the gene expression levels of selected genes in cell cultures.

Total RNA extraction from cells: After the experiment, cells were treated with the Primezol reagent to isolate total RNA. The protocol strictly followed the manufacturer's instructions.

Reverse transcription (RT-PCR): We used the extracted RNA (200 ng) to synthesise complementary DNA (cDNA) with a cDNA synthesis kit containing an oligo(dT)18 primer and reverse transcriptase.

Quantitative Real-Time PCR (RT-qPCR): The second step in quantitative Real-Time PCR analysis involves the real-time amplification of the cDNA using gene-specific primers. The nucleotide sequences of the primers used in the analysis are presented in Table 1:

Table 1. *Nucleotide sequences of the primers for the genes under study*

Gene (*Homo sapiens*)	**Nucleotide sequences**

ACTB	F: 5'- GTGGCCGAGGACTTTGATTG - 3' R: 5'- CCTGTAACAACGCATCTCATA - 3'
RPLP0	F: 5'-AGCCCAGAACACTGGTCTC-3' R 5'-ACTCAGGATTTCAATGGTGCC-3'
COX-2	F: 5'- GAAACAGAGAAGTTGGCAGCA - 3' R: 5'- GGCAGGATACAGCTCCACAG- 3'
TNFα	F: 5'- CTCTTCTGCCTGCTGCACTTT - 3' R: 5'- ATGGGCTACAGGCTTGTCACT - 3'
IL-1β	F: 5' CCACCTCCAGGGACAGGATA - 3' R: 5' AGAATTAGCAAGCTGCCAGGA 3'
IL1RN	F: 5' - GCCCATCCTCAGGACCTTTC - 3' R: 5' - ATGTCCTAGCCATCCCCACT - 3'
IL-6	F: 5' – GGCACTGGCAGAAAACAACC - 3' R: 5' - GCAAGTCTCCTCATTGAATCC - 3'
IL-8	F: 5' – AGCCTTCCTGATTTCTGCAG - 3' R: 5' – GTCCACTCTCAATCACTCTCAG - 3'
NF-kB	F: 5' – GTGGTGCCTCACTGCTAACT R: 5' – GGATGCACTTCAGCTTCTGT
Nrf2	F: 5' - TACTCCCAGGTTGCCCACAT - 3' R: 5' - TGACTGAAACGTAGCCGAAGA - 3'
Caspase 1	F: 5' - TGTGTCTTTCCAGACAGCCC - 3' R: 5' - GCCCCAACAAAGATGTCCTGT - 3'
GPx	F: 5' - GAGGCAAGACCGAAGTAAACTAC - 3' R: 5' - CCGAACTGGTTACACGGGAA - 3'
Sirt1	F: 5' – TAGACACGCTGGAACAGGTTGC - 3' R: 5' – CTCCTCGTACAGCTTCACAGTC - 3'
FOXO4	F: 5' – CCGGAGAAGCGACTGACAC - 3' R: 5' – CGGATCGAGTTCCATCCTG - 3'
SULT1A1	F: 5' – CGGCACTACCTGGGTAAGC - 3' R: 5' – CACCCGCATGAAGATGGGAG- 3'
SULT1A3	F: 5' – GGAACCCTCAGGGCTGGAG - 3' R: 5'– CGTCCTTTGGGTTTCGGG - 3'

3.2.6. Statistical Analysis

We analysed changes in gene expression using the 2^−ΔΔCt method and presented results as relative units of mRNA (RU). The relative expression of target genes was determined by comparison with untreated cells (control, C), which were assumed to have a gene

expression level of 1. The genes for β-actin (ACTB) and RPLP0 were used as endogenous controls.

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

4 RESULTS

4.1 *Effect of SMW and SBAC on cell viability*

The effects of SMW and SBAC on cell viability were assessed using an MTT assay. Only concentrations that yielded cell survival above 80% were considered non-toxic. Based on the MTT assay results (Figs. 1 and 2), three concentrations of SMW and three concentrations of each SBAC were selected for subsequent experiments.

Data are presented as the mean ± SD from three independent experiments, with the corresponding significance level (p) relative to the control (untreated cells).

As shown in Fig. 1, at the highest SMW concentration in the culture medium (20%), cell viability decreased sharply compared with untreated cells ($p = 0.0003$). However, even at this concentration, cell viability remained above 80%. SMW concentrations of 1–16% did not cause statistically significant changes in cell viability. These results indicate that all tested SMW concentrations are non-toxic to the HIEC-6 cell line. Concentrations of 4%, 6%, and 8% were selected for subsequent experiments.

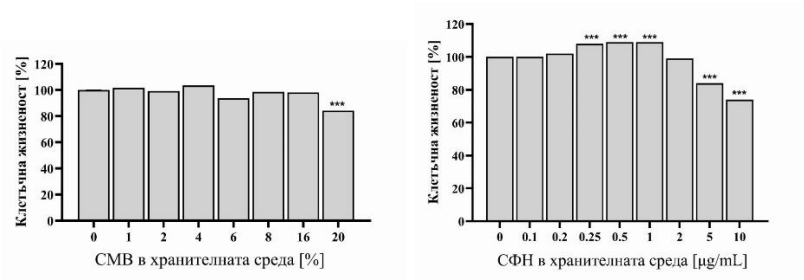


Figure 1. *Effect of SMW (left) and SFN (right) on the viability of HIEC-6 cells (***p ≤ 0.001, vs. C)*

Interestingly, SFN induced a statistically significant increase in cell proliferation at concentrations of 0.25, 0.5, and 1 μg/mL (Fig. 1, right). At 5 μg/mL, a marked decrease in cell viability was observed, and the highest SFN concentration tested reduced viability to 74%, classifying it as cytotoxic ($p < 0.001$). Based on these results, concentrations of 0.5, 1, and 2 μg/mL were selected for subsequent analyses.

As shown in Fig. 2 (left), TAU concentrations of 0.2–5 mM had no significant effect on cell viability. The slight decrease in cell viability after treatment with 10 mM TAU was not statistically significant (95%, $p = 0.2$). A statistically significant reduction was observed only at 20 mM ($p = 0.03$). Nevertheless, even at this concentration, cell viability remained high (89%), suggesting that all tested TAU concentrations are non-toxic to HIEC-6 cells. Concentrations of 1, 2, and 5 mM were selected for subsequent analyses.

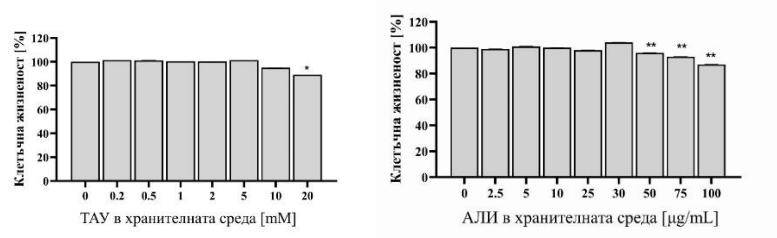


Figure 2. *Effect of TAU (left) and ALI (right) on the viability of HIEC-6 cells; *p ≤ 0.05; **p ≤ 0.01 vs. C.*

As shown in Fig. 2 (right), ALI treatment significantly reduced cell viability at the three highest concentrations tested. Notably, at the highest concentration (100 μg/mL), cell viability approached the 80% threshold. Based on these results, ALI concentrations of 10, 25, and 30 μg/mL were selected for subsequent experimental analyses.

4.2 *Expression of target genes in a model of LPS-induced inflammation*

To investigate the potential of SMW and SBAC to modulate the expression of genes associated with the inflammatory response, the regulation of redox homeostasis, and enzymes involved in detoxification processes, a model of LPS-induced inflammation was employed. Cells were pre-treated for 24 hours with SMW or SBAC, followed by exposure to 1 μg/mL LPS. Some cells were treated with the same concentration of LPS alone. Additionally, 3 mM salicylic acid (SA) was used as a reference substance known for its anti-inflammatory properties. As previously described, cells were treated with three selected concentrations of each test compound, determined by the MTT assay. According to the experimental design, the experimental groups and treatment conditions for the HIEC-6 cells were as follows: untreated cells (C – control); LPS 1 μg/mL; SA 3 mM; SMW % (4, 6, 8); SFN μg/mL (0.5, 1, 2); ALI μg/mL (10, 25, 30); and TAU mM (1, 2, 5).

mRNA levels of NF-kB, TNF-α, IL-6, IL-8, COX-2, Casp1, IL-1β, IL1RN, Nrf-2, GPx, Sirt1, FOXO-4, SULT1A1, and SULT1A3 were compared with those of untreated cells.

The expression of the investigated genes in human intestinal epithelial cells (HIEC-6) was analysed under unstimulated conditions and after induction of inflammation with LPS. In the graphs, mRNA levels are presented in relative units (RU), with LPS (+/-) indicating cells with or without LPS-induced inflammation, and the corresponding significance level (p) relative to the control (untreated cells).

4.2.1 Effect of SMW and SBAC on the expression of NF-kB

NF-κB expression in human intestinal epithelial cells HIEC-6 was analysed under basal conditions and after induction of an inflammatory response with LPS. LPS alone increased NF-κB expression by 1.54 RU compared with untreated cells (p=0.01). By

comparison, SA led to a statistically significant decrease in expression to 0.40 RU (p=0.006 compared with untreated cells) (Fig. 3 and 4).

Among the sulfur sources investigated, SMW produced the most pronounced effect on NF-κB expression (Fig. 3). At 4% SMW, no statistically significant change was observed; however, at 6% and 8%, NF-κB expression was significantly reduced in both unstimulated and LPS-stimulated cells. In unstimulated cells, the relative expression at 6% and 8% SMW was 0.68 and 0.61 RU (p=0.03), respectively, while in LPS-stimulated cells, it was 0.72 RU (p=0.01) and 0.58 RU (p=0.04), respectively. Thus, under both experimental conditions, expression decreased as the concentration of SMW increased.

In summary, SMW exerts a distinct inhibitory effect on NF-κB expression, evident at concentrations of 6% and 8%, and this effect persists under both basal and LPS-stimulated conditions.

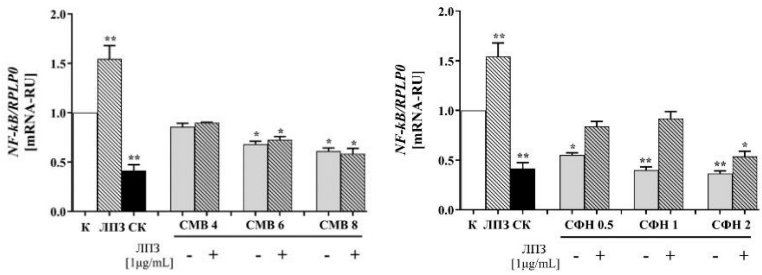


***Figure 3**. Effect of SMW (left) and SFN (right) on NF-kB expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01, vs. C).*

Treatment with SFN also reduced NF-κB expression, with the effect evident at all tested concentrations in unstimulated cells (Fig. 3, right). Relative expression at 0.5, 1, and 2 μg/mL was 0.55 RU (p=0.05), 0.39 RU (p=0.009), and 0.36 RU (p=0.05), respectively. In LPS-stimulated cells, a statistically significant reduction was observed at the highest concentration (2 μg/mL), where expression reached 0.53 RU (p=0.05 compared with untreated cells).

In summary, SFN reduces NF-κB expression at all tested concentrations in unstimulated cells, whereas in LPS-stimulated cells, this effect was observed only at 2 μg/mL.

With TAU, the effect was more limited and condition-dependent (Fig. 4, left). At 2 mM, expression was significantly reduced only in LPS-stimulated cells, reaching 0.70 RU (p=0.03). At the highest concentration, 5 mM, expression was significantly reduced in both unstimulated cells (to 0.55 RU; p=0.005) and LPS-stimulated cells (to 0.46 RU; p=0.002).

In summary, TAU reduces NF-κB expression most clearly at 5 mM, where the effect is observed under both experimental conditions, whereas at 2 mM, it is observed only in LPS-stimulated cells.

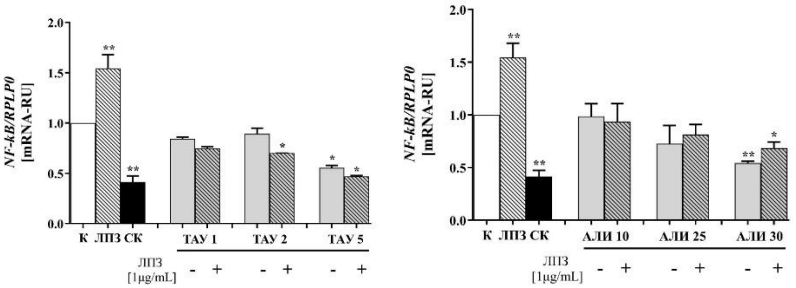


***Figure 4**. Effect of TAU (left) and ALI (right) on NF-kB expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

Compared with the other treatments, ALI affects NF-κB expression only at the highest concentration tested (Fig. 4, right). No statistically significant changes were observed at 10 or 25 μg/mL. At 30 μg/mL, however, expression was significantly reduced in both unstimulated cells (to 0.54 RU; p=0.006) and LPS-stimulated cells (to 0.68 RU; p=0.02).

