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The Potential of Sulfur-Containing Mineral Water and Sulfur-Containing Bioactive Compounds to Modulate Inflammatory Markers in Human Intestinal Epithelial Cells: A Comparative Study

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Abstract

Background: The therapeutic potential of sulfur-containing mineral water (SMW) has been recognised since ancient times. Given the substantial amount of data on the beneficial effects of sulfur-containing active ingredients in foods, interest in SMW has been increasing in recent years, particularly regarding its contribution to human health as part of the daily diet. The anti-inflammatory potential of sulforaphane (SFN), allicin (ALL), and taurine (TAU) is well documented. This study aimed to analyse and compare the anti-inflammatory potential of SMW, SFN, ALL, and TAU on human intestinal epithelial cells (HIEC-6). **Methods:** A cell viability assay was conducted, selecting three concentrations from each sulfur-containing compound for analysis. In a model of lipopolysaccharide-induced inflammation, total RNA was extracted, and the gene expressions of cyclooxygenase-2 (COX-2), tumour necrosis factor-alpha (TNFα), interleukin-1ß (IL-1ß), and IL-1ß receptor antagonist (IL1RN) were analysed. **Results:** With the exception of ALL, all studied compounds significantly decreased the expression of COX-2 in a dose-dependent manner. No change in IL-1ß expression was observed in SMW- and SFN-treated cells. All sulfur compounds, including SMW, significantly decreased TNF-α expression, and this effect was particularly pronounced in TAU-treated cells. SMW, SFN, and ALL, but not TAU, increased the mRNA levels of IL-1RN in unstimulated cells. **Conclusions:** SMW significantly modulated the expression of the studied markers, and these effects were comparable to those of SFN, TAU, and ALL. These results demonstrate the anti-inflammatory potential of SMW and elucidate the underlying mechanisms behind its therapeutic effects.

Keywords: sulfur-containing mineral water; sulforaphane; taurine; allicin; COX-2; TNF-α, IL-1β, IL-1RN; HIEC-6; LPS-induced inflammation

1. Introduction

The healing properties of mineral waters have been recognised since ancient times. Due to the extensive use of mineral waters in balneology, dermatology, and rheumatology, their anti-inflammatory properties are the focus of scientific interest [1–4]. Sulphur-containing mineral waters possess a wide range of therapeutic and balneological applications. The concentration of sulphur-containing compounds in mineral waters is considered a determinant of their anti-inflammatory or pro-inflammatory effects on human metabolism [5]. In a human intervention study, the daily intake of SMW demonstrated its antioxidant and anti-inflammatory potential by improving total thiol levels in plasma and reducing plasma C-reactive protein (CRP) in healthy volunteers [6,7]. Furthermore, SMW, prescribed as a complementary therapy for patients with specific pathological conditions, was

reported to improve blood glucose concentration and to reduce plasma concentration of reactive oxygen metabolites [8].

The health benefits of SMW are attributed to its sulfur content, primarily in the form of hydrogen sulfide (H_2S) and soluble sulfides, which are thought to modulate various biological processes [9,10].

Hydrogen sulfide is a gaseous transmitter that has various biological effects, including immunomodulatory effects by inhibiting the production of interleukin 6 (IL-6) and TNF-α in macrophages via the suppression of histone H3 acetylation at their promoters. This chromatin remodelling results in decreased gene transcription of these pro-inflammatory cytokines, highlighting an epigenetic mechanism through which H_2S exerts anti-inflammatory effects [11] (Rios et al., 2015). Another study suggests that H_2S-mediated sulfhydration of nuclear factor kappa B (NF-κB) p65 plays a crucial role in its transcriptional activity and thus regulates immune responses [12]. A comprehensive review highlighted the dual role of H_2S in inflammation. Under certain conditions, H2S can have pro-inflammatory effects or be anti-inflammatory by modulating mechanisms that underpin the relationship between oxidative stress and inflammation [13]. However, we should not overlook that SMW also contains other components, such as soluble sulfides and micro and macro elements, which may significantly influence metabolism and contribute to the biological effects.

Recently, the number of in vitro studies involving SMW has risen. Essentially, in vitro studies provide a foundation for exploring the mechanisms of action of various biologically active compounds. They enable rapid and detailed monitoring of changes in target markers following specific treatments. In the field of balneotherapy, primary cells or immortalised cell lines are employed to study the biological effects of a single inorganic molecule [5,14,15], an organic component extracted from mineral water [16], or the whole mineral water [5,17,18]. Moreover, it has been suggested that the sulfur-based compounds may exhibit a mild pro-inflammatory hormesis effect, which primes the body for protection against inflammation. However, despite the rising scientific interest in the beneficial effects of mineral waters and the mechanisms underlying these effects, SMW remains underestimated as a part of the daily diet and the molecular mechanisms of its anti-inflammatory effects are not fully elucidated.

Conversely, plant-derived organosulphur compounds and sulphur-containing amino acids attract significant scientific interest, with their biological effects being the focus of numerous in vitro, in vivo, and human interventional studies [19–21]. A wealth of scientific data accumulated in recent decades reveals the potential of organosulfides found in broccoli and garlic to contribute to the prevention of various pathological processes, including chronic inflammation, by suppressing the expression and activity of several inflammatory mediators such as COX-2, nitric oxide (NO), IL-1β, IL-6, TNF-α, among others [22–24]. Furthermore, these compounds have been shown to activate the antioxidant response by stimulating the Nrf2 signalling pathway [25] and display remarkable cytoprotective effects [26].

Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane) is one of the most studied isothiocyanates, primarily due to its antioxidant, anticancer, and anti-inflammatory properties [27]. It is a dietary organosulfur compound found in plants from the Brassica genus, such as cauliflower, broccoli, kale, cabbage, collards, and others [28]. Consuming cruciferous vegetables has been linked to a reduced risk of certain metabolic disorders closely associated with low-grade inflammation, such as diabetes [29]. The anti-inflammatory potential of SFN has been well demonstrated in various types of in vitro models of inflammation, including lipopolysaccharide (LPS)-stimulated expression of COX-2 and TNF-α [30,31]. In addition to this data, the anti-inflammatory effects of SFN were demonstrated in in vivo models of lung injury, muscle inflammation, hypoxic pulmonary hypertension, and diabetes [31–33]. Furthermore, several clinical studies have demonstrated the potential of SFN as an adjuvant to anti-inflammatory therapies in humans. Unlike many other plant-derived biologically active compounds, such as polyphenols, SFN has good bioavailability and relatively low toxicity [34,35].

