https://doi.org/10.31925/farmacia.2026.2.11 **ORIGINAL ARTICLE**

COMBINED CYTOTOXIC EFFECTS OF FISETIN AND GENISTEIN IN DETROIT-562 PHARYNGEAL CARCINOMA CELLS

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Abstract

Pharyngeal cancer is one of the most aggressive malignancies, often diagnosed at advanced stages, with current therapeutic options facing significant limitations in terms of efficacy and tolerability. Therefore, natural compounds, often characterized by a more favorable safety profile, could represent a promising option in the therapeutic management of this disease. In this context, the present study evaluated the cytotoxic potential of two flavonoids, fisetin (FIS) and genistein (GEN), and their combination on a 2D pharyngeal cancer cell model, Detroit-562. The study included an analysis of cell viability using the MTT assay and LDH release, followed by assessment of cell morphology and essential organelles, such as the nuclei and mitochondria, and evaluation of pro-apoptotic and necrotic effects *via* AO/PI double staining. In addition, the combination's irritant potential was analyzed in the HET-CAM *in ovo* model. The results showed that, across all assays, the combinatorial treatment exhibited higher cytotoxicity without causing an irritant effect on the vascular plexus, supporting its potential use as an adjuvant therapy for the treatment of pharyngeal cancer.

Rezumat

Acest studiu a evaluat potențialul citotoxic al a două flavonoide, fisetină (FIS) și genisteină (GEN), precum și al combinației acestora, pe un model celular 2D de cancer faringian, Detroit-562. Studiul a inclus analiza impactului asupra viabilității celulare prin testul MTT și eliberarea de LDH, urmată de evaluarea morfologiei celulare și a organitelor esențiale, precum nucleii și mitocondriile, precum și evaluarea efectelor pro-apoptotice și necrotice prin dublă colorare AO/PI. În plus, potențialul iritant al combinației a fost analizat în cadrul modelului *in ovo* HET-CAM. Rezultatele au arătat că, în toate testele efectuate, tratamentul combinat a prezentat o citotoxicitate superioară fără a produce un efect iritant asupra plexului vascular, susținând potențiala sa utilizare ca terapie adjuvantă în tratamentul cancerului faringian.

Keywords: pharyngeal cancer, fisetin, genistein, combinatorial treatment, cytotoxicity

Introduction

Head and neck cancers primarily affect the upper respiratory tract and the upper segment of the digestive tract, with approximately 500,000 cases reported worldwide each year. Recently, they have been associated with a significant increase in morbidity and mortality (1). Pharyngeal cancer includes malignant tumors of the nasopharynx, oropharynx, and hypopharynx. Although it is less common, it is an aggressive form of cancer that is often diagnosed at advanced stages due to its deep location and nonspecific symptoms (2). Despite advances in conventional treatments, the options still have numerous limitations. For example, radiation therapy has problems with precision and accessibility for patients. Chemotherapy and hormone therapy are associated with resistance and significant side effects, while surgical resection does not always guarantee complete tumor removal (3). Consequently, in recent years, alternatives to these therapies have been explored, such as plant-based treatments (4). Currently, almost half of anticancer drugs are of natural origin. Among the most effective secondary metabolites with anticancer potential are phenolic compounds, such as flavonoids (5).

Flavonoids are derived from various parts of plants and are known for their biological activities, such as antioxidant, anti-inflammatory, and antiviral effects. Based on their chemical structure, they can be classified into multiple subclasses, including flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanidins. Among these, flavonols such as fisetin, quercetin, and kaempferol are well known for their anticancer activity. Studies have shown that they can influence processes involved in carcinogenesis by inhibiting tumor cell proliferation and metastasis and inducing apoptosis (6). Fisetin (FIS) is found in many fruits and vegetables and exhibits multiple biological activities (7). Numerous studies have demonstrated that FIS can reduce cell proliferation and migration, induce apoptosis, and inhibit angiogenesis and oxidative stress. Simultaneously, it targets tumor cells specifically, suggesting high selectivity and minimal impact on normal cells (7,8). Among isoflavones, genistein and daidzein are two of the most studied natural substances for their anticancer potential. Both induce apoptosis and regulate the cell cycle, thereby inhibiting tumor growth in various cancers (9). Meanwhile, genistein (GEN), derived primarily from soy, has demonstrated anticancer effects through several key cellular mechanisms, such as disrupting the epithelial-mesenchymal transition and inhibiting angiogenesis (10). Furthermore, studies in the literature have demonstrated the beneficial effects of flavonoids when used as adjuvants to conventional anticancer therapies (11). However, research on the combined use of multiple flavonoids is limited, and further studies on their efficacy are necessary.

Hence, the present study aimed to evaluate the cytotoxic effects of FIS and GEN, tested both individually and in combination, on the Detroit-562 pharyngeal carcinoma cell line. This objective was accomplished by assessing cell viability using the MTT assay and cytotoxicity by measuring LDH release, followed by a morphological evaluation. In addition, nuclear changes were examined using Hoechst 33342 staining, and the impact on mitochondrial function was evaluated using MitoTracker Red CMXRos. At the same time, apoptotic and necrotic modifications were analyzed by AO/PI double staining. In addition, the irritant potential of the tested compounds was evaluated using the HET-CAM assay to complement the safety profile.