In summary, ALI exerts an inhibitory effect on NF-κB expression, but this effect is limited to the highest concentration tested (30 μg/mL). At this concentration, the change persists under both experimental conditions.

Overall, all four sulfur sources reduced NF-κB expression, though they differed in concentration range and in how the effect depended on the presence of LPS. The most pronounced effect was observed with SMW, whereas for SFN, TAU, and ALI, the

statistically significant reduction was limited to specific concentrations and/or experimental conditions.

4.2.2 Effect of SMW and SBAC on the expression of TNF-α

The effects of the investigated sulfur sources on TNF-α expression are presented in Figures 5 and 6. TNF-α is a pro-inflammatory cytokine, the expression of which is regulated, among other factors, by NF-κB-dependent signalling.

In cells treated with LPS alone, TNF-α expression was significantly increased compared with untreated control cells (p=0.02). SA, used as a reference treatment, resulted in a significant decrease in expression to 0.65 RU compared with untreated cells (p=0.0047).

The most pronounced effect on TNF-α expression was observed with SMW (Fig. 5, left). In LPS-stimulated cells, all three tested concentrations—4%, 6%, and 8%—produced a statistically significant decrease in expression (p=0.011, p=0.007, and p=0.05, respectively). In unstimulated cells, expression changed only at the highest concentration of 8%, where the level reached 0.90 RU compared with untreated cells (p=0.05).

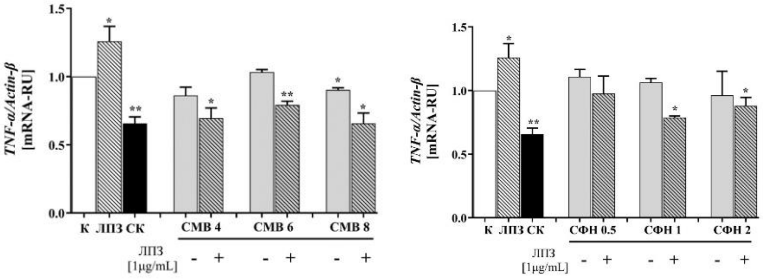


Figure 5. *Effect of SMW (left) and SFN (right) on TNF-α expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

In summary, SMW has a pronounced effect on TNF-α expression; suppression is observed at all tested concentrations in LPS-stimulated cells, whereas in unstimulated cells, suppression is observed only at an 8% SMW concentration.

For SFN, a statistically significant reduction in TNF-α expression in LPS-stimulated cells was observed at 1 and 2 μg/mL (Fig. 5, right). The relative expression at these concentrations was 0.78 RU (p=0.04) and 0.87 RU (p=0.017), respectively. At 0.5 μg/mL, no statistically significant change was observed, with mRNA levels remaining comparable to those of the untreated controls.

In summary, SFN reduces TNF-α expression in LPS-stimulated cells at 1 and 2 μg/mL, whereas the lowest concentration tested shows no statistically significant effect.

TAU showed a broader effect on TNF-α expression, observed in both unstimulated and LPS-stimulated cells (Fig. 6, left). In unstimulated cells, a statistically significant reduction was observed at 1 and 5 mM TAU. At 2 mM, expression decreased to 0.82 RU, but this was not statistically significant. In LPS-stimulated cells, all three concentrations produced a statistically significant reduction in expression. At 1 mM, the relative expression was 0.74 RU (p=0.028), while at 2 and 5 mM, it was 0.65 RU (p=0.001) and 0.70 RU (p=0.001), respectively.

In summary, TAU inhibits TNF-α expression under both experimental conditions; in LPS-stimulated cells, this effect was observed at all tested concentrations.

For ALI, the effect on TNF-α expression was more limited and dependent on the experimental condition (Fig. 6, right). In unstimulated cells, a statistically significant reduction was observed at 10 μg/mL, with relative expression reaching 0.70 RU (p=0.002). In LPS-stimulated cells, a statistically significant effect was observed only at 25 μg/mL, where expression was reduced to 0.75 RU (p=0.003).

In summary, ALI reduces TNF-α expression at different concentrations depending on the experimental condition - specifically, 10 μg/mL in unstimulated cells and 25 μg/mL in LPS-stimulated cells.

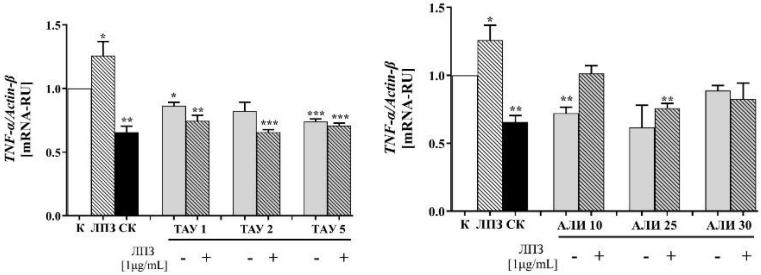


Figure 6. *Effect of TAU (left) and ALI (right) on TNF-α expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 vs. C).*

In summary, all four sulfur sources investigated reduced TNF-α

4.2.3 Effect of SMW and SBAC on the expression of IL-6

We investigated the effect of SMW and SBAC on IL-6 expression in HIEC-6 cells, both under conditions of LPS-induced inflammation and in unstimulated cells (Figs. 13–16). LPS administration alone significantly increased gene expression to 1.22 RU (p=0.003), whereas SA suppressed expression to 0.61 (p=0.006).

The most pronounced inhibitory effect on the expression of this gene was observed with SMW and TAU; this effect was particularly strong under conditions of induced inflammation (Figs. 7 and 8, respectively). As shown in Figure 7, all three tested concentrations of SMW exerted a statistically significant effect in cells with induced inflammation: the expression levels were 0.61 (p=0.007) in cells treated with 4% SMW, and 0.63 (p=0.001) and 0.75 (p=0.04) for the two higher concentrations, respectively. Only the 6% SMW concentration in the culture medium affected IL-6 expression in cells with unaltered homeostasis (p=0.017).

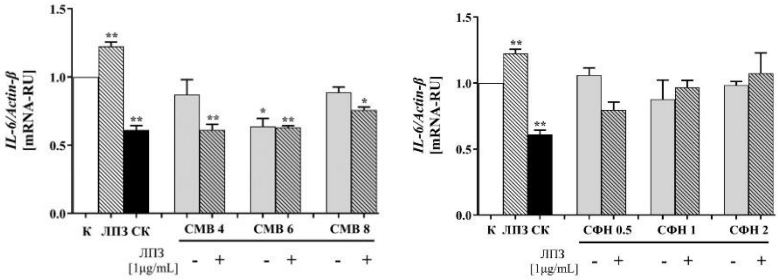


Figure 7. *Effect of SMW (left) and SFN (right) on IL-6 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

Surprisingly, none of the SFN concentrations had a statistically significant effect on IL-6 gene expression, either in LPS-stimulated cells or in the absence of inflammation (Fig. 7 right).

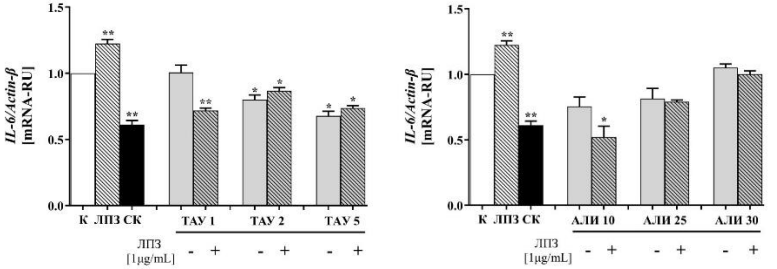


Figure 8. *Effect of TAU (left) and ALI (right) on IL-6 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C)*

Figure 8 shows that TAU, like SMW, has anti-inflammatory potential by inhibiting expression at all applied concentrations under inflammatory conditions. This effect of TAU is also observed in unstimulated cells treated with the two higher concentrations, 2 mM and 5 mM, with expression changes of 0.79 (p=0.002) and 0.67 (p=0.03), respectively.

In cells treated with ALI, an effect was reported only in those treated with the lowest concentration of 10 μg/ml, under inflammatory conditions. In this group of cells, gene expression was statistically

significantly suppressed to 0.52 (p=0.03 vs. untreated cells) (Fig. 8 right)

In summary, all four studied sulfur sources down-regulated IL-6 expression, but to varying extents depending on concentration and experimental conditions. The most pronounced effect was observed with SMW and TAU, which altered IL-6 expression at all studied concentrations under LPS stimulation. In ALI, the effect was limited to the lowest concentration and occurred only in LPS-stimulated cells, whereas in SFN no statistically significant changes were observed.

4.2.4 Effect of SMW and SBAC on the expression of IL-8

Interleukin-8 is a pro-inflammatory chemokine expressed by various tissues and cell types that participates in neutrophil recruitment and activation during inflammation. IL-8 expression is closely linked to inflammatory response regulation and the NF-κB signalling pathway.

Figures 9 and 10 present the results on how the investigated sulfur sources affect IL-8 expression in unstimulated cells and under LPS-induced inflammation. LPS treatment increases IL-8 expression to 1.23 RU (p=0.05), whereas SA significantly reduces it to 0.78 RU (p=0.04).

For SMW, SFN, and ALI, IL-8 expression did not change significantly compared with the respective controls in either unstimulated or LPS-stimulated cells. At certain concentrations, we observed trends toward reduced expression, but they did not reach statistical significance.

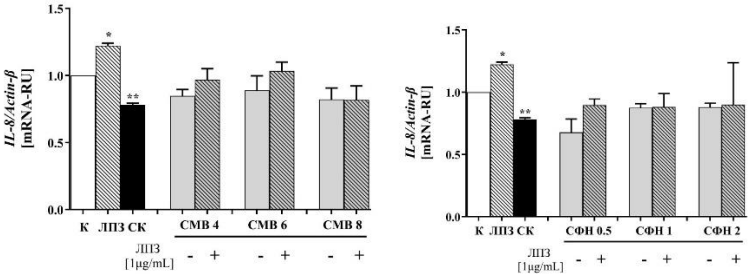


Figure 9. *Effect of SMW (left) and SFN (right) on IL-8 expression in HIEC-6 (*p ≤ 0.05 and **p ≤ 0.01, vs. C)*

Of the four sulfur sources tested, only TAU showed a statistically significant decrease in IL-8 expression. The effect was limited to the highest concentration tested (5 mM) and was observed only in LPS-stimulated cells, where expression was reduced to 0.70 RU (p=0.009) (Fig. 10 left).

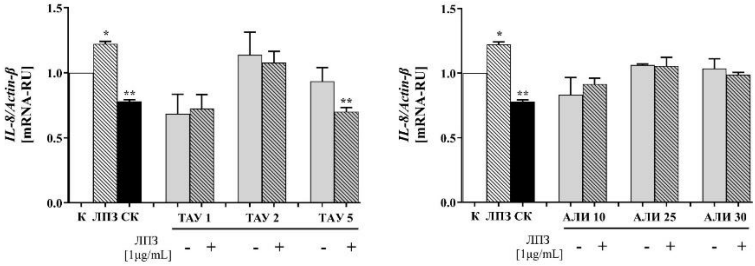


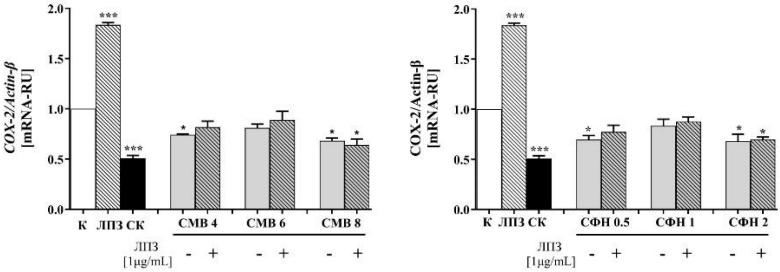
Figure 10. *Effect of TAU (left) and ALI (right) on IL-8 expression in HIEC-6 (*p ≤ 0.05 and **p ≤ 0.01, vs. C)*

In summary, IL-8 expression shows limited sensitivity to the treatments investigated, compared to some of the other pro-inflammatory genes analysed. SMW, SFN, and ALI did not induce statistically significant changes, whereas TAU reduced expression only at the highest concentration and under LPS-induced inflammation.