Allicin [S-(2-propenyl)-2-propene-1-sulfinothioate] is the most bioactive organosulfur compound found in garlic, which is released after crushing, cutting, or chewing the garlic cells.

[21,36,37]. It is a thioester of sulfenic acid and is also known as allyl thiosulfinate, synthesized from alliin (S-allylcysteine sulfoxide) during the mechanical breakdown of garlic tissue [36].

Garlic organosulfur compounds, including allicin, have attenuated the release of proinflammatory mediators from various types of inflammatory cells, thereby reducing chronic inflammatory processes [38,39]. They can also inhibit the transcription factor NF-kB, which plays a key role in inflammation, and the phosphorylation of p38 mitogen-activated protein kinases. Lang et al. demonstrated that allicin has the potential to reduce intestinal inflammation by exerting an inhibitory effect on spontaneous and TNF-α-induced secretion of proinflammatory chemokines in HT-29 and Caco-2 epithelial cell lines in a dose-dependent manner [38].

Taurine is a conditionally essential sulfonated beta-amino acid synthesised in the human body from cysteine, and is often included in food supplements. Despite being one of the few non-protein amino acids, it is among the most abundant amino acids in the human body. It is found in high concentrations in various tissues, particularly in the brain, retina, muscle tissue, and phagocytic cells. Taurine has been reported to act as an antioxidant during oxidative stress-induced inflammation, exhibit cytoprotective effects, and potentially maintain homeostasis during both acute and chronic inflammation [40].

Scientific data accumulated in recent decades reveal the potential of active substances in foods, primarily of plant origin, to suppress inflammatory responses by inhibiting the activity of numerous pro-inflammatory markers in various in vitro models [30,31,41,42]. However, data comparing the biological effects of sulfur-containing active substances of natural origin on intestinal epithelial cell models are scarce. The gastrointestinal tract is likely the first site where bioactive compounds from foods and water exert their biological effects. Therefore, intestinal cell lines may serve as a valuable model for studying the mechanisms of these effects in both normal and pathological conditions, including inflammation models.

The present study aimed to perform a comparative analysis of the biological effects of sulfur-containing biologically active compounds (SBAC) from foods and waters on human intestinal epithelial cells. We investigated and compared the biological impact of SMW from the Varna basin with SBAC entering the body with foods, such as SFN from broccoli, ALL and TAU.

2. Materials and Methods

2.1. Sampling of Mineral Water

Twenty-four hours before the analyses the SMW samples were collected from a public fountain in Varna (Bulgaria) in clean plastic bottles and were stored in the dark at 4 °C in a refrigerator. Two hours before the experiment, the water samples were left at room temperature. Immediately before each experiment, a sample of 50 ml SMW was filtered through a 0.2 µm pore size filter and used to prepare working concentrations with nutritional medium.

2.2. Studied Compounds, Materials and Reagents

L-Sulforaphane (Cat. No. 14797) and Alliin (Cat. No. 14001) were purchased from Cayman Chemical Company, Ann Arbor, MI, USA. Taurine was obtained from Thermo Fisher Scientific, Inc., Waltham, MA, USA (Cat.No. 166540250). Reduced-Serum Medium Opti-MEM™ I with Phenol red (Cat. No. 31985-070), Phenol red-free Reduced-Serum Medium Opti-MEM™ I (Cat. No. 11058-021), Epidermal growth factor (Cat. No. PHG 0311), and GlutaMAX™ Supplement (Cat. No. 35050061) were sourced from Thermo Fisher Scientific Inc. (Gibco, Waltham, MA, USA). Amplifyme SG Universal Mix (Cat. No. AM 02-200) was purchased from Blirt S.A. (Gdansk, Poland). PCR Red Mastermix (Cat. No. M3029.0500) was acquired from Genaxxon Bioscience GmbH (Ulm, Germany). Fetal Bovine Serum (FBS) (Cat. No. F7524), Phosphate Buffered Saline (PBS) (pH 7.4) (Cat. No. 806552), Dulbecco's Phosphate Buffered Saline (DPBS) (Cat. No. 56064C), HEPES (pH 5-6.5) (Cat. No. H3375), and antibiotics were supplied by Sigma-Aldrich (St. Louis, MO, USA). Primezol TM reagent (Cat. No. AN1100) was obtained from Canvax Biotech, S.L. (Cordoba, Spain). 3-(4,5-Dimethylthiazol-

2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Cat. No. A2231.0005) was sourced from ITW Reagents, S.R.L. (AppliChem, Monza (MB), Italy). Dimethyl sulfoxide (DMSO) (Cat. No. BP2311), RevertAid™ First Strand cDNA Synthesis Kit (Cat. No. K1622), and RevertAid™ First Strand cDNA Synthesis Kit (Cat. No. K1622) were purchased from Thermo Fisher Scientific, (Waltham, MA, USA). Trypsin/EDTA 10x (Cat. No. 392-0459) was procured from VWR (Radnor, PA, USA). Salicylic acid, 99.5% (Cat. No. C351) was supplied by Chimtex LTD (Dimitrovgrad, Bulgaria). Lipopolysaccharides (LPS) from Escherichia coli (serotype O26:B6, Cat. No. L8274) were provided by Merck (Sigma Aldrich), Taufkirchen, Germany.

2.2. Cell Line and Subcultivation

The human intestinal epithelial cells HIEC-6 (ATCC® CRL3266™) were obtained from the American Type Culture Collection (ATCC) and cultured in OptiMEM reduced serum medium, containing 20 mM HEPES, 10 mM GlutaMAX, 10 ng/mL epidermal growth factor (EGF), 4% fetal bovine serum (FBS), and a penicillin/streptomycin mixture to final concentrations of 1% for each component. Cells were cultured in 75 cm² flasks and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to determine cell viability. The cells were collected and seeded in 12-well flasks using phenol red-free Opti-MEM™ I medium at a density of 6.5×10^4 cells per well. Following 24 hours of incubation, the culture medium was removed and the cells were incubated in fresh medium containing increasing percentages of mineral water (v/v): 1%, 2%, 4%, 8%, 16%, and 20%, or sulfur-containing bioactive compounds, as follows: SFN (g/ml) 0.05, 0.1, 0.2, 0.25, 0.5, 1, 2, 5, 10; ALL (g/ml) 2.5, 5, 10, 25, 30, 50, 75, 100; TAU (mM/L) 0.1, 0.25, 0.5, 1, 2, 4, 5. After 20 hours of incubation, 100 μL of MTT working solution (2 mg/mL in phosphate buffer saline) was added to each well, and incubation continued for 4 hours. Following the incubation period, the MTT solution was removed, and 1 ml of dimethyl sulfoxide (DMSO) was added to each well to lyse the cells and dissolve the formazan crystals. Finally, absorbance was determined using a Synergy 2 plate reader (BioTek) at a wavelength of 550 nm. The viability of the treated cells was expressed as a percentage of the viability of the non-treated cells (controls), which was considered 100%. All treatments were performed in triplicate, and data were presented as mean ± SEM. Based on the MTT assay, three values of SMW percentage content in the culture medium, along with concentrations of SFN, ALL, and TAU, were selected for further analyses.