Materials and Methods

Reagents and Instruments

All reagents and materials were obtained from the following suppliers. Eagle's Minimum Essential Medium (EMEM, 30-2003™), fetal bovine serum (FBS), penicillin/streptomycin mixture, and trypsin–EDTA solution were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Acridine Orange, Propidium Iodide, and sodium lauryl sulfate (SLS) were obtained from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). Hoechst 33342 dye and MitoTracker™ Red CMXRos were acquired from Thermo Fisher Scientific (Waltham, MA, USA). The equipment used in this study included the Cytation 5 plate reader and Lionheart FX automated microscope (BioTek Instruments Inc., Winooski, VT, USA), the Olympus IX73 inverted microscope with cellSens Dimensions v.1.8 software (Olympus, Tokyo, Japan), and the SteREO Discovery.V8 stereomicroscope (ZEISS, Jena, Germany) for *in ovo* evaluation.

Cell culture protocol

The *in vitro* experiments were performed using the Detroit 562 cell line (CCL-138™; ATCC, Manassas, VA, USA) as a 2D cell model. A specific cell culture medium was used for cell cultivation (Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. During the experiments, the cells were incubated under specific conditions (37°C, 5% CO_2).

Detroit 562 Cell Treatment Protocol

The FIS stock solution was 20 mM, and the GEN stock solution was 50 mM, both dissolved in DMSO, with the maximum concentration not exceeding 0.5%. Dilutions corresponding to the tested concentrations were prepared from the stock solutions: for FIS, 5, 10, 15, and 20 µM; for GEN, 50, 100, 150, and 200 µM. In addition, combination treatments

using FIS + GEN were performed at the following concentrations: 5 μM + 50 μM, 10 μM + 100 μM, 15 μM + 150 μM, and 20 μM + 200 μM. For the MTT, LDH, and cellular morphology assays, all concentrations were tested. For nuclear and mitochondrial assessments and AO/PI staining, only the highest concentrations were selected: FIS (20 μM), GEN (200 μM), and their combination (20 μM + 200 μM).

The concentrations for FIS and GEN were selected based on data from previous studies on the cytotoxicity of these two compounds (12–14).

Cell Viability Assay (The MTT Test)

To assess cell viability using the MTT assay, Detroit 562 cells were cultured in 96-well plates at a density of 1×10^4 cells *per* well and, after reaching approximately 70% confluence, were exposed for 24 hours to the selected concentrations of FIS, GEN, and combinations of the two compounds (FIS+GEN). At the end of the treatment period, the culture medium was replaced with fresh medium (100 μl/well), followed by the addition of 10 μL MTT kit 1, and the plates were re-incubated for 3 h. Subsequently, 100 μL of MTT solubilizing solution (kit 2) was added, and the plates were incubated at room temperature in the dark for 30 min. The absorbance was then measured at 570 and 630 nm using a Cytation 5 microplate reader (15).

Lactate Dehydrogenase (LDH) Release Quantification

The cytotoxic potential of FIS, GEN, and their combination on Detroit-562 cells was investigated using the lactate dehydrogenase (LDH) release test. Briefly, 1×10^4 cells/well were cultured in 96-well plates and treated with FIS at 5, 10, 15, and 20 μM, GEN at 50, 100, 150, and 200 μM, and FIS+GEN at 5 μM + 50 μM, 10 μM + 100 μM, 15 μM + 150 μM, and 20 μM + 200 μM for 24 hours. At the end of the treatment interval, 50 μL of culture medium from each well was removed, transferred to new 96-well plates, mixed with 50 μL of reaction mixture, and incubated at room temperature for 30 minutes. Finally, 50 μL of stop solution was added to each well, and absorbance was measured at 490 and 680 nm using a Cytation 5 device from BioTek Instruments Inc. (Winooski, VT, USA) (16).

Morphological examination under bright-field microscopy

The cellular morphology of the Detroit-562 cells was examined using a Lionheart FX automated microscope after 24 hours of treatment with FIS (5, 10, 15, 20 μM), GEN (50, 100, 150, 200 μM), and their combination FIS + GEN (5 μM + 50 μM, 10 μM + 100 μM, 15 μM + 150 μM, 20 μM + 200 μM).

Nuclear fluorescence staining using Hoechst 33342

The effects of the tested compounds on nuclear morphology were assessed by Hoechst staining. Detroit-562 cells were seeded into 12-well plates at a density of 1×10^5 cells/well and treated with FIS (20 μM), GEN (200 μM), and their combination FIS + GEN (20 μM + 200 μM) for 24 h. After the incubation period, the culture medium was removed, and a Hoechst staining solution (1:2000 dilution in PBS) was added to each well, with the plate protected from light. The cells were incubated in the dark for 7 minutes, followed by three wash steps with PBS to remove excess dye. All experiments were performed in triplicate. Nuclear images were acquired using a Lionheart FX automated microscope and analyzed using Gen5™ Microplate Data Collection and Analysis software (version 3.14; BioTek Instruments Inc., Winooski, VT, USA).

Mitochondrial fluorescence staining using MitoTracker™ Red CMXRos.

Mitochondrial morphology was assessed in Detroit-562 cells cultured in 12-well plates (1×10^5 cells/well) and allowed to reach proper confluence. The cells were treated for 24 h with FIS (20 μM), GEN (200 μM), or the combination FIS + GEN (20 μM + 200 μM). For staining, MitoTracker Red CMXRos was prepared as a 1 mM stock solution in DMSO and diluted to a final concentration of 300 nM in culture medium. The cells were incubated with the staining solution for 30 minutes under standard culture conditions, then washed with PBS to remove excess dye. Subsequently, the cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and washed again with PBS. Fluorescent images were acquired using a Lionheart FX automated microscope (17).