4.2.5 Effect of SMW and SBAC on the expression of COX-2

COX-2 is an inducible enzyme associated with the inflammatory response, and its gene expression can be regulated by NF-κB-dependent signalling mechanisms. In the present study, the influence of the investigated sulfur sources on COX-2 expression was evaluated in unstimulated and LPS-stimulated HIEC-6 cells (Figs. 11 and 12).

In LPS-stimulated cells, COX-2 expression increased 1.8-fold compared with untreated cells (p=0.0003), whereas salicylic acid (SA) reduced it significantly to 0.51 RU (p=0.0003).



***Фигура 11**. Ефект на СМВ (ляво) и СФН (дясно) върху експресията на COX-2 в HIEC-6, (*p ≤ 0,05; ***p ≤ 0,001 спрямо К).*

With SMW, the lowest concentration tested (4%) significantly reduces COX-2 expression in unstimulated cells (p=0.05). At the highest concentration (8%), expression was significantly reduced in both unstimulated and LPS-stimulated cells to 0.68 RU (p=0.05) and 0.64 RU (p=0.03), respectively. No statistically significant change was noted at the intermediate concentration. Thus, the effect of SMW is characterised by a reduction in expression at both the low and high concentrations, with the change showing no consistent linear dependence on concentration (Fig. 11, left).

SFN exhibits a similar, though more limited, profile of action (Fig. 11, right). The highest concentration (2 μg/mL) significantly reduces COX-2 expression in both unstimulated and LPS-stimulated cells (p=0.04 and p=0.037, respectively). In unstimulated cells, the lowest concentration (0.5 μg/mL) also showed a statistically significant reduction (p=0.05). No statistically significant change was found for the intermediate concentration.

The most pronounced effect on COX-2 expression among the studied sulphur sources was observed with TAU (Fig. 12, left). At 2 mM, expression decreased significantly in both unstimulated cells (to 0.66 RU, p=0.02) and LPS-stimulated cells (to 0.62 RU, p=0.03). At 5 mM, expression decreased to 0.79 RU under both experimental conditions, with the change significant in unstimulated cells (p=0.04) and LPS-stimulated cells (p=0.02). The lowest concentration (1 mM) resulted in a statistically significant decrease only in LPS-stimulated

cells (p=0.002). In unstimulated cells at this concentration, a decrease in expression was also observed to 0.83 RU, but it was not statistically significant.

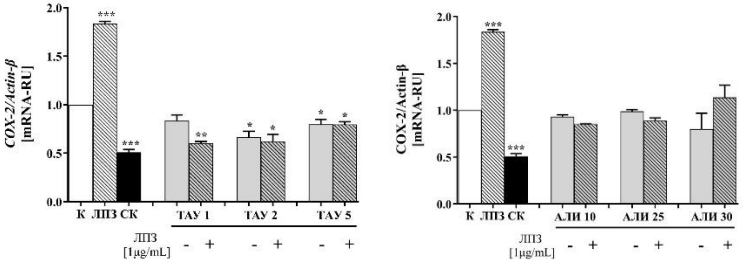


Figure 12. *Effect of TAU (left) and ALI (right) on COX-2 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01, ***p ≤ 0.001 vs. C).*

In contrast to SMW, SFN, and TAU, no statistically significant change in COX-2 expression was observed at any of the tested concentrations of ALI relative to the respective controls (Fig. 12, right).

In summary, SMW, SFN, and TAU reduce COX-2 expression at specific concentrations and under the experimental conditions tested, whereas no statistically significant changes were observed with ALI. The most pronounced inhibition of COX-2 expression was observed with TAU; this effect was noted at all three concentrations under at least one of the experimental conditions tested.

4.2.6 Effect of SMW and SBAC on the expression of CASP1

Caspase-1 is a component of the inflammasome response, associated with IL-1β maturation and regulation of the inflammatory response. In the present study, the influence of the investigated sulfur sources on *CASP1* expression was evaluated in unstimulated and LPS-stimulated HIEC-6 cells (Figs. 13 and 14).

Treatment with LPS significantly increased *CASP1* expression to 2.34 RU (p=0.04) compared with untreated cells, whereas SK decreased it to 0.42 RU (p=0.009).

With SMW, a statistically significant decrease in CASP1 expression was observed at all tested concentrations; at 4%, this effect was observed only in LPS-stimulated cells (p=0.012) (Fig. 13, left). At 6% SMW, expression decreased to 0.68 RU (p=0.03) in unstimulated cells and to 0.50 RU (p=0.002) in LPS-stimulated cells. At 8% SMW, expression decreased to 0.60 RU (p=0.03) in unstimulated cells and to 0.40 RU (p=0.005) in LPS-stimulated cells. These results indicate that SMW exerts a pronounced effect on CASP1 expression across all tested concentrations and both experimental conditions, with the change at 4% being statistically significant only under LPS stimulation.

Treatment with SFN resulted in a statistically significant reduction in CASP1 expression at concentrations of 1 and 2 μg/mL (Fig. 13, right). At 1 μg/mL, this effect was observed only in LPS-stimulated cells, with expression decreasing to 0.40 RU (p=0.02). At 2 μg/mL, a reduction was observed in both unstimulated and LPS-stimulated cells, to 0.40 RU (p=0.02) and 0.30 RU (p=0.022), respectively. No statistically significant change was observed at the lowest concentration. Consequently, under the conditions studied, the effect of SFN is more pronounced at higher concentrations; however, the available data do not allow a definitive determination of a concentration-dependent pattern.

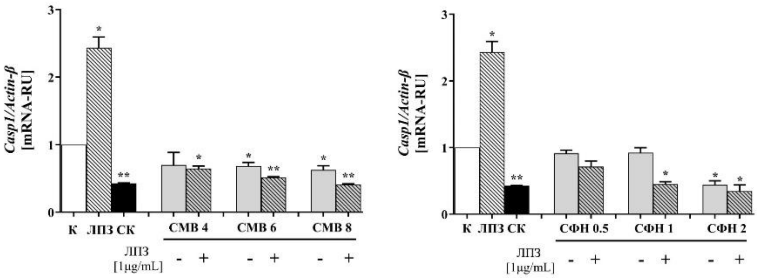


Figure 13. *Effect of SMW (left) and SFN (right) on CASP1 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

With TAU, a statistically significant decrease in CASP1 expression was observed only in LPS-stimulated cells at concentrations of 2 and 5 mM (Fig. 14, left). Expression decreased to

0.59 RU (p=0.05) and 0.30 RU (p=0.03), respectively. No statistically significant changes were observed under the other conditions and concentrations.

The effect of ALI on CASP1 expression is shown in Figure 14 (right). As with SFN, the highest concentration, 30 μg/mL, significantly suppressed gene expression in both unstimulated cells and those under induced inflammation. The observed expression levels under these two conditions were 0.52 RU (p=0.04) and 0.35 RU (p=0.0014), respectively. The intermediate concentration of ALI (25 μg/mL) also significantly suppressed expression, but only in unstimulated cells (0.75 RU, p=0.05 compared to controls).

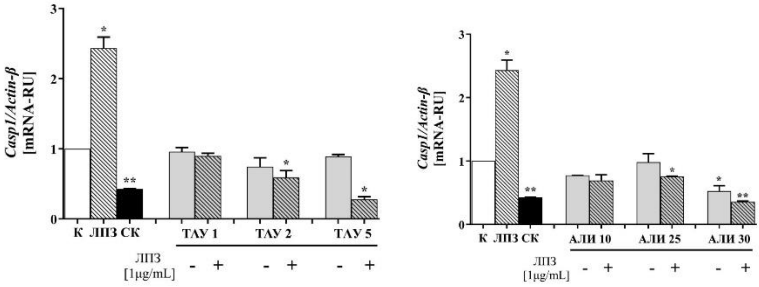


Figure 14. *Effect of TAU (left) and ALI (right) on CASP1 expression in HIEC-6 (*p ≤ 0.05, **p ≤ 0.01, vs. C).*

In summary, all four investigated sulfur sources reduce CASP1 expression at specific concentrations and under the experimental conditions tested, with these changes more pronounced in LPS-stimulated cells. The most extensive and consistent profile is observed with SMW, for which a statistically significant reduction was found at all tested concentrations, whereas for SFN, TAU, and ALI, the effect is limited to specific concentrations and conditions.

4.2.7 Effect of SMW and SBAC on the expression of IL-1β

IL-1β is a key pro-inflammatory cytokine, the expression of which is linked to NF-κB-dependent signalling and the inflammasome response.

Figures 15 and 16 present the effect of the investigated sulfur sources on IL-1β expression. As with other pro-inflammatory genes,

LPS treatment significantly increased IL-1β expression to 1.25 RU ($p=0.03$), whereas SA treatment significantly reduced it to 0.58 RU ($p<0.001$) compared with the respective controls.

In SMW-treated cells, IL-1β expression did not change significantly in either unstimulated or LPS-stimulated cells (Fig. 15, left).

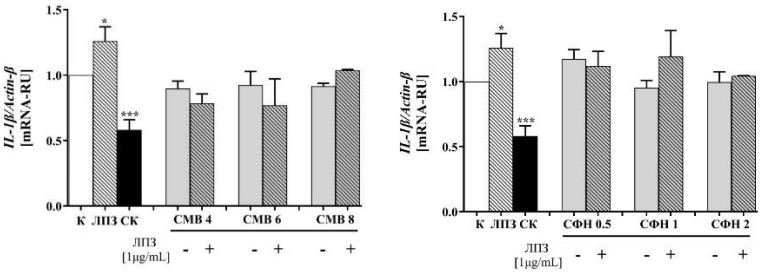


Figure 15. *Effect of SMW (left) and SFN (right) on IL-1β expression in HIEC-6 (*p ≤ 0.05; ***p ≤ 0.001 vs. C).*

Treatment with SFN also failed to produce statistically significant changes in IL-1β expression at any of the tested concentrations or under either experimental condition (Fig. 15, right).

Cells treated with TAU showed the most pronounced effect on IL-1β expression (Fig. 16, left). All three tested concentrations—1, 2, and 5 mM, resulted in a statistically significant reduction in expression, but only in LPS-stimulated cells. The expression levels were 0.67 RU ($p=0.05$), 0.58 RU ($p=0.02$), and 0.63 RU ($p=0.036$), respectively. Unstimulated cells showed no statistically significant changes.

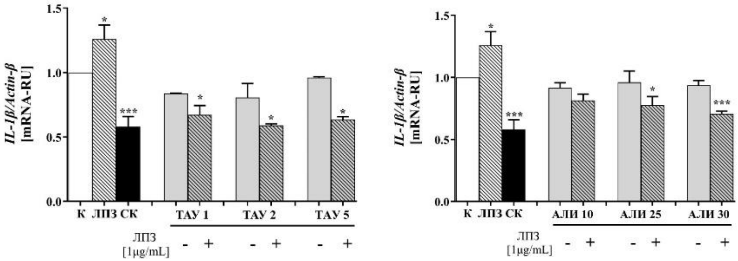


Figure 16. *Effect of TAU (left) and ALI (right) on IL-1β expression in HIEC-6 (*p ≤ 0.05; ***p ≤ 0.001 vs C).*

Treatment with ALI produced a statistically significant reduction in IL-1β expression at 25 and 30 μg/mL; this effect was observed only in LPS-stimulated cells. Expression levels decreased to 0.77 RU (p=0.05) and 0.70 RU (p=0.01), respectively. No statistically significant changes were observed in unstimulated cells at the tested concentrations (Fig. 16, right).

In summary, SMW and SFN did not induce statistically significant changes in IL-1β expression under the tested conditions. TAU had the most pronounced effect, reducing expression at all three concentrations, but only in LPS-stimulated cells. With ALI, a reduction in expression was observed at the medium and highest concentrations, again limited to LPS-stimulated cells. Thus, among the treatments investigated, TAU and ALI showed the most pronounced changes in IL-1β expression, whereas no statistically significant changes were observed for SMW and SFN.

4.2.8 Effect of SMW and SBAC on the expression of IL1RN

Figures 17 and 18 present the effects of the investigated sulfur sources on *IL1RN* expression. In cells treated with LPS alone, IL1RN mRNA levels decreased significantly to 0.77 RU (p=0.02) compared with untreated cells. SA did not cause a statistically significant change in *IL1RN* expression; mRNA levels remained comparable to untreated cells.