2.4. Induction of Inflammation

HIEC cells were seeded in Opti-MEM™ I medium using 12-well flasks at a density of 6.5×10^4 cells per well. After 24 hours, the cells were pre-treated and incubated for another 24 hours with selected concentrations of SMW (4, 6, and %) and sulfur-containing compounds: SFN [μg/ml] 0.5, 1, and 2; ALL [μg/ml] 10, 25, and 30; TAU [mM/ml] 1, 2, and 5. The cells were treated with 1 μg/ml LPS to induce inflammation. Additionally, SA was used as a reference compound with an anti-inflammatory effect (100 μM per well). Each treatment was conducted in triplicate.

2.5. Gene Expression Analysis

Two-step quantitative Real-Time PCR was employed to determine the gene expression levels of selected genes in the cell culture. The nucleotide sequences of the primers used for analysing the selected genes are presented in Table 1. The primers were designed using the Real-Time PCR gene expression Design Tool and were synthesised by Sigma–Aldrich and Integrated DNA Technologies.

Table 1. Primers nucleotide sequences.

Target gene (Human)	Nucleotide sequence (5`-3`)
Actin beta	F GTG GCC GAG GAC TTT GAT TG
	R CCT GTA ACA ACG CAT CTC ATA
COX-2	F GAA ACA GAG AAG TTG GCA GCA
	R GGC AGG ATA CAG CTC CAC AG
TNF-α	F CTC TTC TGC CTG CTG CAC TTT
	R ATG GGC TAC AGG CTT GTC ACT
IL-1β	F CCA CCT CCA GGG ACA GGA TA
	R AGA ATT AGC AAG CTG CCA GGA
IL1RN	F GCC CAT CCT CAG GAC CTT TC
	R ATG TCC TAG CCA TCC CCA CT

2.5.1. Total RNA Extraction

After the experiment, the cells were treated with Primezol reagent to isolate total RNA. The protocol adhered fully to the manufacturer's guidelines. Before proceeding to cDNA synthesis, the concentration of isolated RNA from each sample was measured using a multifunctional reader (Synergy 2, BioTek) and was utilised for cDNA synthesis.

2.5.2. Reverse Transcription

Extracted RNA (200 ng) was reverse transcribed and employed for cDNA synthesis using the First Strand cDNA Synthesis Kit, which contained oligo (dT)18 primer, reaction buffer, nuclease-free water, RNase inhibitor, nucleotide mix (dNTP), and reverse transcriptase enzyme. The reaction was performed according to the manufacturer's instructions on the GeneAmp PCR System 9700 (Applied Biosystems), with a final reaction volume of 20 µl. Following synthesis, each cDNA sample was diluted with 60 µL of molecular biology-grade water and stored at -20°C.

2.5.3. Quantitative Real-Time Polymerase Chain Reaction (RT PCR)

As a template for quantitative real-time PCR, a preamplifier of 0.50 µL cDNA was used in a final reaction volume of 5 µl. The final concentration of the primers in the reaction mixture was 300 nm. Two-step RT PCR analysis was performed with the AMPLIFYME SG Universal Mix kit (Blirt S.A.) using Rox Low fluorescent binding dye. Reactions were conducted in 96-well plates with the following reaction parameters: enzyme activation and denaturation at 95°C for 3 minutes; denaturation at 95 °c for 5 seconds for 40 cycles; annealing at 60°C for 10 seconds for 40 cycles; extension and fluorescence detection at 72 °c for 18 seconds for 40 cycles, followed by melting curve analyses. The analyses were performed on the QuantStudio 5 (Applied Biosystems). For the calculation of gene expression levels, the 2-$\Delta\Delta$Ct method was applied [43] and mRNA levels were presented as relative units (RU). The expression of genes of interest was assessed by comparison with untreated cells (controls), where the mRNA levels were considered equal to 1. Actin beta was used as an endogenous control gene.

2.6. Statistical Analysis

Statistical analyses were conducted using Microsoft Excel and GraphPad Prism 10.0. An unpaired two-tailed Student's t-test was employed to compare the differences between treated cells and the controls. All samples were measured in triplicate, and the mean values were utilised for analysis. The data are presented as mean ± standard error of the mean (SEM). A p value of less than 0.05 indicated a significant difference between the groups.

3. Results

3.1. Cell Viability

The effect of SMW and sulfur-containing bioactive compounds on cell viability was assessed using the MTT assay. Cells were treated with increasing concentrations of the compounds under investigation. Only concentrations that resulted in a survival rate exceeding 80% were deemed nontoxic. Based on the MTT results (Figure 1A–D), three concentrations of SMW and each sulfur-containing compound were chosen for further analyses.

The effect of SMW on HIEC-6 viability is illustrated in Figure 1A. It was observed that cell viability decreased sharply compared to the controls (p = 0.0003) at the highest percentage of SMW content in the culture medium (20%). However, even at this high concentration of SMW, cell viability remained above 80%. Values between 1% and 16% SMW content did not significantly alter cell viability. Based on these results, all applied concentrations of SMW were considered non-toxic for HIEC-6. Three concentrations of SMW in the culture medium were selected for further analyses: 4%, 6%, and 8%.
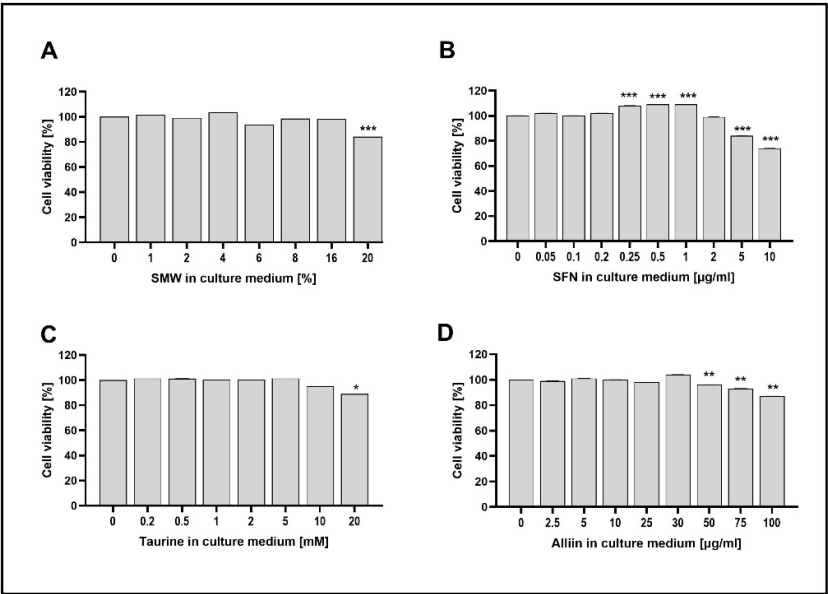