Microscopic evaluation of Cell Viability and Apoptosis/Necrosis Assessment

Apoptotic and necrotic changes in Detroit-562 cells were assessed by double staining with acridine orange/propidium iodide (AO/PI) following a 24-hour exposure to FIS (20 μM), GEN (200 μM), and their combination, FIS + GEN (20 μM + 200 μM). The cells were seeded in 12-well plates (1×10^5 cells/well) and maintained under standard culture conditions until adequate adhesion was achieved. After treatment, 100 μL of a staining solution, prepared in the appropriate culture medium and containing 10 μg/mL AO and 10 μg/mL PI, was added to each well. The plates were incubated for 10 minutes at room temperature, protected from light. Fluorescence images were subsequently obtained using a Lionheart FX automated microscope. Image acquisition and subsequent quantitative analysis were performed using Gen5 Microplate Data Collection and Analysis software (18).

Evaluation of Irritation Potential Using the HET-CAM Assay

Based on the *in vitro* concentration–response analysis, the combined treatment was subsequently evaluated using the highest tested concentrations. The potential irritant and vascular toxic effects of the combination were evaluated using the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM). The eggs

were washed, disinfected with 70% alcohol, and placed in an incubator. On the fourth day of incubation, the eggshells were pierced with scissors, and approximately 7-8 ml of albumen was removed, depending on the egg size, to facilitate separation of the chorioallantoic membrane from the inner shell, thereby allowing better visualization of the blood vessels. On the fifth day, a small window was cut in the upper part of each egg, which was then sealed with adhesive tape, and the eggs were kept in the incubator until the day of the experiment.

The experiment was performed on the eighth day of incubation by applying 600 µL of FIS + GEN at 20 µM + 200 µM to the chorioallantoic membrane. In parallel, 600 µL of the positive control (SLS 1%) and the negative control (H_2O) were applied. The CAM was subsequently observed for 5 minutes to detect any vascular responses, including hemorrhage, coagulation, or vessel lysis. Microscopic images taken before treatment (T0) and after 5 minutes of exposure (T5) using a Zeiss Discovery V8 stereomicroscope with an Axio CAM 105 camera. The acquired images were processed and analyzed with ZEN Core 3.8 software to assess the vascular alterations induced by the tested sample. The severity of irritation was quantified by calculating the irritation score (IS) according to the formula shown below:

$$IS = 5 \times \frac{301 - H}{300} + 7 \times \frac{301 - L}{300} + 9 \times \frac{301 - C}{300}$$

The irritation score (IS) was calculated to assess the irritant potential of the tested samples, based on the time to the appearance of vascular responses, *i.e.*, hemorrhage (H), lysis (L), and coagulation (C). Based on the calculated IS values, the tested substances were classified as non-irritating (0–0.9), irritating (1–8.9), or severely irritating (9–21) (19). The assay was performed in triplicate, so a total of 9 eggs (3 *per* group) were used.

Statistical Analysis

Results are reported as mean values ± standard deviation (SD). Data distribution normality was verified using the Shapiro–Wilk test. As the dataset showed a normal distribution, statistical analysis was performed using one-way ANOVA, followed by Dunnett's multiple-comparison test as a post hoc test. Statistical processing was conducted with GraphPad Prism software (version 10.2.3; GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at *$p < 0.05$, with increasing levels of significance indicated as **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. All experiments were conducted in three independent biological replicates, each including three technical replicates.

Results and Discussion

Head and neck cancers are considered among the most aggressive tumors worldwide (20). This category of cancers includes a group of malignancies such as pharyngeal cancer, laryngeal cancer, salivary gland cancer, and tumors in the nasal and oral areas (21). Pharyngeal cancer continues to experience unsatisfactory mortality rates. Comparatively, nasopharyngeal cancer has the best prognosis, while oropharyngeal and hypopharyngeal cancer rates are poorer, even considering the advances in treatment (22). However, histological and molecular variability, as well as the lack of specific diagnostic markers, contribute to increased mortality and difficulties in therapeutic approaches to these malignancies (23). In this context, investigating new treatment options is essential. Natural compounds may represent promising candidates for addressing this issue, given their proven efficacy over time in alleviating or even treating various diseases, as well as their advantage of higher tolerability (24).

In vitro cellular models are relevant systems for investigating the antitumor potential of compounds, providing valuable insights into the mechanisms of action involved in inhibiting tumor proliferation and invasion (25). Accordingly, Detroit-562 is a human pharyngeal squamous cell carcinoma cell line derived from the pleural fluid of a patient with primary pharyngeal cancer. It is widely used to study pharynx cancer pathogenesis, metabolism, and therapeutic treatments, characterized by adherent epithelial growth and a mutant p53 (R175H) (26). Therefore, in this study, the Detroit-562 cell line was used as a 2D *in vitro* model to evaluate the cytotoxic potential of two natural compounds, namely FIS and GEN, as well as their combination. Additionally, the irritant potential of the combined substances was assessed using the HET-CAM assay.