Figure 17 (left) presents the results of SMW treatment. In unstimulated cells, 6% and 8% SMW significantly increased *IL1RN* expression, reaching 1.38 RU (p=0.05) and 1.39 RU (p=0.03), respectively. In LPS-stimulated cells, gene expression did not change significantly at any tested SMW concentration.

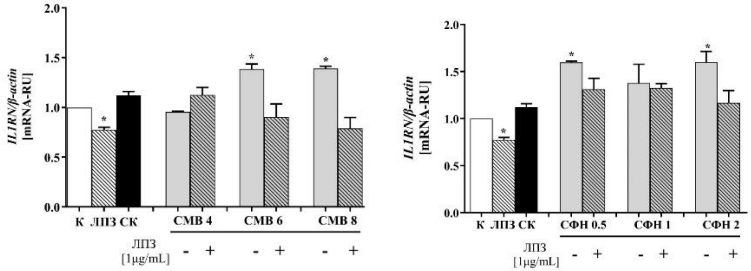


Figure 17. *Effect of SMW (left) and SFN (right) on IL1RN expression in HIEC-6 (*p ≤ 0.05; vs C).*

A similar profile was also observed with SFN (Fig. 17, right). In unstimulated cells, 0.5 and 2 μg/mL SFN produced statistically significant increases in IL1RN expression, reaching 1.59 RU (p=0.03) and 1.60 RU (p=0.05), respectively. No statistically significant change was observed at 1 μg/mL. In LPS-stimulated cells, SFN did not produce a statistically significant change in IL1RN expression at any of the concentrations tested.

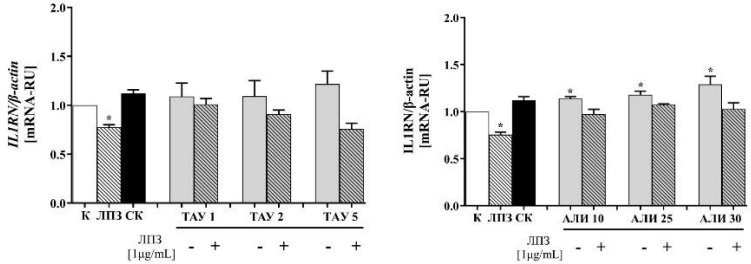


Figure 18. *Effect of TAU (left) and ALI (right) on IL1RN expression in HIEC-6 (*p ≤ 0.05; vs. C).*

Unlike the other sulfur sources investigated, TAU caused no statistically significant change in IL1RN expression at any concentration or under any experimental condition (Fig. 18, left). In LPS-stimulated cells, expression trended downward (to 0.84 RU) at 5 mM TAU, but this change did not reach statistical significance (p=0.20).

With ALI, a more pronounced pattern of changes in IL1RN expression was observed (Fig. 18). All three concentrations tested resulted in a statistically significant increase in expression in unstimulated cells: 1.13 RU at 10 μg/mL (p=0,03), 1.17 RU at 25 μg/mL (p=0,02), and 1.29 RU at 30 μg/mL (p=0,04). In LPS-stimulated cells, no statistically significant change was observed at any of the concentrations applied. Although mean expression levels increased gradually with higher concentrations, the data obtained are insufficient to definitively establish a concentration-dependent relationship.

In summary, SMW, SFN, and ALI increase IL1RN expression in unstimulated cells, though the concentration-response patterns differ. The effect of ALI is the most pronounced, with all three concentrations tested producing a statistically significant increase, whereas for SMW and SFN, a change is observed only at specific concentrations. In LPS-stimulated cells, none of the four sulfur sources investigated induce a statistically significant change in IL1RN expression. For TAU, no statistically significant effect was found under any of the tested conditions.

4.2.9 Effect of SMW and SBAC on the expression of NRF2

The effects of the investigated sulfur sources on Nrf2 expression are shown in Figures 37–40. Application of LPS alone did not produce a statistically significant change in gene expression compared with untreated cells. In contrast, SA led to a statistically significant increase in expression to 1.15 RU (p=0.04).

Following SMW treatment, a statistically significant increase in Nrf2 expression was observed only at the highest concentration of 8%, with expression reaching 1.85 RU (p=0.05) (Fig. 19, left). This effect was observed exclusively in unstimulated cells. In LPS-stimulated cells, no statistically significant change in Nrf2 expression was detected at any SMW concentration tested.

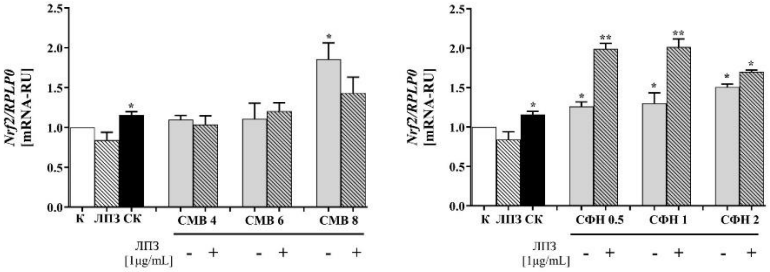


Figure 19. *Effect of SMW (left) and SFN (right) on Nrf2 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01, vs. C).*

Treatment with SFN resulted in a statistically significant increase in Nrf2 expression at all tested concentrations in both unstimulated and LPS-stimulated cells (Fig. 19, right). At 0.5 μg/mL, expression reached 1.26 RU (p=0.02) in unstimulated cells and 1.88 RU (p=0.006) in LPS-stimulated cells. At 1 μg/mL, the corresponding values were 1.30 RU (p=0.05) and 2.00 RU (p=0.002), while at 2 μg/mL, they were 1.50 RU (p=0.02) and 1.69 RU (p=0.012). Higher expression levels than the respective controls were observed under both experimental conditions, with statistically significant changes at all applied concentrations.

Cells treated with TAU also showed a statistically significant increase in Nrf2 expression (Fig. 20, left). In unstimulated cells, all applied concentrations increased expression, whereas in LPS-stimulated cells, only the two higher concentrations showed a statistically significant effect. At 1 mM TAU, expression in unstimulated cells increased to 1.64 RU (p=0.007). At 2 mM TAU, unstimulated cells showed the highest expression (3.70 RU; p<0.001), while LPS-stimulated cells showed 2.80 RU (p<0.001). At 5 mM TAU, the corresponding values were 3.30 RU (p=0.01) and 1.29 RU (p=0.05). The data indicate that the effect magnitude varies across concentrations, with no consistent concentration-dependent trend.

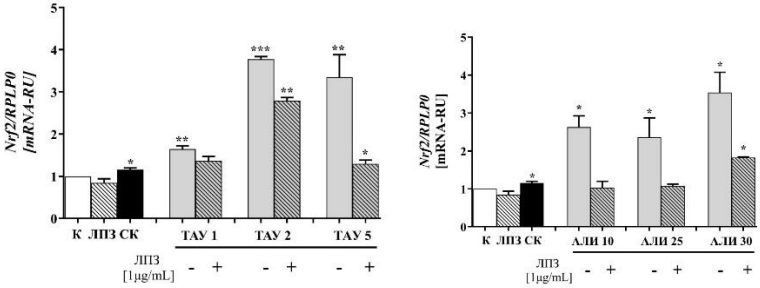


Figure 20. *Effect of TAU (left) and ALI (right) on Nrf2 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 vs. C).*

ALI also led to a statistically significant increase in Nrf2 expression, observed primarily in unstimulated cells (Fig. 20, right). At 10 μg/mL, expression increased to 2.62 RU (p=0.012); at 25 μg/mL, to 2.36 RU (p=0.04); and at 30 μg/mL, to 3.53 RU (p=0.05). At the highest concentration of 30 μg/mL, a statistically significant increase was also observed in LPS-stimulated cells, with expression reaching 1.80 RU (p=0.014). At the other concentrations, no statistically significant change was observed under LPS-induced inflammatory conditions.

In summary, all investigated sulfur sources increased Nrf2 expression under certain experimental conditions, though with varying profiles. SFN showed the most consistent effect, with a statistically significant increase in expression at all tested concentrations in both unstimulated and LPS-stimulated cells. For TAU, the effect was pronounced in unstimulated cells and at the two higher concentrations under LPS-induced inflammation. ALI increased expression predominantly in unstimulated cells, with an effect in LPS-stimulated cells observed only at the highest concentration. In the case of SMW, a change was observed in only 8% of cases, and exclusively in unstimulated cells.

4.2.10 Effect of SMW and SBAC on the expression of GPx

Glutathione peroxidase is an enzyme involved in cellular antioxidant defence. Figures 21 and 22 present the effects of natural sulfur sources on GPx expression. Treatment with LPS alone resulted

in a statistically significant decrease in gene expression to 0.63 RU (p=0.05) compared to untreated cells, whereas treatment with SA led to a statistically significant increase in expression to 1.66 RU (p=0.05).

All natural sulfur sources increased GPx expression, though the magnitude and profile of the effect varied by concentration and experimental conditions.

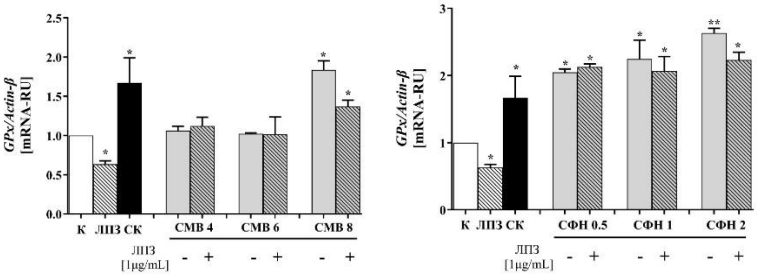


Figure 21. *Effect of SMW (left) and SFN (right) on GPx expression in HIEC-6 (*p ≤ 0.05; vs. C).*

With SMW, a statistically significant increase in GPx expression was observed at the highest tested concentration of 8% in both unstimulated and LPS-stimulated cells (Fig. 21). In unstimulated cells, expression reached 1.83 RU (p=0.03), whereas under LPS-induced inflammation it reached 1.36 RU (p=0.015). At the other tested concentrations, no statistically significant change in GPx expression was observed.

The most consistent pattern of increased expression was observed with SFN and TAU, with statistically significant changes at all applied concentrations under at least one experimental condition (Figs. 21 and 22).

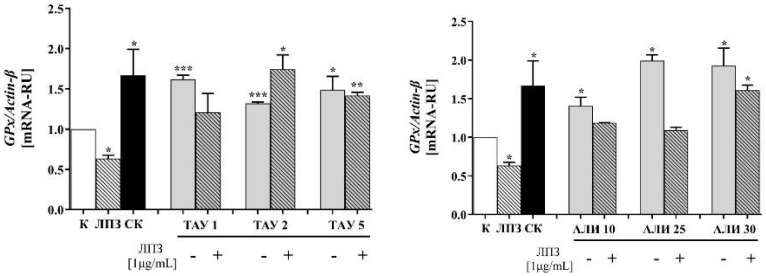


Figure 22. *Effect of TAU (left) and ALI (right) on GPx expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01, and ***p ≤ 0.001 vs. C).*

ALI also led to an increase in GPx expression, though the effect was more pronounced in unstimulated cells (Fig. 22). At 10 μg/mL, the expression level reached 1.40 RU (p=0.05); at 25 μg/mL, it reached 2.00 RU (p=0.018); and at 30 μg/mL, it reached 1.91 RU (p=0.04). In LPS-stimulated cells, expression increased significantly only at the highest concentration of 30 μg/mL (p=0.04). The exact value of the change in this group is not specified in the provided data.

In summary, all investigated natural sources of sulfur demonstrated an increase in GPx expression under certain experimental conditions, though with varying profiles. SFN showed the most consistent effect, with all three concentrations resulting in a statistically significant increase in expression in both unstimulated and LPS-stimulated cells. For TAU, all concentrations increased expression significantly in unstimulated cells, and the two higher concentrations did so in LPS-stimulated cells. For SMW, the effect was limited to the highest concentration but was present under both experimental conditions. In ALI, expression increased at all concentrations in unstimulated cells, whereas in LPS-stimulated cells, the change reached statistical significance only at the highest concentration.

4.2.11 Effect of SMW and SBAC on the expression of SIRT1

SIRT1 regulates several cellular processes related to metabolism, redox homeostasis, and responses to cellular stress. Figures 23 and 24 present the results of the investigation into the effects of natural sulfur sources on SIRT1 expression. Treatment of

cells with LPS alone resulted in a statistically significant decrease in SIRT1 expression to 0.70 RU (p=0.03) compared with untreated cells. In contrast, the application of SA led to a statistically significant increase in expression—approximately a two-fold increase compared with untreated cells (p=0.015).