Figure 1. Effect of SMW (A), SFN (B), TAU (C) and A (D) on HIEC-6 cells viability. Data are presented as mean ± SD of three independent experiments; *p<0.05, **p<0.01 and ***p≤0.001, versus control (untreated cells).

3.2. Expression of Target Genes in a Model of LPS-Induced Inflammation

To explore the anti-inflammatory potential of SMW and SBAC on HIEC-6, we employed a model of LPS-induced inflammation. As detailed in the previous chapter, cells were pre-treated for 24 hours with the substances under investigation, after which they were exposed to 1 µg/ml LPS. Some of the cells were also exposed to the same concentration of LPS alone, without any pretreatment. Furthermore, we utilised 3 mM salicylic acid as a reference substance known for its anti-inflammatory effects. As previously described, cells were treated with three selected concentrations of each studied compound, in accordance with the MTT assay. The mRNA levels of COX-2, TNF-α, IL-1β, and IL1RN were compared with those of untreated cells.

Interestingly, SFN significantly stimulated cell proliferation at 0.25, 0.5, and 1 µg/ml (Figure 1B). However, cell viability noticeably decreased at 5 µg/ml SFN. Furthermore, the highest concentration of SFN markedly inhibited cell viability (74%) and may be deemed cytotoxic (p<0.001). Concentrations of 0.5, 1, and 2 µg/ml SFN were employed in the subsequent analyses.

As demonstrated in Figure 1C, the TAU concentrations of 0.2-5 mM did not affect cell viability. The slight decrease in viability of cells treated with 10 mM TAU was not statistically significant (95%,

p=0.2). A statistically significant reduction in cell viability was observed in cells treated with 20 mM TAU (p=0.03). Nevertheless, even at this concentration, cell viability remained sufficiently high (89%) to conclude that all TAU concentrations applied were non-toxic to HIEC-6. The TAU concentrations used in the subsequent analyses were 1, 2, and 5 mM.

Cell viability in ALL-treated cells (Figure 1D) significantly decreased at the three highest concentrations. Notably, the viability of cells treated with the highest concentration of 100 μg/ml approached the 80% threshold. Based on these results, the chosen concentrations of allicin for the subsequent analyses were 10, 25, and 30 μg/ml.

3.2.1. Effect of SMW and SBAC on COX-2 Expression

We explored the potential of SMW and SBAC to influence COX-2 expression. Data are presented in Figure 2A–D. As observed, in cells treated with LPS, the gene expression was 1.8-fold higher (p=0.0003). Conversely, SA significantly inhibited the expression of COX-2.

Figure 2A illustrates the mRNA levels of the COX-2 gene in SMW-treated cells. We observed that pretreatment with SMW significantly reduced COX-2 transcription levels in LPS-stimulated HIEC-6. The lowest concentration of SMW significantly decreased mRNA levels in unstimulated cells (p=0.05). Conversely, the highest concentration of 8% SMW significantly suppressed the expression of the enzyme, both in unstimulated cells and under conditions of induced inflammation, suggesting a concentration-dependent effect of SMW on COX-2 expression.
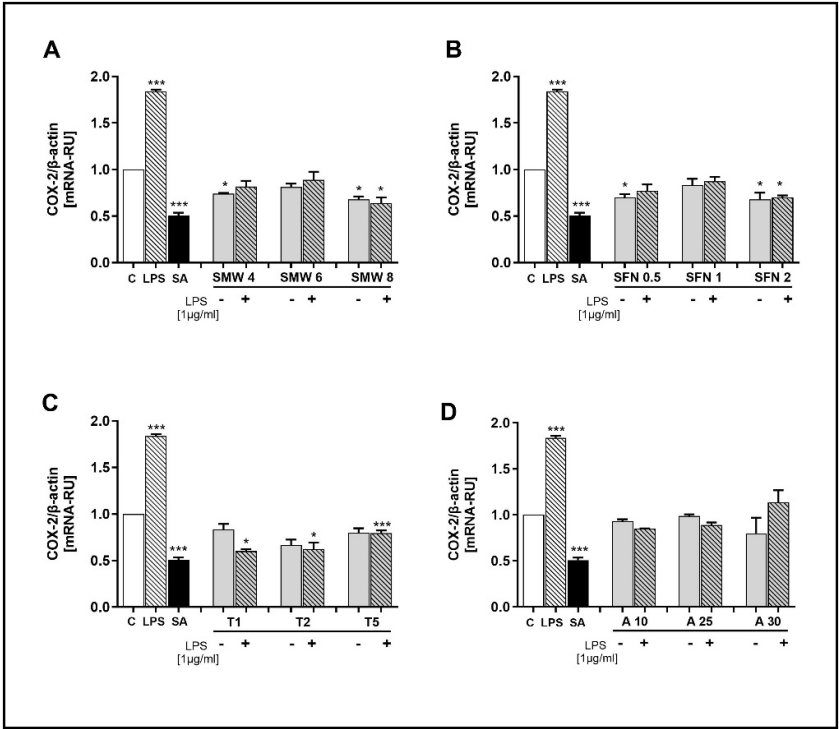

Figure 2. Effect of SMW and SBAC on COX-2 expression in HIEC-6, under conditions of LPS-induced inflammation. Levels mRNA are presented as relative units (RU), with untreated cells – controls taken as 1; C – control (untreated cells); LPS – lipopolysaccharides; SA – salicylic acid; SMW4, SMW6, SMW8 – cells pretreated with 4%, 6% or 8% sulfur-containing mineral water, respectively (A); SFN0.5, SFN 1, SFN 2 – cells pretreated with sulforaphane 0.5, 1 or 2 μg/ml, respectively (B); T1, T2, T5 – cells pretreated with 1, 2 or 5 mM taurine, respectively (C); A10, A25, A30 – cells pretreated with 10, 25 or 30 μg/ml alliin (D); LPS ⁻/+ cells without/with LPS-induced inflammation (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 versus C).