Dose-dependent decrease in cell viability following FIS, GEN and their combined treatment

For the evaluation of cytotoxic effects, an MTT assay was performed. The results (Figure 1) indicated the following cell viability values: In the case of FIS, at concentrations of 5 µM, 10 µM, 15 µM, and 20 µM, cell viability was 93.08%, 86.21%, 75.72%, and 67.75%, respectively. For GEN**,** the recorded cell viability values at 50 µM, 100 µM, 150 µM, and 200 µM were 90.12%, 84.26%, 73.23%, and 50.01%**,** respectively. Regarding the combinations of the two compounds (FIS 5 µM + GEN 50 µM, FIS 10 µM + GEN 100 µM, FIS 15 µM + GEN 150 µM, and FIS 20 µM + GEN 200 µM), the following cell viability values were observed: 83.64%, 71.42%, 49.25%, and 39.08%**,** respectively. These results indicate that cytotoxicity was slightly more pronounced in the case of GEN compared to FIS. The greatest decline in cell viability was observed for the combined treatments, with the most evident effects at FIS 15 µM + GEN 150 µM and FIS 20 µM + GEN 200 µM.

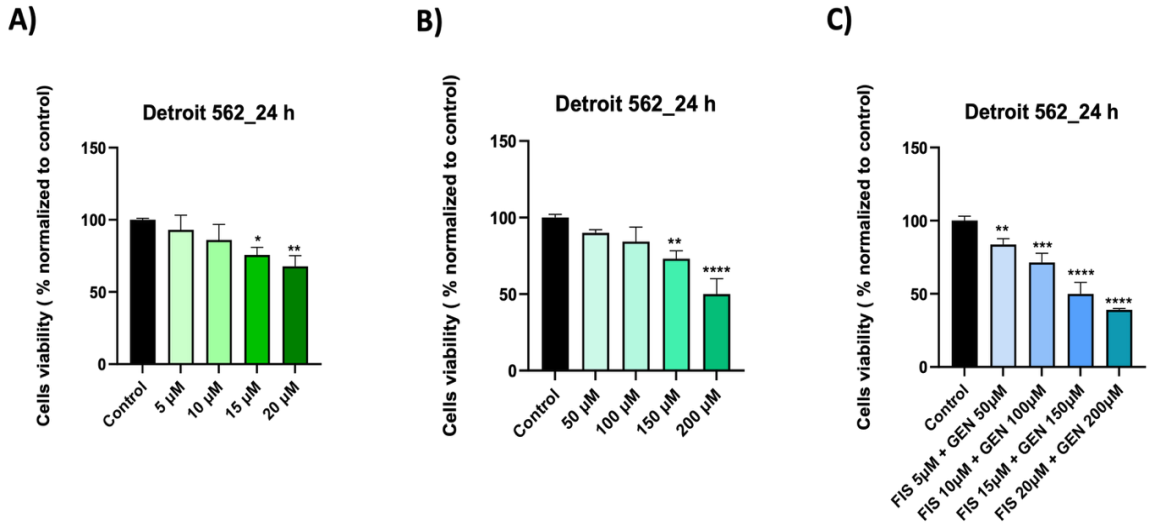

Figure 1.
Graphical representation of the percentage of viable Detroit-562 cells after 24 hours of treatment with (A) FIS, (B) GEN, (C) FIS + GEN. The results were presented as viability percentages (%) normalized to control (non-treated cells) and expressed as means ± standard deviation of three experiments performed in triplicate. The statistical differences between the control and the treated groups were analyzed using the one-way ANOVA and Dunnett's multiple comparisons tests (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)

The MTT assay is a widely used colorimetric test for assessing cell viability, based on the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals using mitochondrial enzymes. This technique is used to determine the percentage of metabolically active cells. The amount of formazan formed is directly proportional to the percentage of live cells and can be quantified by measuring absorbance (27, 28). In line with the results of the present study, FIS at 10–20 µM was evaluated by Afzal *et al*. in the triple-negative breast cancer cell line (MDA-MB-231), where a significant reduction in viability was observed at higher doses (29). In another study using the SNU-308 bile duct cancer cell line, FIS enhanced gemcitabine's effect, but when administered alone, it showed a lower effect (30). GEN has also been reported to reduce cell viability in a dose-dependent manner in other studies. In HeLa cells, exposure for 24 h to concentrations ranging from 6.25 to 200 µM resulted in a progressive decrease in viability, with more evident effects at higher concentrations (13). In line with these findings, our study found that GEN tested at 50–200 µM also induced a concentration-dependent reduction in cell viability. In addition, Ruçińska *et al*. reported a decrease in NIH 3T3 cell viability at concentrations above 20 µM, confirming the compound's cytotoxic potential (31).

Dose-dependent increase in LDH release following FIS, GEN, and their combination treatment

Given that the MTT assay primarily reflects mitochondrial function and metabolic activity following treatment, the cytotoxicity evaluation was further extended by performing the LDH assay to assess cell membrane integrity. The results of this determination indicated an increase in LDH release compared to the control, in a dose-dependent manner, for all tested samples. Regarding treatment with FIS, the following percentages of LDH release relative to the control were obtained: 4.49%, 6.73%, 12.73%, and 20% at concentrations of 5, 10, 15, and 20 µM, respectively. After GEN treatment, the percentages were as follows: 6.28%, 9.89%, 17.20%, and 29.01% at concentrations of 50, 100, 150, and 200 µM, respectively. In the case of combination treatments (FIS 5 µM + GEN 50 µM, FIS 10 µM + GEN 100 µM, FIS 15 µM + GEN 150 µM, and FIS 20 µM + GEN 200 µM), LDH release was more pronounced, following the same dose-dependent trend observed with the individual compounds, with the following percentage values: 11.33%, 17.03%, 32.33%, and 42.33%. These results indicate that the greatest impact on cell membrane integrity, as reflected by increased LDH release, was observed following the combined treatment (Figure 2).