In the case of SMW, no statistically significant change in SIRT1 expression was observed at any of the concentrations tested or under any of the experimental conditions (Fig. 23).

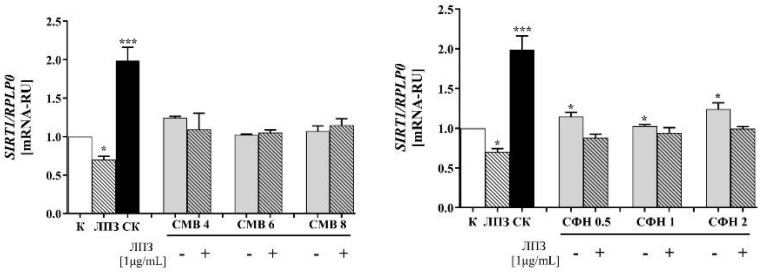


Figure 23 *Effect of SMW (left) and SFN (right) on SIRT1 expression in HIEC-6 (*p ≤ 0.05; ***p ≤ 0.001 vs. C).*

Among the natural sources of sulfur investigated, the most pronounced change in SIRT1 expression was observed with SFN (Fig. 23, right). In unstimulated cells, all concentrations tested produced a statistically significant increase in expression. At 0.5 μg/mL, expression reached 1.14 RU (p=0.05); at 1 μg/mL, it was 1.10 RU (p=0.05); and at 2 μg/mL, it was 1.24 RU (p=0.05). In LPS-stimulated cells, no statistically significant change in SIRT1 expression was observed at any concentration tested.

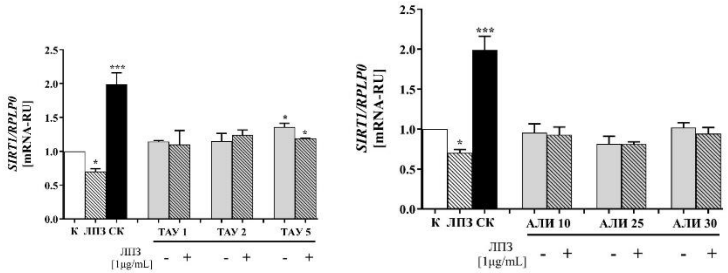


Figure 24. *Effect of TAU (left) and ALI (right) on SIRT1 expression in HIEC-6 (*p ≤ 0.05; ***p ≤ 0.001 vs. C).*

For TAU, a statistically significant increase in SIRT1 expression was observed only at 5 mM, in both unstimulated and LPS-stimulated cells (Fig. 24). In unstimulated cells, expression reached 1.42 RU (p=0.04), whereas in LPS-stimulated cells it reached 1.12 RU (p=0.03). At lower TAU concentrations, no statistically significant change in SIRT1 expression was observed.

For ALI, no statistically significant change in SIRT1 expression was observed at any of the tested concentrations in unstimulated or LPS-stimulated cells (Fig. 24, right).

In summary, among the natural sulfur sources investigated, SFN showed the most pronounced effect on SIRT1 expression, with a statistically significant increase at all tested concentrations in unstimulated cells. For TAU, a change was observed only at the highest concentration under both experimental conditions, whereas no statistically significant changes were found for SMW or ALI. Under LPS-induced inflammatory conditions, none of the four sulfur sources investigated produced a statistically significant increase in SIRT1 expression at any of the concentrations tested.

4.2.12 Effect of SMW and SBAC on the expression of FOXO 4

Graphs 25 and 26 present the effects of the studied natural sulfur sources on the expression of FOXO4 – a transcription factor associated with the regulation of the cellular stress response and processes supporting cellular homeostasis. Administration of LPS alone did not result in a statistically significant change in FOXO4 expression, whereas SA, used as a reference control, increased expression almost twofold to 1.84 RU (p<0.001).

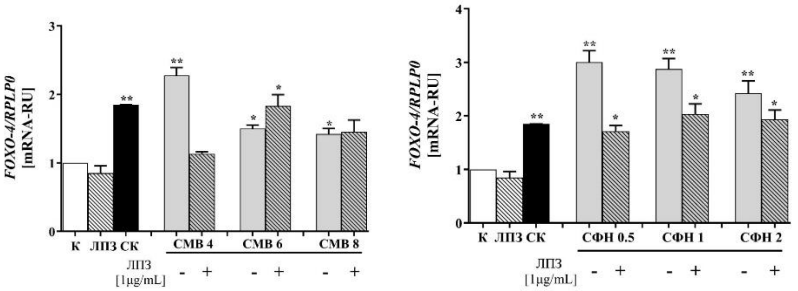


Figure 25. *Effect of SMW (left) and SFN (right) on FOXO4 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

As shown in Figure 25, CM increased FOXO4 expression in unstimulated cells at all concentrations tested, with the effect being statistically significant. Expression levels at 4%, 6%, and 8% CM were 2.27 RU (p=0.006), 1.50 RU (p=0.04), and 1.42 RU (p=0.05), respectively. Under LPS-induced inflammatory conditions, a statistically significant increase was observed at 6% CM, with expression reaching 1.80 RU (p=0.011). At 8% CM, a trend towards increased expression (up to 1.45 RU) was noted, but the difference did not reach statistical significance (p=0.07).

Among the treatments investigated, SFN produced the most pronounced increase in FOXO4 expression. In unstimulated cells, SFN increased expression by 2.5- to 3-fold; a statistically significant increase was also observed under LPS-induced inflammatory conditions, although to a lesser extent (Fig. 25).

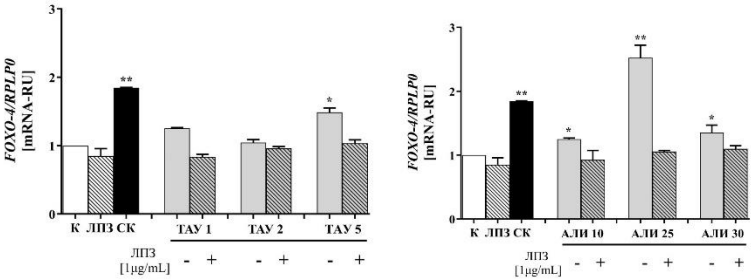


Figure 26. *Effect of TAU (left) and ALI (right) on FOXO4 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

For TAU, a statistically significant increase in FOXO4 expression was observed only at the highest applied concentration (5 mM) in unstimulated cells, with expression levels reaching 1.48 RU (p=0.02). Under LPS-induced inflammatory conditions, no statistically significant effect of TAU was observed. Lower concentrations (1 mM and 2 mM) also failed to produce statistically significant changes in expression under either experimental condition (Fig. 26).

For ALI, a statistically significant increase in FOXO4 expression was observed at all applied concentrations, but only in unstimulated cells (Fig. 26). Expression levels at 10, 25, and 30 μg/mL were 1.25 RU (p=0.05), 2.50 RU (p=0.01), and 1.35 RU (p=0.04), respectively. Under LPS-induced inflammation, none of the ALI concentrations resulted in a statistically significant change in FOXO4 expression.

In summary, all four natural sulfur sources increased FOXO4 expression in unstimulated cells, though the magnitude and concentration-dependence profiles varied. SFN demonstrated the most pronounced effect—including in LPS-stimulated cells—whereas for SMW and ALI, the increase was primarily limited to unstimulated cells. In the TAU group, a change was observed only at the highest concentration in unstimulated cells.

4.2.13 Effect of SMW and SBAC on the expression of SULT1A1

SULT1A1 encodes a phase II biotransformation enzyme that catalyses the sulfation of numerous endogenous and exogenous compounds, including steroid and thyroid hormones, as well as pharmaceutical drugs.

The effects of the investigated natural sulfur sources on SULT1A1 expression are shown in Figures 27 and 28. Administration of LPS alone resulted in a statistically significant decrease in SULT1A1 expression to 0.78 RU (p=0.002), whereas SA, used as a reference control, reduced expression to 0.60 RU (p=0.01).

For SMW, the effect profile varied by experimental condition and concentration (Fig. 27, left). In unstimulated cells, 6% and 8% SMW produced statistically significant increases in SULT1A1

expression to 1.15 RU (p=0.02) and 1.20 RU (p=0.03), respectively. Under LPS-induced inflammation, the lowest concentration—4% SMW—produced a statistically significant decrease in expression to 0.70 RU (p=0.02). No statistically significant changes were observed at the other SMW concentrations.

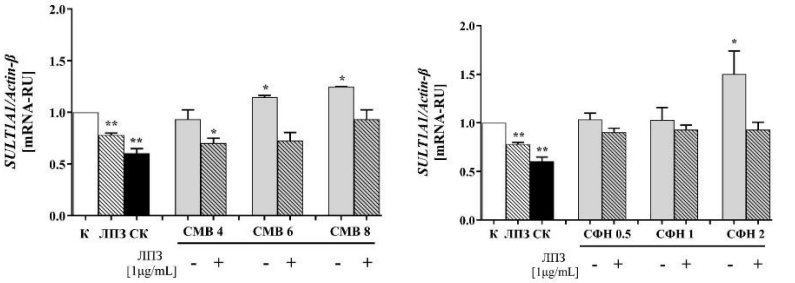


Figure 27. *Effect of SMW (left) and SFN (right) on SULT1A1 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

SFN showed a more limited effect on SULT1A1 expression (Fig. 27, right). A statistically significant increase was observed only at the highest concentration of 2 μg/mL in unstimulated cells, where expression reached 1.50 RU (p=0.05). At concentrations of 0.5 and 1 μg/mL, no statistically significant changes in expression were observed under either experimental condition.

A similarly limited profile was observed for TAU (Fig. 28, left). The highest concentration of 5 mM resulted in a statistically significant increase in SULT1A1 expression to 1.42 RU (p=0.02) in unstimulated cells. At lower concentrations and under conditions of LPS-induced inflammation, no statistically significant changes were observed.

ALI did not lead to statistically significant changes in SULT1A1 expression at any of the concentrations applied or under either of the two experimental conditions (Fig. 28, right).

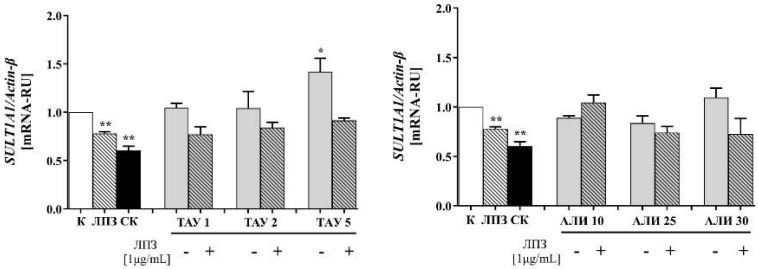


Figure 28. *Effect of TAU on SULT1A1 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C)*

In summary, SMW showed the broadest range of changes in SULT1A1 expression, which varied by experimental condition; this included an increase in unstimulated cells and a decrease in LPS-stimulated cells at specific concentrations. For SFN and TAU, an increase in expression was observed only at the highest concentration tested and exclusively in unstimulated cells, whereas ALI did not induce any statistically significant changes.

4.2.14 Effect of SMW and SBAC on the expression of SULT1A3

SULT1A3 encodes a phase II biotransformation enzyme, catecholamine sulfotransferase, which catalyses the sulfation of catecholamines and other substrates.

The effects of the studied natural sulfur sources on SULT1A3 expression are presented in Figures 29 and 30. A single application of SA and LPS led to statistically significant decreases in gene expression to 0.59 RU (p=0.007) and 0.80 RU (p=0.05), respectively.

In SMW, no statistically significant change in SULT1A3 expression was found at any of the applied concentrations and under any of the two experimental conditions (Fig. 29 left).

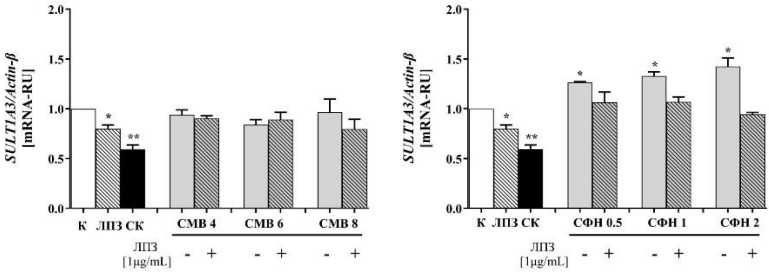


Figure 29. *Effect of SMW (left) and SFN (right) on SULT1A3 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

Among the natural sulfur sources investigated, SFN showed the most pronounced increase in SULT1A3 expression (Fig. 29, right). All three tested concentrations resulted in a statistically significant increase in expression in unstimulated cells, with levels of 1.26 RU (p=0.04), 1.33 RU (p=0.02), and 1.42 RU (p=0.011) at 0.5, 1, and 2 μg/mL, respectively. Under LPS-induced inflammation, we observed no statistically significant changes at any concentration.