The effect of SFN on COX-2 expression was comparable (Figure 2B). Notably, the highest concentration of 2 µg/ml inhibited gene expression in both LPS-stimulated and unstimulated cells (p=0.037 and p=0.04, respectively).

Taurine pretreatment significantly reduced COX-2 transcription levels in LPS-stimulated HIEC-6 across all three concentrations tested, with this effect being markedly more pronounced in cells treated with the highest concentration (Figure 2C).

None of the applied concentrations of ALL changed the expression levels of the COX-2 gene (Figure 2D).

3.2.2. Effect of SMW and SBAC on TNF-α Expression

Another target pro-inflammatory gene in our study was TNF-α. The results illustrating the effect of SMW and SBACon on TNF-α expression under conditions of LPS-induced inflammation are summarised in Figure 3A–D. Regarding COX-2, in cells treated with LPS alone, the TNF-α gene was significantly overexpressed compared to the control cells (p=0.02). Salicylic acid inhibited the expression of the gene (p=0.0047).

The inhibitory effect of SMW on TNFα expression was significant and dependent on concentration (Figure 3A). Notably, while unstimulated cells show that only the highest concentration exhibits an inhibitory effect on gene expression, all three SMW concentrations demonstrate such an effect under induced inflammation.
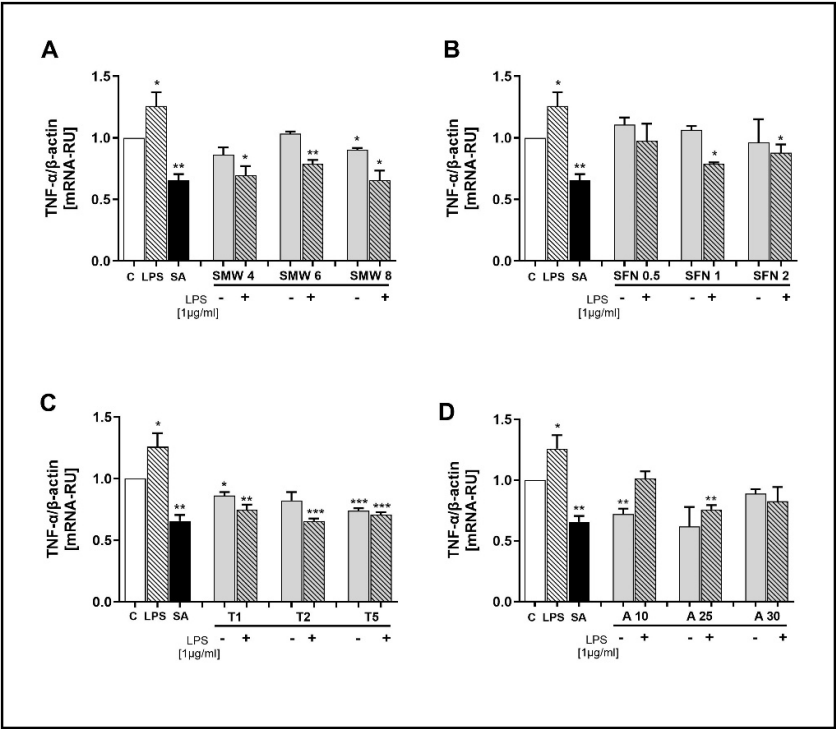

Figure 3. Effect of SMW and SBAC on TNF-α expression in HIEC-6, under conditions of LPS-induced inflammation. Levels mRNA are presented as relative units (RU), with untreated cells – controls taken as 1; C – control (untreated cells); LPS – lipopolysaccharides; SA – salicylic acid; SMW4, SMW6, SMW8 – cells pretreated with 4%, 6% or 8% sulfur-containing mineral water, respectively (A); SFN0.5, SFN 1, SFN 2 – cells pretreated with sulforaphane 0.5, 1 or 2 µg/ml, respectively (B); T1, T2, T5 – cells pretreated with 1, 2 or 5 mM taurine, respectively (C); A10, A25, A30 – cells pretreated with 10, 25 or 30 µg/ml alliin (D); LPS ⁻/+ cells without/with LPS-induced inflammation (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 versus C).

TNF-α expression was significantly inhibited in stimulated cells that were pretreated with 1 and 2 µg/ml SFN (Figure 3B). A concentration of 0.5 µg/ml SFN did not influence the expression of this gene, and its mRNA levels remained comparable to those measured in untreated controls.

Taurine demonstrated significant potential to inhibit TNF-α expression, both in conditions of induced inflammation and in unstimulated cells, and this effect was concentration-dependent (Figure 3C)

The effect of ALL on TNF-α expression was also investigated. In the absence of inflammation, we observed a statistically significant decrease in cytokine expression in cells treated with 10 µg/ml allicin compared to the control. Under conditions of induced inflammation, a concentration of 25 µg/ml significantly reduced mRNA levels (Figure 3D).