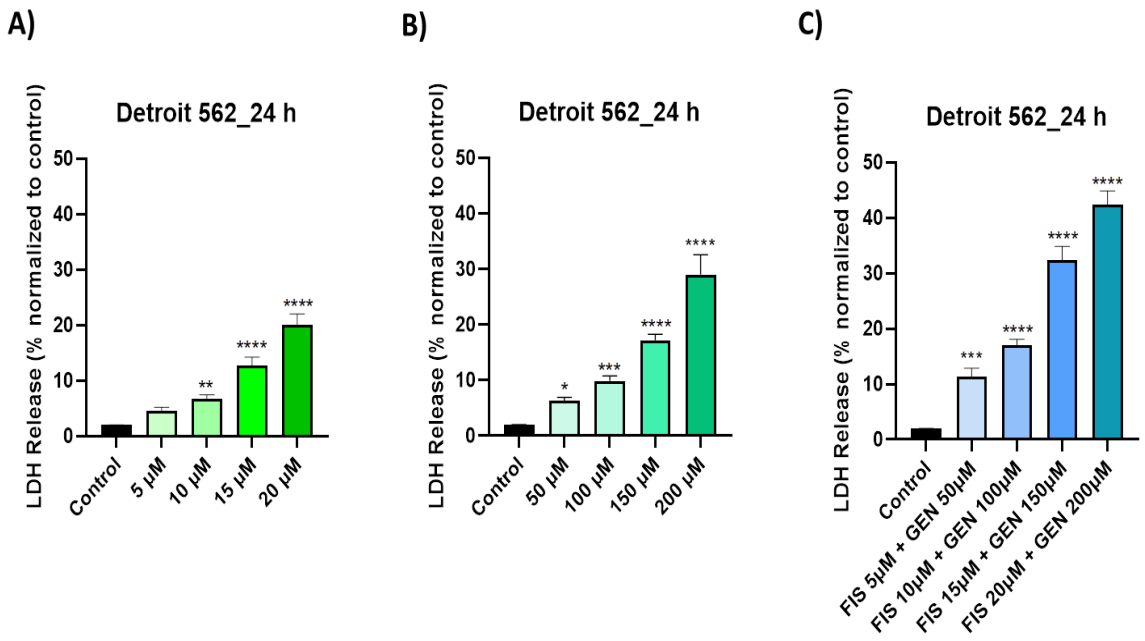

Figure 2.
Lactate dehydrogenase (LDH) release percentages in Detroit-562 cells after treatment with (A) FIS, (B) GEN, (C) FIS + GEN, for 24 hours. The findings were expressed as means ± standard deviation of three experiments performed in triplicate. The statistical differences between the control and the treated groups were analyzed using the one-way ANOVA and Dunnett's multiple comparisons tests (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001)

The LDH assay is one of the most commonly used tests for assessing cytotoxicity, alongside the MTT assay. The basic principle of this assay is that the enzyme lactate dehydrogenase (LDH) is released into the extracellular medium as a result of disruption of the cell membrane's integrity. LDH release is determined by spectrophotometric quantification of a colored reaction product resulting from a secondary reaction involving the LDH-mediated reduction of NAD^+ to NADH. The intensity of the recorded signal is directly proportional to the amount of LDH released and, consequently, to the extent of cell damage (32). The use of this assay to investigate cytotoxicity following *in vitro* treatment of various cancer cell lines of different types and origins is common in the literature. For example, Kim evaluated the effect of FIS at concentrations ranging from 10 to 200 µM on liver cancer cell lines (HepG2, Hep3B, and Huh-7). The results of this evaluation, conducted 24 hours after treatment, indicated a dose-dependent increase in LDH release (33). Meanwhile, Choi *et al*. determined the cytotoxicity of GEN at concentrations of 1–100 µM on SK-OV-3 ovarian cancer cells. After 48 hours of exposure, it was evident that the greatest cytotoxic effect occurred with the 100 µM treatment (34). In neuroblastoma cells (SK-N-MC), Ismail *et al*. reported investigating the effect of GEN at concentrations of 1, 12.5, 25, 50, 75, and 100 µM at multiple time points (24, 48, 72 hours), and observed a dose- and time-dependent effect (35).

Dose-dependent morphological alterations induced by FIS, GEN, and their combined treatment

The cytotoxicity analysis was continued by evaluating the impact of 24 hours of treatment with the tested samples on cellular morphology (Figure 3). After exposure to FIS at the tested concentrations, a decrease in confluence was observed, particularly at 15 and 20 µM. At these concentrations, notable morphological changes were observed, including cell blebbing and shrinkage. Meanwhile, GEN induced a decrease in confluence starting at 100 µM. The most predominant morphological changes were cell rounding and cell shrinkage. In the combined treatment, as with the individual compounds, a decrease in confluence and increased cell rounding compared to the control were observed, even at the lowest tested dose. Furthermore, following treatment with FIS 15 µM + GEN 150 µM and FIS 20 µM + GEN 200 µM, an additional effect of cellular fragmentation was observed alongside the morphological changes noted at lower concentrations.