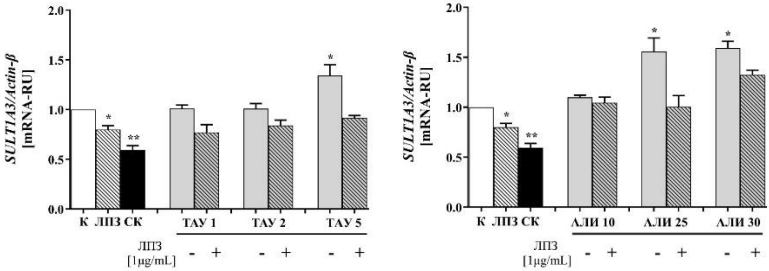


Figure 30. *Effect of TAU (left) and ALI (right) on SULT1A3 expression in HIEC-6 (*p ≤ 0.05; **p ≤ 0.01 vs. C).*

For TAU, SULT1A3 expression increased only at the highest applied concentration (5 mM) in unstimulated cells, reaching 1.34 RU (p=0.05). At lower concentrations and under conditions of LPS-induced inflammation, no statistically significant changes were found (Fig. 30, left).

For ALI, SULT1A3 expression increased significantly in unstimulated cells at the two higher concentrations—25 and 30 μg/mL—reaching 1.55 RU (p=0.04) and 1.59 RU (p=0.05),

respectively. Under the other conditions, no statistically significant change was found (Fig. 30, right).

In summary, the natural sulfur sources investigated showed distinct profiles of SULT1A3 expression changes. SFN induced an increase at all applied concentrations in unstimulated cells, whereas for TAU and ALI, the effect was limited to the highest concentration and the two higher concentrations, respectively. For SMW, no statistically significant change was observed. A characteristic feature of SFN, TAU, and ALI was that the increase in expression was observed only in unstimulated cells, with no statistically significant changes under conditions of LPS-induced inflammation.

4.3 *CORRELATION ANALYSES*

A correlation analysis of the expression of all 14 genes was performed for the four sulfur sources, both in unstimulated HIEC-6 cells and under LPS-induced inflammation. Pearson's linear correlation (r) was used, and the data are presented as colour-coded correlation matrices.

4.3.1 Comparison of correlation dependencies under the influence of microwave radiation, in unstimulated cells, and under conditions of induced inflammation

In cells treated with SMW, LPS-induced inflammation is associated with a selective restructuring of expression dependencies. Key pro-inflammatory genes, specifically NF-κB, TNF-α, IL-6, IL-8, COX-2, and Caspase-1, show tighter coordination, though this does not reflect uniform amplification across all interconnections; instead, it indicates a specific reorganisation of the inflammatory expression module. Concurrently, negative correlations between NF-κB and genes linked to the antioxidant-adaptive response strengthen, while Nrf2, GPx, SIRT1, and FOXO4 show a more coordinated expression profile. These findings point to a distinct structuring of the inflammatory and adaptive expression responses under LPS stimulation.

Of particular interest are IL1RN and SULT1A1; their characteristic associations with the two primary expression modules—observed under non-stimulated conditions are either weakened or altered in the presence of LPS. Thus, the correlation analysis reveals an LPS-associated reorganisation of expression relationships in SMW-treated

cells, characterised by a clearer demarcation between the pro-inflammatory and antioxidant-adaptive profiles. These associations are exploratory in nature and do not establish causal or mechanistic relationships.

4.3.2 Comparison of correlation dependencies under the influence of SFN, in unstimulated cells, and under conditions of induced inflammation

The primary effect of LPS on expression relationships during SFN treatment is a shift from relatively heterogeneous correlation patterns to more clearly organised, distinct functional modules. Under induced inflammation, pro-inflammatory markers form a more coordinated profile, whereas antioxidant-adaptive genes cluster into a separate, internally coherent module. Notably, the Nrf2/GPx axis maintains an exceptionally strong positive correlation regardless of the inflammatory context ($r = 0.97$ without LPS and $r = 0.98$ with LPS). This identifies coordination between Nrf2 and GPx as one of the most robust features of the expression response to SFN, consistent with the preservation of a tight link between the Nrf2-mediated transcriptional response and antioxidant defence in the presence of an inflammatory stimulus.

At the same time, LPS not only preserve this axis; it also entails a broader restructuring of the relationships between the inflammatory and protective profiles. In the interpretation, emphasise specific changes in the correlations of NF-κB, TNF-α, IL-6, IL-8, COX-2, Caspase-1, and IL-1β relative to Nrf2, GPx, SIRT1, and FOXO4. This is because strengthening internal interdependencies within the pro-inflammatory module, coupled with strengthening negative associations between this module and antioxidant/adaptive genes, would support the conclusion that the two profiles are more functionally distinct. In this context, the stability of the Nrf2/GPx relationship during the transition from a basal state to an LPS-stimulated state indicates that the inflammatory stimulus affects the antioxidant module's connections to the broader expression network more than it affects the internal coordination between Nrf2 and GPx.

The correlation matrix for the sulforaphane condition (in the absence of LPS stimulation) reveals a pronounced correlation among the genes involved in antioxidant defence and stress adaptation. As shown in Figure 63, the transcription factor Nrf2 exhibits a strong

positive association with GPx (r = 0.97), as well as with the expression of SIRT1 (r = 0.81), FOXO4 (r = 0.79), SULT1A1 (r = 0.77), and, most notably, SULT1A3 (r = 0.98). Furthermore, GPx expression is strongly positively correlated with FOXO4 (r = 0.91) and is virtually identical to SULT1A3 (r = 1.00). IL1RN can also be included in this module, based on positive correlations with Nrf2 (r = 0.89), GPx (r = 0.93), SIRT1 (r = 0.83), FOXO4 (r = 0.91), and SULT1A3 (r = 0.91).).

4.3.3 *Comparison of correlation dependencies under the influence of TAU, in unstimulated cells, and under conditions of induced inflammation*

In a direct comparison of the two correlation matrices under the influence of TAU, the most noticeable radical change is in the expression profile of IL1RN relative to pro-inflammatory and antioxidant genes. In unstimulated cells, IL1RN was clearly opposed to the main pro-inflammatory markers, with significant negative correlations with NF-κB, TNF-α, and IL-6. At the same time, it is positively associated with antioxidant and stress-adaptive genes - Nrf2, GPx, SIRT1 and FOXO4. Under conditions of LPA-induced inflammation, this configuration is reversed to a significant extent. IL1RN began to correlate positively with NF-κB (r = 0.93), TNF-α (r = 0.75), IL-6 (r = 0.98) and Caspase-1 (r = 0.98), and the relationship with IL-1β increased from virtually absent under basal conditions (r = -0.09) to strongly positive (r = 0.74). In parallel, the dependencies of IL1RN on Nrf2, GPx, SIRT1 and FOXO4 changed direction and became negative.

In summary, under TAU action without an inflammatory stimulus, IL1RN expression correlates with the antioxidant and stress-adaptive profile and is inversely related to key pro-inflammatory genes. Upon LPS stimulation, the expression of this marker is repositioned to a profile that changes in parallel with a large number of inflammatory markers and is opposite to Nrf2/GPx/SIRT1/FOXO4. This can probably be explained by the natural role of IL1RN as an antagonist of the IL-1 receptor in conditions of an activated inflammatory response.

4.3.4 *Comparison of correlation dependencies under the influence of ALI, in unstimulated cells, and under conditions of induced inflammation*

Upon treatment with ALI, LPS-induced inflammation is associated with a distinct reorganisation of the correlation pattern shifting from a relatively heterogeneous profile in unstimulated cells to a more clearly polarised structure, characterised by a sharper distinction between pro-inflammatory and antioxidant-adaptive expression relationships. Inflammation enhances coordination among key pro-inflammatory markers, including NF-κB, TNF-α, Caspase-1, and IL-1β, while their correlations with the Nrf2/GPx-related profile become strongly negative. Notably, the NF-κB/Nrf2 expression relationship shifts, with the negative correlation intensifying from r = -0.70 in unstimulated cells to r = -0.92 after LPS exposure. A similar opposition is observed between NF-κB and GPx (shifting from r = -0.82 to r = -0.93), as well as between NF-κB and FOXO4 (from r = -0.41 to r = -0.95); this indicates that, under an inflammatory stimulus, the expression variations of these protective markers become far more distinctly opposed to the expression of NF-κB.

Concurrently, the internal coordination of the antioxidant-adaptive profile is strengthened. The Nrf2/GPx relationship remains strongly positive (r = 0.86 in unstimulated cells and r = 0.90 in the presence of LPS), while the Nrf2/FOXO4 correlation strengthens significantly (from r = 0.42 to r = 0.81). The inclusion of SULT1A3 in this correlation profile is particularly notable: the Nrf2/SULT1A3 expression dependency increases from r = 0.74 to r = 0.96, while the GPx/SULT1A3 relationship remains extremely strong, further increasing under inflammatory conditions from r = 0.96 to r = 0.98. At the same time, SULT1A3 develops stronger negative associations with certain pro-inflammatory markers, including Caspase-1 (from r = -0.48 in unstimulated cells to r = -0.97 under induced inflammation) and IL-1β (r = -0.32 and r = -0.87, respectively). Consequently, inflammation does not merely strengthen individual correlations; it is associated with more pronounced polarisation in the expression patterns of the two primary profiles—pro-inflammatory and antioxidant.

4.3.5 Integrated comparative analysis of expression patterns under the influence of the investigated dietary sources of sulfur

A comparative analysis of the eight correlation matrices identified two functionally distinct expression modules: an inflammatory module—comprising primarily NF-κB, TNF-α, IL-6, IL-8, COX-2, Caspase-1, and IL-1β—and a redox/adaptive module centred around Nrf2 and GPx, which, depending on the treatment, also includes SIRT1, FOXO4, IL1RN, and SULT1A1/SULT1A3. While LPS-induced inflammation generally reinforces the unidirectional relationships among markers within the inflammatory module, the specific expression profile varies by applied agent. In SFN, a robust Nrf2/GPx module stands out; in SMW, NF-κB and Nrf2 are clearly opposed; and in ALI, a pronounced polarisation emerges between the inflammatory and redox/adaptive panels. TAU exhibits dynamic relationships between IL1RN and the other markers.

The most consistent positive correlation is between Nrf2 and GPx, a relationship preserved across all eight conditions, though its strength varies. Conversely, NF-κB and Nrf2 consistently show a negative correlation across all matrices. These findings suggest coordinated expression profiles whose manifestation depends on the agent and cellular state. This correlation analysis is exploratory in nature and does not establish causal or mechanistic links.

***Table 2**. Comparative analysis of the most significant expression relationships*

Treatment	**- LPS**	**+LPS**
Nrf2/GPx		
SMW	0.99 (*p=0,01*)	0.90 (*p=0,04*)
SFN	0.98 (*p=0,03*)	0.97 (*p=0,004*)
TAU	0.89 (*ns)*	0.54 (*p=0.04*)
ALI	0.90 (*ns)*	0.86 (*p=0.04*)
NF-κB/Nrf2		
SMW	−0.79 (*ns)*	−0.99 *(p<0.001*)
SFN	−0.96 (*p=0.04*)	−0.71 (*ns)*
TAU	−0.58 (*ns)*	−0.57 (*ns)*

ALI	−0.70 (*ns)*	−0.92 *(p=0.03*)

5 DISCUSSION

In this study, SMW and SBAC were compared for their potential to modulate pro- and anti-inflammatory markers in an in vitro model of LPS-induced inflammation in human intestinal epithelial cells. To the best of our knowledge, this is the first study to investigate the anti-inflammatory potential of SMW from the Varna Basin in comparison with well-studied sulfur-containing compounds.

Effects of SMW and SBAC on cell viability

Assessing cell viability allowed us to determine the concentration range suitable for subsequent investigation. All SMW and TAU concentrations maintained HIEC-6 cell viability above 80%, indicating no significant cytotoxicity under the tested conditions. Similarly, ALI viability did not fall below this threshold, despite statistically significant changes at higher concentrations. These results align with published data on differences in the cytotoxic potential of garlic-derived organosulfides toward normal versus tumour cells (Mansingh et al. 2018; Zhang et al. 2020).

SFN showed a more complex profile, with viability increasing at intermediate concentrations, then decreasing at the highest concentration. This response aligns with the reported dependence of SFN's effects on concentration, cell type, and experimental model (Baralić et al. 2024). Overall, the results indicate that the concentration ranges used for SMW, TAU, and ALI maintain cell viability, whereas SFN's effect is more strongly concentration-dependent. These data provide an experimental basis for interpreting subsequent changes in gene expression..