3.2.3. Effect of SMW and SBACon IL-1β Expression

The effect of SMW and SBACon IL-1β expression is illustrated in Figure 4A–D. Similarly to the previous target genes, LPS significantly stimulated IL-1β gene expression, whereas in cells treated with SA, mRNA levels were notably lower than those in the controls. No change in IL-1β gene expression was observed in SMW and SFN-treated cells at any of the applied concentrations (Figure 4A and B, respectively). In cells with LPS-induced inflammation, taurine demonstrated a statistically significant inhibitory effect on mRNA levels for the IL-1β gene (Figure 4C). At concentrations of 25 and 30 µg/ml, ALL exhibited an inhibitory effect on IL-1β expression, but only in cells with induced inflammation. In other instances, no effect of allicin on gene expression was found (Figure 4D).
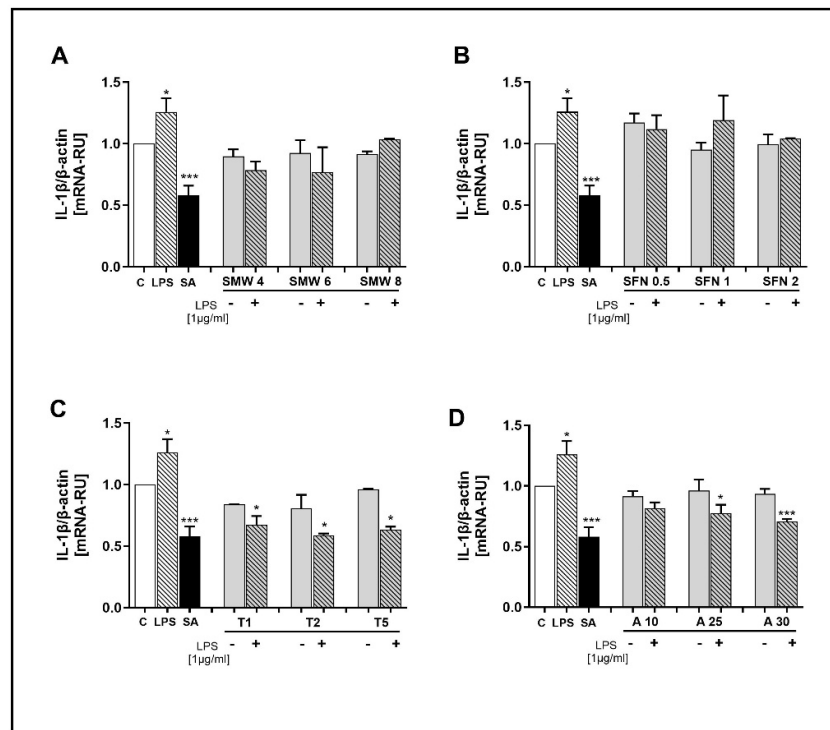

Figure 4. Effect of SMW and SBAC on IL-1β expression in HIEC-6, under conditions of LPS-induced inflammation. Levels mRNA are presented as relative units (RU), with untreated cells – controls taken as 1; C – control (untreated cells); LPS – lipopolysaccharides; SA – salicylic acid; SMW4, SMW6, SMW8 – cells pretreated with 4%, 6% or 8% sulfur-containing mineral water, respectively (A); SFN0.5, SFN 1, SFN 2 – cells pretreated with sulforaphane 0.5, 1 or 2 µg/ml, respectively (B); T1, T2, T5 – cells pretreated with 1, 2 or 5 mM taurine, respectively (C); A10, A25, A30 – cells pretreated with 10, 25 or 30 µg/ml alliin (D); LPS ⁻/+ cells without/with LPS-induced inflammation (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001 versus C).

3.2.4. Effect of SMW and SBACon IL-1RN Expression

We investigated the effect of SMW and SBAC on the gene expression for the anti-inflammatory marker IL-1RN (Figure 5A–D). In cells treated with LPS alone, significantly lower mRNA levels for the gene were observed compared to untreated cells. Salicylic acid had no effect on the gene expression of this marker.
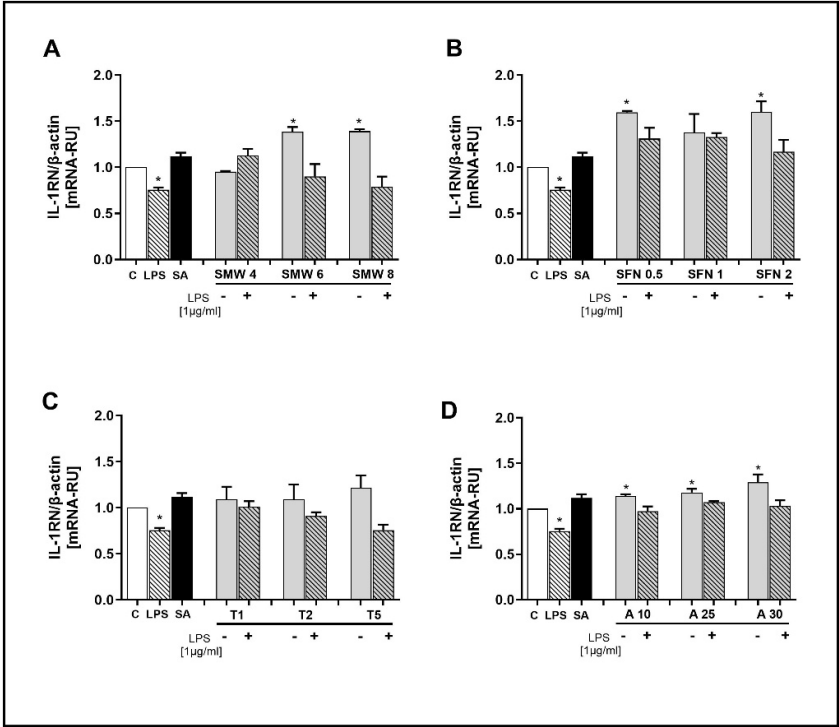

Figure 5. Effect of SMW and SBAC on IL-1RN expression in HIEC-6, under conditions of LPS-induced inflammation. Levels mRNA are presented as relative units (RU), with untreated cells – controls taken as 1; C – control (untreated cells); LPS – lipopolysaccharides; SA – salicylic acid; SMW4, SMW6, SMW8 – cells pretreated with 4%, 6% or 8% sulfur-containing mineral water, respectively (A); SFN0.5, SFN 1, SFN 2 – cells pretreated with sulforaphane 0.5, 1 or 2 µg/ml, respectively (B); T1, T2, T5 – cells pretreated with 1, 2 or 5 mM taurine, respectively (C); A10, A25, A30 – cells pretreated with 10, 25 or 30 µg/ml alliin (D); LPS −/+ cells without/with LPS-induced inflammation (*p ≤ 0.05; versus C).

The results presented in Figure 5 indicate that SMW, SFN, and ALL increased the expression of IL-1RN, but only in unstimulated cells. It is worth noting that for allicin, this effect is pronounced; the IL-1RN gene was significantly overexpressed at all applied concentrations (Figure 5A,B,D). No changes in mRNA levels were observed in cells treated with taurine (Figure 5C).

4. Discussion

For many years, scientists have concentrated their efforts on functional foods and nutrition, primarily from plant sources, as providers of active substances that contribute to human health. In this respect, it can be said that mineral waters have long been underestimated as a source of biologically active substances within the human body, with knowledge of their beneficial effects relying chiefly on empirical data.

Since H_2S has been identified as a signalling gas molecule with numerous biological effects, interest in SMW has grown significantly. A 2013 study demonstrated the immunomodulatory and antioxidant properties of SMW and compared them to other exogenous donors of H2S in patients

with respiratory diseases [44]. A physico-chemical analysis reported that the dominant microelement in Varna mineral waters is sulphur in the form of soluble sulfides and H_2S [45].