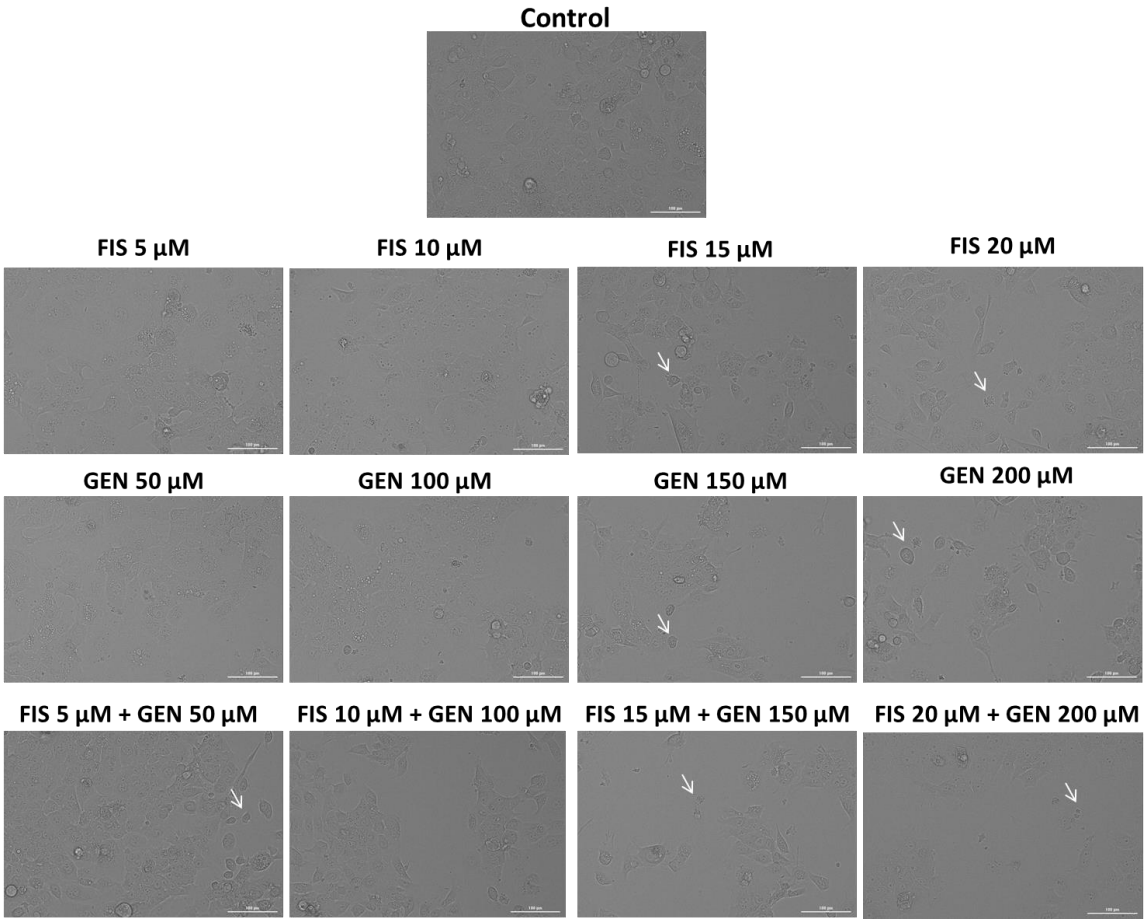

Figure 3.
Illustrative images of the morphology of Detroit-562 cells after treatment with FIS (5, 10, 15, 20 µM), GEN (50, 100, 150, 200 µM), and their combination FIS + GEN (5 µM + 50 µM, 10 µM + 100 µM, 15 µM + 150 µM, 20 µM + 200 µM) for 24 hours. The white arrows show morphological alterations. The scale bar was 100 µm.

Nuclear morphological changes induced by FIS, GEN, and their combined treatment

The cytotoxicity analysis was further extended by examining the impact of the tested compounds on nuclear morphology using Hoechst 33342 staining (Figure 4A). Since the most pronounced toxic effects were observed at the highest tested concentrations, the evaluation of nuclear features focused on these concentrations. In treatments with FIS (20 µM) and GEN (200 µM) alone, nuclear changes were observed, including chromatin condensation and nuclear rounding. However, the most evident morphological changes at the nuclear level were observed following the combined treatment (FIS 20 µM + GEN 200 µM), with a more pronounced decrease in cell confluence, along with nuclear rounding, chromatin condensation, and nuclear fragmentation. FIS treatment increased the apoptotic index to 22.03%, while GEN treatment increased it to 29.43%. The combined FIS+CIS treatment induced a more pronounced increase, reaching 34.13% (Figure 4B).