Effects of SMW and SBAC on gene expression of NF-kB

The LPS-induced increase in NF-κB expression confirms the development of a pro-inflammatory transcriptional response in the HIEC-6 model used, while the reduction observed following treatment with salicylic acid demonstrates the model's sensitivity to anti-inflammatory modulation. When interpreting these results, note that the study assesses expression of an NF-κB-related transcript, not the functional activity of the entire NF-κB signalling pathway.

SMW (sulfurous mineral water) shows the most consistent profile; 6% and 8% concentrations reduce NF-κB expression in both unstimulated and LPS-stimulated cells, with a trend towards a more pronounced effect at higher concentrations. These findings align with published data showing that H_2S and sulfide compounds can modulate NF-κB-associated inflammatory processes (Sen et al. 2012; Rios et al. 2015; Bhatia and Gaddam 2021; Shahid and Bhatia 2024; Xu et al. 2026). At the same time, the distinct profile observed for the complex sulfurous thermal water suggests that the effects of the mineral water cannot be attributed solely to H_2S (Prandelli et al. 2013). The complex mineral composition of SMW must also be taken into account; consequently, the relative contribution of individual components cannot be determined within the current model. Treatment with SFN suppresses NF-κB expression at all tested concentrations in unstimulated cells, whereas under LPS stimulation, a significant effect is observed only at 2 μg/mL. This profile is consistent with data on the anti-inflammatory and redox-modulating effects of SFN in intestinal and other cellular models, while also highlighting the dependence of the effect on concentration and experimental conditions (Heiss et al. 2001; Heiss and Gerhäuser 2005; Zhang and Wu 2021). For TAU, the effect also depends on concentration and the presence of LPS, which aligns with reported modulation of TLR/NF-κB-related processes in epithelial models (Lin et al. 2025; Xiao et al. 2025). These results should be distinguished from effects attributed to TauCl, as the present study was conducted using TAU.

ALI shows the most limited effect on NF-κB, with a significant reduction observed at 30 μg/mL under both experimental conditions. These data are consistent with published reports on the anti-inflammatory activity of ALI, while also aligning with findings that indicate varying sensitivity depending on the cellular model (Shi et al. 2017; Wang et al. 2017; Quintero-Fabián et al. 2013; Ho and Su 2014). Therefore, the effects of ALI in HIEC-6 cells should not be directly extrapolated to more reactive organosulfides derived from garlic.

A comparative analysis reveals that the four natural sulfur-containing agents modulate NF-κB expression with varying dependencies on concentration and context. SMW exhibits the most consistent effect; SFN demonstrates a broad effect in unstimulated cells; TAU shows a marked dependence on both concentration and

inflammatory stimulus; and ALI displays an effect primarily at the highest concentration tested. The literature search yielded no directly comparable studies in HIEC-6 cells using an analogous experimental design; consequently, these results complement existing data on how various natural sulfur-containing sources modulate NF-κB expression.

Effects of SMW and SBAC on gene expression of TNF-α

TNF-α is a key mediator of the inflammatory response; therefore, changes in its expression serve as an important indicator of the inflammatory profile of the cellular model under study. In the present study, SMW and all sulfur-containing agents investigated show potential to suppress TNF-α expression. The effects of SMW, SFN, and TAU are concentration-dependent and become more pronounced under LPS-induced conditions. At 8% SMW and 5 mM TAU, suppression is observed in both unstimulated and LPS-stimulated cells, whereas ALI shows a significant effect only at specific concentrations and conditions.

The results for SFN, TAU, and ALI align with published data on their ability to limit pro-inflammatory mediator expression. Anti-inflammatory effects of SFN have been documented in intestinal cell models, including under LPS-induced conditions (Zhang and Wu 2021), while TAU has been reported to influence inflammatory cytokines across various experimental models (Ma et al. 2022). Data from ALI also support its potential to modulate pro-inflammatory gene expression (Ruhee et al. 2019; Lv et al. 2021). While these findings are consistent with the changes observed in the present study, they do not allow direct deduction of a specific mechanism within the HIEC6 model. Of particular interest is the SMW profile, which shows concentration-dependent suppression of TNF-α expression under various experimental conditions. As a complex natural source, SMW contains sulfur compounds and mineral components; however, the current experimental design does not allow for the determination of the relative contribution of individual constituents. These results supplement existing data on the biological effects of SMW and underscore the need for further investigation into the specific components and mechanisms underlying the observed changes in the expression of inflammatory genes.

Effects of SMW and SBAC on gene expression of IL-6

In the present study, TAU and SMW most strongly suppress IL-6 expression, particularly under LPS-induced conditions. For ALI, a significant effect is observed only at 10 μg/mL, whereas SFN does not significantly affect IL-6 expression, regardless of the presence of an inflammatory stimulus. These results indicate that the individual sulfur-containing agents exhibit distinct profiles regarding their influence on this inflammatory marker.

The effect of TAU is consistent with published data regarding the suppression of IL-6 in various experimental models of inflammation (SAhaffer et al. 2014; Ma et al. 2022). The similar profile observed in HIEC-6 cells adds to the existing evidence of its activity in intestinal cell models. SMW also suppresses IL-6 expression under LPS stimulation. This aligns with findings on the anti-inflammatory properties of sulfide mineral waters in other cell models, where IL-6 and other inflammatory mediators have been reduced (CheleSAhi et al. 2020; Carbajo et al. 2025). However, as a complex natural source, SMW does not allow for the determination of the relative contribution of its individual sulfur and mineral components.

The limited effect of ALI, observed at a single concentration, underscores the dependence of the response on both concentration and the specific cell model. Available data on other organosulfides, including allicin, indicate a similar sensitivity to experimental conditions, yet they do not allow direct extrapolation to ALI. Particularly noteworthy is the lack of a significant effect of SFN on IL-6, which contrasts with the suppression of this cytokine reported in other cellular models (Zhang and Wu 2021; Lv et al. 2021). This discrepancy likely reflects the specificity of the cellular model and demonstrates that the absence of change in a single inflammatory transcript does not preclude modulation of other components of the cellular response.

In summary, IL-6 emerges as a sensitive transcriptional marker for TAU and SMW in HIEC-6 cells, whereas ALI exhibits a limited effect, and SFN shows no significant effect under the conditions studied. These differences underscore that their effects depend on gene expression, which is governed by the compound's chemical nature, concentration, and cellular context, and highlight the need to

monitor a range of inflammatory and redox-related genes simultaneously.

Effects of SMW and SBAC on gene expression of IL-8

In the present study, TAU is the only sulfur-containing agent among those investigated that suppresses IL-8 expression, with the effect more pronounced under LPS stimulation. In contrast, SFN, ALI, and SMW did not significantly change IL-8 expression under the conditions studied. The effect of TAU aligns with published *in vitro* data regarding IL-8 suppression during inflammatory stimulation and adds to existing evidence of its anti-inflammatory effects in intestinal cell models (Barua et al., 2001; SAhaffer et al., 2014; Ma et al., 2022). Taken together with the observed suppression of IL-6, this result distinguishes TAU from the other agents studied because of its broad influence on inflammatory cytokine expression.

The absence of a significant effect of SFN on IL-8 and IL-6 differs from findings in other cell models; this likely reflects the dependence of the expression response on cellular context and experimental conditions (Zhang and Wu, 2021; Ruhee et al., 2019; Lv et al., 2021). The similar lack of change observed with ALI and SMW indicates that the investigated agents' effects are not uniform across inflammatory transcripts. Limited data on the effect of SMW on IL-8, combined with discrepancies between expression and protein levels observed in other models, suggest that this result should be interpreted with caution (CheleSAhi et al., 2020; Carbajo et al., 2025). Similarly, data on ALI indicate that its anti-inflammatory effects depend on the specific cellular model and experimental conditions (Quintero-Fabián et al., 2013; Shi et al., 2017).

In summary, IL-8 shows variable sensitivity to the agents investigated; TAU has the most pronounced effect, suppressing both IL-6 and IL-8 expression. These findings underscore the importance of cellular context and the simultaneous monitoring of multiple inflammatory genes when evaluating the effects of natural sulfur-containing sources on gene expression.

Effects of SMW and SBAC on gene expression of COX-2

In the present study, SMW, SFN, and TAU show variable, though predominantly inhibitory, effects on COX-2 expression, whereas ALI causes no significant changes. SMW suppresses COX-2 expression at the highest concentration tested in both unstimulated and

LPS-stimulated cells, whereas TAU shows a concentration-dependent effect only under LPS stimulation. These findings align with published data showing SFN suppresses COX-2 in the context of LPS-induced inflammation (Shan et al. 2010; Lee et al. 2012; Ruhee et al. 2020) and contribute to the existing body of evidence on the influence of TAU on the expression of inflammatory genes (Miao et al. 2011; Swiderski et al. 2023).

The effect of SMW is particularly noteworthy, given the limited data on how natural sulfur-containing mineral waters affect COX-2 expression in intestinal cell models. However, as a complex natural source, SMW does not allow for the determination of the specific contributions of individual sulfur or mineral components. The lack of an observed effect with ALI indicates that its influence on inflammatory gene expression is not uniform across all markers and does not necessarily mirror the profile of more reactive organosulfides, such as allicin. In summary, COX-2 shows varying sensitivity to the agents investigated; SMW, SFN, and TAU can suppress its expression under specific concentration and stimulation conditions, whereas ALI showed no such effect. These differences underscore the importance of an agent's chemical nature, its concentration, and the cellular context in determining the expression profile.

Effects of SMW and SBAC on gene expression of Caspase 1

In the present study, all four natural sulfur sources suppress CASP1 expression in HIEC-6 cells, with a more pronounced effect under LPS stimulation, though they show distinct concentration- and context-dependent profiles. SMW demonstrates the most consistent effect, suppressing expression at all tested concentrations in both unstimulated and LPS-stimulated cells. SFN shows effects at higher concentrations, TAU primarily under LPS stimulation, and ALI at 25 and 30 μg/mL. The concurrent suppression of CASP1 and NF-κB by SMW and SFN suggests a similar transcriptional profile, though it does not establish a causal link.

These results align with published data on the effects of H_2S, SFN, and TAU on inflammasome-related inflammatory processes (Qin et al. 2019; Greaney et al. 2016; Nam et al. 2017). Regarding ALI, direct evidence for CASP1 is limited, as most research focuses on allicin. However, allicin’s capacity to modulate LPS-induced

inflammatory signalling supports the effect observed here (Shi et al. 2017; Quintero-Fabián et al. 2013). For SMW, no directly comparable data in HIEC-6 cells were identified, which further strengthens the value of the expression profile obtained. Importantly, the current data reflect transcriptional modulation of CASP1 and do not allow conclusions about caspase-1 activity or the functional activation of the NLRP3 inflammasome. In this context, the results extend the characterisation of the inflammatory expression response to include a gene associated with inflammasome regulation.

Effects of SMW and SBAC on gene expression of IL-1β

In the present study, TAU and ALI suppress IL-1β expression only under LPS-induced conditions; this indicates that the effect depends on the inflammatory context and complements existing data on the anti-inflammatory properties of both agents. In contrast, SMW and SFN did not significantly change IL-1β expression under the conditions examined. These differences highlight the specificity of the expression response, which varies by cellular model and stimulation conditions.

In HIEC-6 cells, the IL-1β expression response may differ from that observed in cells with a well-defined NLRP3 inflammasome. Data from LPS-stimulated HIEC-6 cells suggest NLRP3-independent IL-1β activation mediated by caspase-4 (Chan et al. 2023), which may explain the context-dependent response to TAU and ALI. This interpretation remains hypothetical, as caspase-4 and its associated mechanisms were not investigated in the current study. These findings further characterise the IL-1β expression profile in HIEC-6 cells following treatment with natural sulfur-containing compounds.

Effects of SMW and SBAC on gene expression of IL1RN

IL1RN is a natural antagonist of the IL-1β receptor and an important regulator of the inflammatory response; the balance between IL-1β and IL1RN helps limit this response (Cardoso et al. 2022; Maculewicz et al. 2022). In the present study, SMW, SFN, and ALI stimulate IL1RN expression in unstimulated HIEC-6 cells, whereas LPS stimulation does not. This discrepancy indicates that the IL1RN expression response depends on the cellular state and stimulation conditions.