In the present study, we compared the SMW and SBAC in terms of their potential to modulate pro- and anti-inflammatory markers from foods in human intestinal epithelial cells. To our knowledge, this is the first study to explore the molecular mechanisms behind the anti-inflammatory potential of SMW in comparison with well-studied sulfur-containing compounds.

4.1. Effects of SMW and SBAC on Cell Viability

The first part of our study involved determining the range of non-toxic concentrations of all tested substances (Figure 1A–D). As shown in Figure 1A, the highest concentration of SMW significantly decreased cell viability. However, even at this concentration of SMW in the culture medium, cell viability did not fall below the threshold of 80%. For the subsequent experiments, we selected three medium concentrations, aiming to ensure 100% cell viability while maintaining a sufficient presence of active ingredients in the water [45].

An intriguing result emerged from the SFN cytotoxicity test, revealing that three of the concentrations had a proliferative effect, increasing cell viability to approximately 109% (Figure 1B). SFN has been extensively researched for its anticancer potential, demonstrated as cytotoxic activity on cancer cell lines [27]. Low doses of SFN may enhance cell survival, while high doses of the compound may inhibit cell viability. In this study, the intermediate concentrations stimulated cell proliferation. However, the cell line used in the present study is derived from normal intestinal epithelial cells with intact homeostasis, which likely influences the effects of SFN on cell viability, suggesting that the biological effects of SFN depend on its concentration, cell type, and conditions.

Regarding taurine, cell viability remained unchanged across all applied concentrations. Even at the highest concentration, where a statistically significant decrease was noted, approximately 90% cell survival was observed (Figure 1C). Based on these results, we can infer that all taurine concentrations are non-toxic. The last three concentrations of allicin, 50, 75, and 100 g/ml, significantly reduced cell viability to 96% (p=0.002), 93% (p=0.002), and 87% (p=0.006), respectively (Figure 1D). Allicin has been documented to exhibit high cytotoxic activity in vitro across various cancer cell lines, similar to sulforaphane. [21,46]. However, in our study, even these high concentrations of allicin did not reduce cell viability below 80%.

In summary, all applied concentrations of SMW, TAU, and Allicin can be considered non-toxic to the tested cells. Conversely, SFN exhibits a complex effect on cell viability, stimulating proliferation at the medium concentrations used while displaying cytotoxicity at the highest concentration.

4.2. Effects of SMW and SBAC on COX-2 Gene Expression

We investigated the potential of SMW to influence the expression of COX-2 in a model of LPS-induced inflammation. The results indicated that a 4% concentration of SMW in the nutrient medium inhibited COX-2 expression in unstimulated cells only. However, at the highest concentration, SMW significantly suppressed the enzyme's expression in both unstimulated and LPS-stimulated cells. This suggests a concentration-dependent effect of SMW on COX-2 gene expression. Interestingly, the effect of SMW closely resembled that of SFN on COX-2 expression (Figure 2B). This similarity is not surprising, as SFN's anti-inflammatory properties are well established and considered fundamental to its anticancer potential as an organosulfur compound [30,47].

Dietary TAU is believed to significantly prevent cardiovascular disease and type 2 diabetes due to its beneficial biological effects, including reducing pro-inflammatory cytokines [40,48]. In our study, TAU suppressed the expression of COX-2 solely in LPS-stimulated cells, and this effect was concentration-dependent (Figure 2C). Studies suggest that one potential mechanism underpinning the anti-inflammatory effects of TAU relates to its ability to decrease mRNA expression of Toll-Like Receptor-2 (TLR-2), thereby inhibiting the expression of pro-inflammatory cytokines [48,49]. Therefore, TAU could be regarded as a therapeutic agent with the potential to mitigate inflammatory signals.

Cyclooxygenase-2 is the inducible isoform of the enzyme involved in the synthesis of prostanoids (prostaglandins, prostacyclins, and thromboxanes) from arachidonic acid [50]. Its expression is activated in response to various pro-inflammatory stimuli. Unlike the constitutive isoform COX-1, the inducible isoform is not subject to biochemical regulation, causing the prostaglandins produced under the action of COX-2 to lose their beneficial physiological effects and instead act as mediators of acute and chronic inflammation and pain perception [41]. For these reasons, COX-2 is a primary target in the development of anti-inflammatory drugs—the therapeutic effect of non-steroidal anti-inflammatory drugs (NSAIDs) stems from their inhibitory effect on cyclooxygenase. Most synthetic NSAIDs lack selectivity for the inducible isoform; hence, a significant undesirable effect of their use is ulcer formation. Scientists are focused on creating NSAIDs with high selectivity for COX-2 and a safer profile to address this issue [51]. In this context, scientific data on the anti-inflammatory effects of active substances from natural sources, which can potentially suppress the activity or expression of the enzyme, could form the basis for developing new generations of selective NSAIDs without undesirable side effects.

Surprisingly, ALL pretreatment did not affect COX-2 expression in HECs at any of the concentrations used. Alongside SFN, allicin has been extensively studied for its anti-inflammatory properties, both in vitro and in vivo. The effects are likely to vary depending on the cell type and the stimulus applied.

4.3. Effects of SMW and SBAC on TNF-α Gene Expression

Tumour necrosis factor alpha is a cytokine recognised as a pivotal regulator of inflammatory processes and is known to be involved in the pathogenesis of several inflammatory and autoimmune diseases [52]. TNF-α can mediate various aspects of inflammation, such as enhancing endothelial cell permeability, thereby facilitating leukocyte migration and releasing other cytokines [53].

In this study, SMW and all SBAC demonstrated a potential to suppress TNF-α expression; for SMW, SFN, and TAU, this effect was concentration-dependent and pronounced under induced inflammation conditions. The highest concentrations of SMW and TAU suppressed the expression of the marker in both unstimulated cells and LPS-stimulated inflammation conditions (Figure 3A and C, respectively). Compared to the control cells, ALL significantly decreased TNF-α expression in unstimulated cells treated with 10 μg/ml and in LPS-stimulated cells treated with 25 μg/ml (Figure 3D).

There is a substantial body of data in the scientific literature supporting the potential of SFN, TAU, and ALL to suppress TNF-α expression. Organosulfur compounds from garlic and cruciferous vegetables exhibit significant anti-inflammatory properties through multiple mechanisms. Sulforaphane, for example, was shown to alleviate LPS-induced cell damage in Caco-2 cells by activating the AMPK/SIRT1/PGC-1α cascade [54]. Furthermore, SFN and ALL have been reported to attenuate the expression of pro-inflammatory cytokines by downregulating transcription factors such as NF-kB and Nrf-2 [55,56].