Assessing nuclear morphology using fluorescent dyes is an important, rapid, and efficient step in studying the potential cytotoxic effects of various compounds. Among these, Hoechst 33342 is one of the most frequently used dyes due to its good cellular permeability. It highlights significant apoptotic changes, such as nuclear fragmentation and chromatin condensation (36,37). A similar assay for assessing nuclear morphology using DAPI staining was used by Shih *et al*. to evaluate changes after treatment with FIS at 40 µM over different time intervals. The most significant effect was observed after 24 hours of treatment, when the nuclei presented the most condensed chromatin (38). Shi *et al*. also assessed nuclear morphology in human lung cancer cells (HCC827 and H358) using Hoechst 33342 staining. The study evaluated the effects of FIS treatment as monotherapy (40 µM) and in combination with carnosic acid. The results showed changes in nuclear morphology following both individual and combined treatments, but the effects were more pronounced with the combination (39). Similarly, Liu *et al*. investigated the effects of GEN on S180 cells (sarcoma cells) after 24 hours of treatment at 10 µM. After treatment, the cells were irradiated with X-rays for 2 and 12 hours. The impact on nuclear morphology was more pronounced after 12 hours of

irradiation (40). In addition, Moore *et al*. evaluated the effects of GEN at a concentration of 50 µM on human uterine leiomyoma (UtLM) and myometrial cells (UtSMC) after 168 hours of incubation from the perspective of nuclear alterations assessed using Hoechst 33342 staining, the results showed the presence of apoptotic and necrotic nuclei, with a more pronounced effect observed in UtSMC cells (41).

Mitochondrial alterations induced by FIS, GEN, and their combined treatment

The impact of the treatment on mitochondrial network structure was further evaluated using Mito-Tracker Red CMXRos staining (Figure 5). Morphological changes after 24 hours of exposure to FIS at 20 µM were moderate, represented by limited structural disruptions. In contrast, treatment with GEN at 200 µM induced more pronounced effects, including mitochondrial fragmentation and chromatin condensation. In the case of the combination, these changes were more significant.

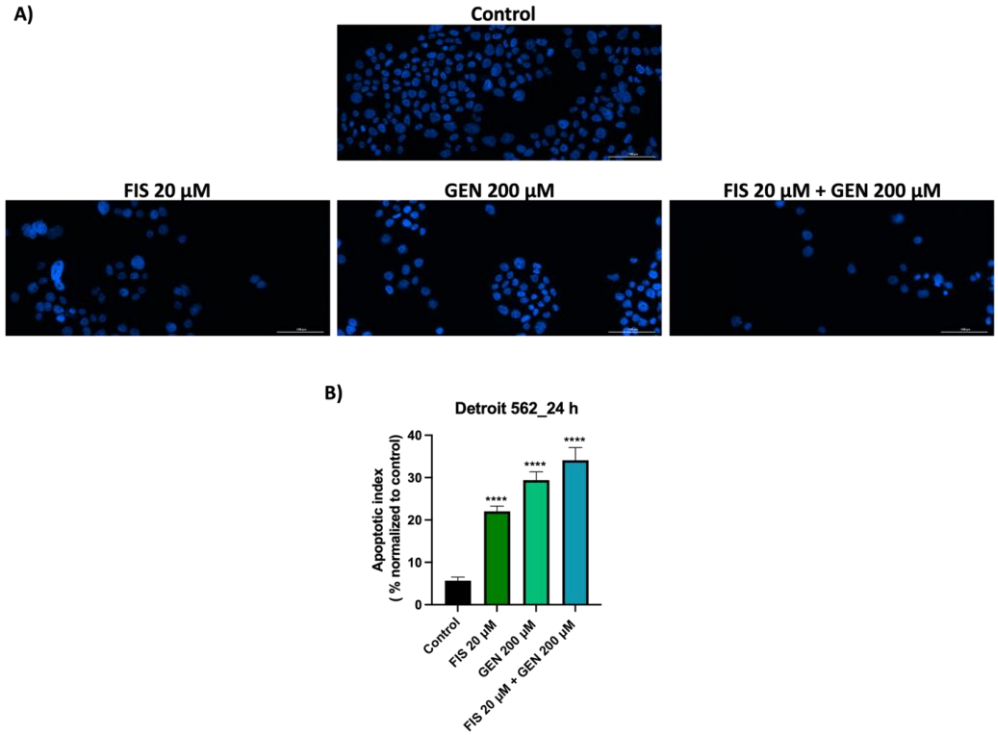

Figure 4.

(A) Representative images showing the aspect of nuclei of Detroit-562 cells and (B) Apoptotic Index (%) post-treatment with FIS (20 µM), GEN (200 µM), and their combination FIS + GEN (20 µM + 200 µM) for an interval of 24 hours. The scale bar was 100 µm. The statistical differences between the control and treated groups were analyzed using a one-way ANOVA and Dunnett's multiple-comparison test (****$p < 0.0001$)

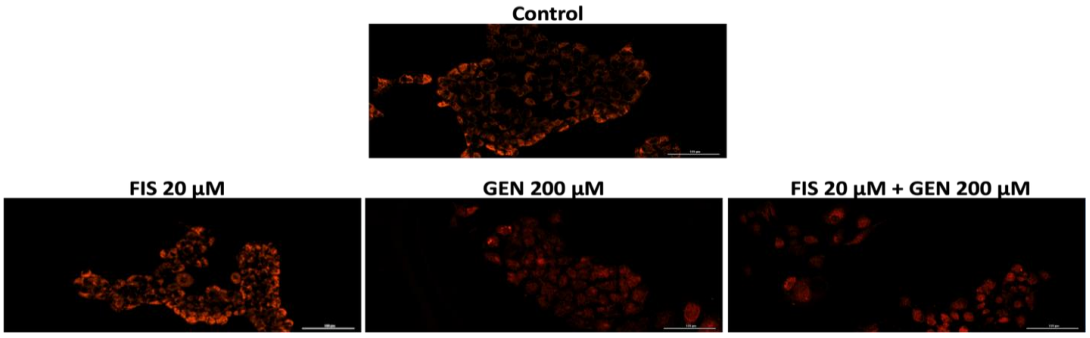

Figure 5.