The observed basal response to SMW, SFN, and ALI complements the previously noted suppression of several pro-inflammatory genes, demonstrating that the agents under investigation can modulate the expression of regulatory components of the inflammatory response. The limited existing data on the transcriptional regulation of IL1RN in intestinal cell models treated with natural sulfur-containing compounds further support these findings. Nevertheless, the observed changes in IL1RN and IL-1β levels do not prove a functional shift in the ratio between these two mediators, nor do they establish a direct causal regulatory relationship. These results expand the expression profile associated with the investigated agents by providing data on a gene linked to anti-inflammatory regulation. Effects of SMW and sulfur-containing compounds on Nrf2 gene expression

NRF2 expression varied significantly among the sulfur-containing agents investigated. SFN showed the strongest effect, increasing NRF2 expression at all concentrations, with a stronger response under LPS stimulation. These findings align with the established role of SFN as a natural activator of the Keap1–NRF2 signalling pathway, as well as with evidence regarding its associated antioxidant and anti-inflammatory activities (Cuadrado et al., 2019; Zhang and Wu, 2021; Suzuki et al., 2023; Chen et al., 2024; Sezgin-Bayindir et al., 2025; Zhang, 2025).

TAU also increased NRF2 expression, more markedly in unstimulated cells; this indicates that the response depends on cellular state and is consistent with data suggesting involvement of PI3K/Akt- and MAPK-dependent mechanisms (SAhaffer et al., 2014; Ma et al., 2022). For SMW, a significant increase was observed only at 8% and exclusively in the absence of LPS. Limited literature on sulfur-containing mineral waters (SMW) and similar mineral waters, as well as studies involving H_2S donors, support the potential involvement of sulfur compounds in regulating Keap1–NRF2 signalling (Sen et al., 2012; Burguera et al., 2017; CheleSAhi et al., 2020; Bhatia & Gaddam, 2021; Carbajo et al., 2025).

Effects of SMW and SBAC on gene expression of GPx

GPx expression varied with the sulfur-containing agent and cellular state. SFN and TAU induced the most consistent increases in GPx levels in both unstimulated and LPS-stimulated HIEC-6 cells.

For SFN, this result aligns with evidence that GPx2 is regulated by NRF2, as well as with SFN's established capacity to activate the antioxidant transcriptional response (Banning et al., 2005; Dinkova-Kostova et al., 2002). The similar response observed with TAU is consistent with findings suggesting that its mechanism of action involves NRF2-dependent antioxidant regulation, including under inflammatory stimulation (Xu et al., 2023; Xiao et al., 2025).

For SMW, GPx increased only at the highest concentration, whereas ALI stimulated expression at all concentrations in unstimulated cells but only at the highest concentration after LPS stimulation. These differences indicate that the transcriptional response depends on both the chemical nature of the agents and the cellular state. Although literature regarding ALI in this context is limited, the observed effect is consistent with data suggesting an influence on the glutathione-dependent antioxidant system (Sangeetha and Darlin Quine, 2009). The concomitant changes in NRF2 and GPx suggest a coordinated antioxidant transcriptional response, though they do not definitively establish a direct causal regulatory relationship. Effects of SMW and SBAB on SIRT1 Gene Expression

SIRT1 expression varies in sensitivity to the sulfur-containing agents under investigation and to the cellular state. SFN stimulates expression in unstimulated HIEC-6 cells; this aligns with evidence regarding the role of SIRT1 in cellular adaptation to oxidative and metabolic stress, as well as with documented effects of SFN on SIRT1-related signaling pathways in intestinal cells (Gezer, 2018; Zhang and Wu, 2021; Sezgin-Bayindir et al., 2025). The lack of a significant effect under LPS stimulation, contrary to some findings in the literature, may reflect differences between the cellular models and the specific parameters measured, as this study evaluates gene expression rather than protein levels or SIRT1 activity.

TAU increases SIRT1 expression only at the highest concentration, an effect that persists even in the presence of LPS. This profile differs from SFN and is consistent with data on TAU's influence on cellular redox regulation (SAhaffer et al., 2014; Ma et al., 2022). In SMW and ALI, we observed no significant changes in SIRT1, despite their effects on other genes examined in the study. This indicates that SIRT1 modulation is not a universal component of the

transcriptional response to natural sulfur-containing factors and highlights the specificity of the reaction to individual agents.

Effects of SMW and SBAC on gene expression of FOXO4

FOXO4 expression responds specifically to the sulfur-containing agents under investigation, depending on the cellular state. SFN increases FOXO4 expression at all tested concentrations in both unstimulated and LPS-stimulated HIEC-6 cells. This consistent response aligns with published data on the influence of SFN on FOXO-related adaptive and redox-regulatory processes (Davis et al. 2009; Liu et al. 2020; Zhang et al., 2021; Sezgin Bayindir et al., 2025). A comparison with SIRT1 reveals a distinct expression profile: while SIRT1 levels increase only in unstimulated cells, FOXO4 is upregulated even in the presence of LPS; this indicates that changes in FOXO4 mRNA are not necessarily linked to an increase in SIRT1 expression. This does not, however, rule out the previously described post-translational interaction between the two regulators (van der Horst et al. 2004; Kobayashi et al. 2005).

TAU, SMW, and ALI demonstrate a more conditional effect. TAU and ALI stimulate FOXO4 primarily in unstimulated cells, whereas for SMW, the response persists under LPS stimulation only at the intermediate concentration. This profile underscores that the transcriptional response depends on the agent's chemical nature, its concentration, and the cellular state. For SMW, the lack of a linear concentration-response relationship is consistent with the concentration-dependent—and potentially hermetic regulation described for sulfur compounds, though it does not identify a specific mechanism (Davinelli et al. 2019; Shahid and Bhatia 2024). ALI stimulates FOXO4 across all tested concentrations in unstimulated cells, in the absence of changes to SIRT1, adding to the limited data on its biological effects and constituting a novel observation within the cellular model employed.

Effects of SMW and SBAC on gene expression of SULT1A1 and SULT1A3

The expression of SULT1A1 and SULT1A3 shows distinct responses to the investigated sulfur-containing agents, which depend on the cellular state. In unstimulated cells, S-methyl-L-cysteine (SMC) increases SULT1A1 mRNA levels at concentrations of 6% and 8%, whereas in the presence of 4% LPS, it causes a decrease;

SULT1A3 expression does not change significantly. S-allyl-L-cysteine (SAC) increases SULT1A1 expression only at the highest concentration, while it increases SULT1A3 expression at all tested concentrations—but exclusively in unstimulated cells. These findings align with existing data on the modulation of sulfotransferases by natural bioactive compounds and underscore the fact that the response depends on both the tissue type and the cellular state (Lněničková et al. 2017; Suiko et al. 2017).

Alliin increases the expression of both genes, but only at the highest concentration and in unstimulated cells. While ALLI does not alter SULT1A1 levels, it increases SULT1A3 mRNA at the two higher concentrations in unstimulated HIEC-6 cells. The limited literature regarding the direct effect of ALI on SULTs precludes a mechanistic explanation; however, this result contributes to our understanding of its biological activity and points to a specific regulation of SULT1A3. The similarity of these findings with data on other organosulfur compounds derived from garlic, which are known to modulate phase II enzymes, underscores the need for further investigation (Song et al., 2021). Notably, LPS and salicylic acid suppress the expression of both genes. This highlights how an inflammatory state affects sulfotransferase expression and likely contributes to the lack of stimulatory responses to some of the agents tested. The data expand the understanding of SULT regulation in an intestinal cell model, with the observed effect of LPS on SULT1A3 being particularly noteworthy.

6 CONCLUSIONS

1. In the experimental model used, sulfur-rich mineral water, sulforaphane, alliin, and taurine maintained cell viability within the tested concentration ranges, allowing for subsequent analysis of their biological effects.

2. The natural sulfur sources investigated differ in their ability to modulate the inflammatory response in human intestinal epithelial cells.

3. Sulfur-rich mineral water exerts a broad-spectrum effect on the expression of key genes encoding pro-inflammatory markers, highlighting its potential to influence molecular mechanisms associated with the cellular inflammatory response.

4. As expected, sulforaphane has the most significant impact on the expression of genes involved in antioxidant defence and cellular adaptation, whereas the effects of taurine and alliin on these signalling pathways are more limited and highly dependent on the concentration applied.

5. 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.

6. The sulfur sources studied show varying selectivity in their effects on sulfotransferase expression; sulfur-rich mineral water primarily affects SULT1A1 expression, whereas sulforaphane and alliin have a more pronounced effect on SULT1A3.

7. The most consistent expression relationship is Nrf2/GPx; it is positive, with both components changing in the same direction across all experimental treatments, both in unstimulated cells and under LPS-induced inflammation.

8. The negative expression relationship between NF-κB and Nrf2 is maintained across all treatments, both in unstimulated cells and under conditions of LPS-induced inflammation...

7 CONTRIBUTIONS

ORIGINAL CONTRIBUTIONS

1. New experimental data have been obtained regarding the effects of sulfur-containing mineral water from the Varna Basin on the expression of genes associated with the inflammatory and redox-related cellular response, as well as biotransformation.
2. Knowledge regarding the molecular effects of the poorly studied organosulfide alliin has been expanded, with the identification of its potential to modulate the expression of genes associated with the inflammatory and redox-related cellular response, as well as biotransformation.

APPLIED CONTRIBUTION

1. A comparative cellular approach has been developed and applied for the preliminary assessment of the molecular effects of natural sulfur sources in HIEC-6 cells under basal and LPS-stimulated conditions.

CONFIRMATIVE CONTRIBUTION

1. The results obtained for sulforaphane and taurine complement and support existing literature data regarding their influence on the expression of genes associated with the inflammatory and redox-related cellular response.

8 LIST OF PUBLICATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION THESIS

8.1 List of publications related to the dissertation

Sokrateva T, Tasinov O, **Stoeva S,** Ivanova D, Nazifova-Tasinova N, Nashar M. Sulfur-containing mineral water as a dietary factor challenging the Phase II detoxification enzymes in human intestinal cells. J of IMAB. 2022 Jul-Sep;28(3):4541-4547. DOI: 10.5272/jimab.2022283.4541

Stoeva, S., Sokrateva, T., Vankova, D., Pasheva, M., & Nashar, M. (2026). Anti-inflammatory potential of sulfurous mineral water and sulfur-containing bioactive compounds: a comparative study. Pharmacia, 73. https://doi.org/10.3897/pharmacia.73.e187811

8.2 Participation in scientific forums in connection with the dissertation

Stefka Stoeva, Deyana Vankova, Milena Pasheva, Todorka Sokrateva, Milka Nashar. Anti-inflammatory effect of sulfur-containing mineral water and sulforaphane in lipopolysaccharide-stimulated human intestinal epithelial cells: A comparative study. Годишна конференция по нутригеномика - NUGOWEEK 2023 “Impact of nutrition during different life stages – tracing the impact of diet on human health”, 5-8 September, 2023, Senigallia, Italy (*Poster*)

Stefka Stoeva, Deyana Vankova, Milena Pasheva, Todorka Sokrateva, Milka Nashar. A comparative study of the biological effects of natural food sources of sulfur on human intestinal cells. 9th Pharmaceutical Business Forum and SAientific-Practical Conference "Faculty of Pharmacy – 15 Years of Traditions, Innovations, and Perspectives," October 20–21, 2023, Varna (Presentation/Paper) – 1st Prize for Best PhD Student Presentation in the "Short Presentations" Session

Stoeva S, Vankova DG, Sokrateva T, Nashar M. The potential of sulfur-containing mineral water and sulfur-containing bioactive compounds to modulate inflammatory markers in human intestinal epithelial cells: a comparative study. Science and Technologies in Biomedicine, 29-31 October, 2025, Varna, Bulgaria (*Poster*)

Stefka Stoeva, Deyana Vankova, Milena Pasheva, Todorka Sokrateva and Milka Nashar, "Anti-inflammatory effect of sulfur-containing mineral water and sulforaphane in lipopolysaccharide-stimulated human intestinal epithelial cells: a comparative study".

Tenth National Scientific Meeting on Biochemistry 2024, Mountain Rehabilitation Centre "Tsigov Chark", 04-06. 10.2024 (Poster)

Todorka Sokrateva, **Stefka Stoeva,** Deyana Vankova, Diana Ivanova, Milka Nashar. Anti-inflammatory potential of mineral water, sulforaphane, allicin, and taurine in an in vitro model of LPS-induced inflammation. 48th ISM World Congress, 12-16 May 2026, Banja Koviljača, Republic of Serbia (invited lecture T. Sokrateva)

Milka Nashar, Diana Ivanova, **Stefka Stoeva,** Todorka Sokrateva. Sulfur-Containing Mineral Water Modulates Redox-Regulating Gene Expression in Human Cells. 48th ISM World Congress, 12-16 May 2026, Banja Koviljaca, Republic of Serbia (Poster)

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