The anti-inflammatory potential of TAU in inhibiting TNF-α is also well documented. Taurine supplementation has been shown to reduce cytokine levels in various contexts, including sepsis models and in response to LPS stimulation [57]. It has been reported that in stimulated pulmonary macrophages, the TAU metabolite taurine chloride inhibits NF-κB migration to the nucleus by stabilising IκB in the cytoplasm [58].

As was mentioned before, the main active component in the SMW we studied is H_2S. There is sufficient scientific evidence for its anti-inflammatory effects. A recently published review summarises the data revealing the mechanisms by which this transmitter gas regulates inflammation. In specific inflammatory conditions, H_2S alleviates inflammation by decreasing the expression of pro-inflammatory mediators, including TNF-α, and by inhibiting important signalling pathways, including phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and NF-κB [59].

This data suggests that SMW and SBACs may affect specific targets in multiple signalling pathways while also modulating common markers of pro-inflammatory signals.

4.4. Effects of SMW and SBAC on IL-1β Gene Expression

Interleukin-1 beta (IL-1β) is a multifunctional pro-inflammatory cytokine that plays a crucial role in both acute and chronic inflammation [60]. It exhibits a pyrogenic effect and has the potential to induce pain signals [61]. IL-1β is expressed in a wide range of tissues and various cell types, particularly in macrophages found in lymphoid organs such as the thymus, spleen, lymph nodes, and bone marrow. In non-lymphoid organs, IL-1β is present in tissue macrophages of the lung, digestive tract, and liver and in various specific cell types, including neutrophils, keratinocytes, epithelial and endothelial cells, lymphocytes, smooth muscle cells, and fibroblasts [60]. The binding of IL-1β to its receptor activates several intracellular signalling pathways in target cells. One of the primary effects of transducing these signals is the creation of a pro-inflammatory environment within the cell through the release of a broad network of pro-inflammatory mediators, including cytokines, chemokines, metalloproteinases, acute phase proteins, prostaglandins, and nitric oxide [62].

In our study, TAU and ALL significantly inhibited gene expression of IL-1β, but only under conditions of LPS-induced inflammation (Figure 4B,D). This result supports the data reported by other studies on the therapeutic potential of both SBACs. On the other hand, neither SMW nor SFN demonstrated such potential. The anti-inflammatory potential of the studied biologically active substances in vitro is likely dependent on the target signalling pathways, the model, and the cell type. A class of receptors, known as NLR family pyrin domain containing 3 (NLRP3), expressed in macrophages, is activated by various harmful stimuli, such as reactive oxygen species, crystalline uric acid, and LPS-induced inflammation, subsequently leading to the release of IL-1β. The HIEC-6 cell line used in our study is fetal, and as a result, specific proteins are expressed very weakly or not at all in these cells, including NLRP3. A recent study reported that in LPS-stimulated HIEC-6, activation of IL-1β is possible through an NLRP3-independent pathway involving caspase-4 [63]. It can be expected that caspase-4 could also be a target for the anti-inflammatory effects of TAU and ALL, which remains to be confirmed in future studies.

4.5. Effects of SMW and SBAC on IL1RN Gene Expression

The IL-1β receptor antagonist (IL1RN; IL-1Ra) is a natural inhibitor of the cytokine's binding to its receptor and, therefore, plays an important role as an anti-inflammatory mediator [64,65]. The balance between IL-1β and IL1RN is a crucial regulatory factor in determining the outcome of the inflammatory process activated by IL-1β [66,67].

The antagonist IL1RN is synthesised in response to various proinflammatory stimuli, acting as an IL-1β inhibitor by competitively binding to its receptor [60]. Once bound to the receptor, IL1RN prevents the binding of IL-1β, thereby inhibiting the signalling pathway and reducing the release of several proinflammatory factors, including IL-6, IL-8, and TNF-α [62].

Research has demonstrated that the inhibition of IL1RN results in an increase in proinflammatory signals and tissue damage. Thus, the balance between IL-1β and IL1RN is crucial for maintaining a robust anti-inflammatory status [68]. Given the scientific evidence supporting a physiological relationship between these two markers and the regulatory role of IL1RN on IL-1β activity, it can be inferred that IL1RN itself could serve as a potential target for the development of new anti-inflammatory drugs [60].

In our study, overexpression of IL1RN was detected in cells pretreated with SMW, SFN, and ALL, but only in unstimulated cells. This result highlights the potential of these natural compounds to enhance immune defence in uncompromised cells, which is likely to increase their resistance and survival when proinflammatory stimuli are present.

Studies examining the factors influencing transcriptional regulation and the relationship between IL-1β and IL1RN are relatively scarce. In the present study, we analysed the effects of the investigated SMW and SBAC on the expression of the genes for IL-1β and IL1RN. We believe that the

results obtained provide a valuable contribution to clarifying this relationship and enhancing our understanding of the anti-inflammatory potential of biologically active substances of natural origin.

In summary, this study examined the potential of SMW from the Varna basin to influence the expression of markers related to the inflammatory response. The results discussed above indicated that the anti-inflammatory effects of SMW are comparable to those of organosulfur compounds known for their well-established anti-inflammatory properties.

5. Conclusions

The results obtained confirm the data on the anti-inflammatory potential of sulforaphane, allicin, and taurine, revealing for the first time the ability of sulfur-containing mineral water to inhibit the expression of genes related to the inflammatory response, such as COX-2, TNF-α, and IL-1β, both under LPS-induced inflammation and in unstimulated cells. In some cases, the effect of the SMW is even more pronounced than that of SFN and ALL. For SMW, these represent the first scientific data of their kind supporting our understanding of its anti-inflammatory effect and its potential to modulate gene expression related to the inflammatory response in intestinal cells.

The results obtained in this study contribute to enhancing our understanding of the effects of active substances from food and mineral waters on the markers associated with the inflammatory response. These findings may also assist in developing new strategies for personalised nutrition, with implications for human health.

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Abbreviations

The following abbreviations are used in this manuscript:

A	Alliin
COX-2	Inducible cyclooxygenase
CRP	C-reactive protein
H$_2$S	Hydrogen sulfide
IL-1RN	IL-1β receptor antagonist
IL-1β	Interleukin -1β
IL-6	Interleukin 6
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
PGE$_2$	Prostaglandin E2
SBAC	Sulfur-containing biologically active compounds
SFN	Sulforaphane
SMW	Sulphur-containing mineral waters

TAU   Taurine

TNF-α Tumour necrosis factor alpha

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