Representative images showing the mitochondrial morphology of Detroit-562 cells following 24 h exposure to FIS (20 µM), GEN (200 µM), and their combination FIS + GEN (20 µM + 200 µM). The scale bar was 100 µm.

Mitochondria play a significant role in the etiology of head and neck cancers through their involvement in cellular metabolism, energy production, and the generation of reactive oxygen species (ROS). Furthermore, mitochondria modulate cellular survival and induce cell death through multiple mechanisms, including apoptosis. Mutations in mitochondrial DNA, especially in genes responsible for encoding the COI, COII, and COIII subunits of complex IV, are among the most frequently reported alterations in head and neck cancers. In addition, genetic disorders that affect mitochondrial ribosomal proteins have also been linked to the development of malignant diseases in this region (42). These findings underline the critical importance of assessing mitochondrial integrity in head and neck cancers, including pharyngeal tumors. Mitochondrial dysfunction is frequently associated with phenotypic and morphological changes in cells as part of the response to cellular stress or damage. These changes often involve actin microfilaments, which undergo structural changes or alterations in nucleotide interactions under stress conditions (43). Overall, evaluating naturally derived compounds from this perspective is common in the literature. For example, silibinin, a natural flavonoid compound, was investigated in Detroit 562 cells. After 48 hours of treatment with concentrations from 25 to 200 μM, dose-dependent changes in mitochondrial structure were observed, including chromatin condensation and fragmentation of the mitochondrial network architecture (44). Similarly, Nica *et al*. evaluated the impact of thymol on Detroit 562 cells at concentrations ranging from 100 to 300 μM using the same method. After 24 hours of treatment, mitochondrial fragmentation and chromatin condensation were observed (18). FIS was studied in oral cancer cells (HSC-3) at a concentration of 40 μM over several time intervals (6, 24, and 48 hours of treatment). The results showed that the greatest decrease in mitochondrial membrane potential occurred after 24 hours of treatment (38). Also, Hsiao *et al*. evaluated the effects of GEN on human leukemia cells (HL-60) treated with concentrations of 20–50 μM for 48 hours, observing the same dose-dependent effects (45).

Apoptosis and necrosis induced by FIS, GEN, and their combined treatment

The evaluation of apoptotic and necrotic effects following treatment was carried out using double staining with Acridine Orange/Propidium Iodide (AO/PI), focusing on the highest tested concentrations (Figure 6A). Microscopic analysis using this method revealed the presence of necrotic nuclei (characterized by red fluorescence, indicating PI-positive cells) following FIS treatment at 20 μM. In addition, at 200 μM, both necrotic cells (red fluorescence) and apoptotic cells (identified by uneven green fluorescence associated with chromatin condensation) were observed. Overall, both necrotic and apoptotic effects were more pronounced in the case of the combined treatment at FIS 20 μM + GEN 200 μM, where a marked decrease in uniform green fluorescence was evident, along with a predominance of chromatin condensation and an increased number of PI-positive cells compared to those with green fluorescence (AO-positive). Quantitative analysis of this assay regarding the cell survival after treatment with FIS (20 μM), GEN (200 μM), and the combined treatment FIS 20 μM + GEN 200 μM indicates the following results: 74.71%, 62.52%, and 29.79%, respectively. In contrast, apoptotic/necrotic rates increased to 25.29%, 37.48%, and 70.21%, confirming a more pronounced effect of the combined treatment (Figure 6B).

The AO/PI double-staining method is commonly used to assess cell viability and apoptotic/necrotic changes by fluorescence microscopy. AO is a permeable dye that permeates both live and dead cells, binding to nucleic acids and emitting green fluorescence. In contrast, PI is impermeable to viable cells and penetrates only cells with compromised membranes, where it binds to DNA and produces red fluorescence. Hence, this method allows for the rapid differentiation between living and dead cells. In addition, AO has metachromatic properties and can accumulate in acidic intracellular compartments, making it useful for highlighting cellular changes associated with stress or degradation processes (46-48). The assessment of the apoptotic and necrotic effects of FIS and GEN using the AO/PI assay has also been described in other studies. In a study conducted by Jafarzadeh *et al*., the apoptotic potential of FIS was explored at a concentration of 50 μg/mL, either alone or in combination with cisplatin (FIS 50 μg/mL + cisplatin 0.1 μg/mL) in ovarian cancer cells (A2780 cells). The evaluation results indicated apoptosis induction in both individual treatments, with the combined treatment producing more pronounced effects than the single treatments (49). In addition, Kaleli *et al*. assessed the impact of 100 μM GEN treatment on MCF-7 and MDA-MB-231 cells after 24 hours. The results of the AO assay indicated the presence of apoptotic features, characterized by cell blebbing and chromatin condensation following treatment (50). Furthermore, Hsiao *et al.* investigated the pro-apoptotic effects of GEN at concentrations of 20-50 μM after 48 hours of treatment in HL-60 cells. The results showed that this compound increased the expression of pro-apoptotic proteins, including Bax, PARP cleavage, caspase-9, and caspase-3, while decreasing the levels of anti-apoptotic proteins such as Bcl-2 and Bid. Furthermore, propidium iodide and FITC staining were used to confirm the potential effects of 40 μM genistein on the expression of proteins such as Calpain 1, GRP78, and GADD153, which are associated with endoplasmic reticulum stress. The results confirmed an increased expression of these proteins following treatment (45).

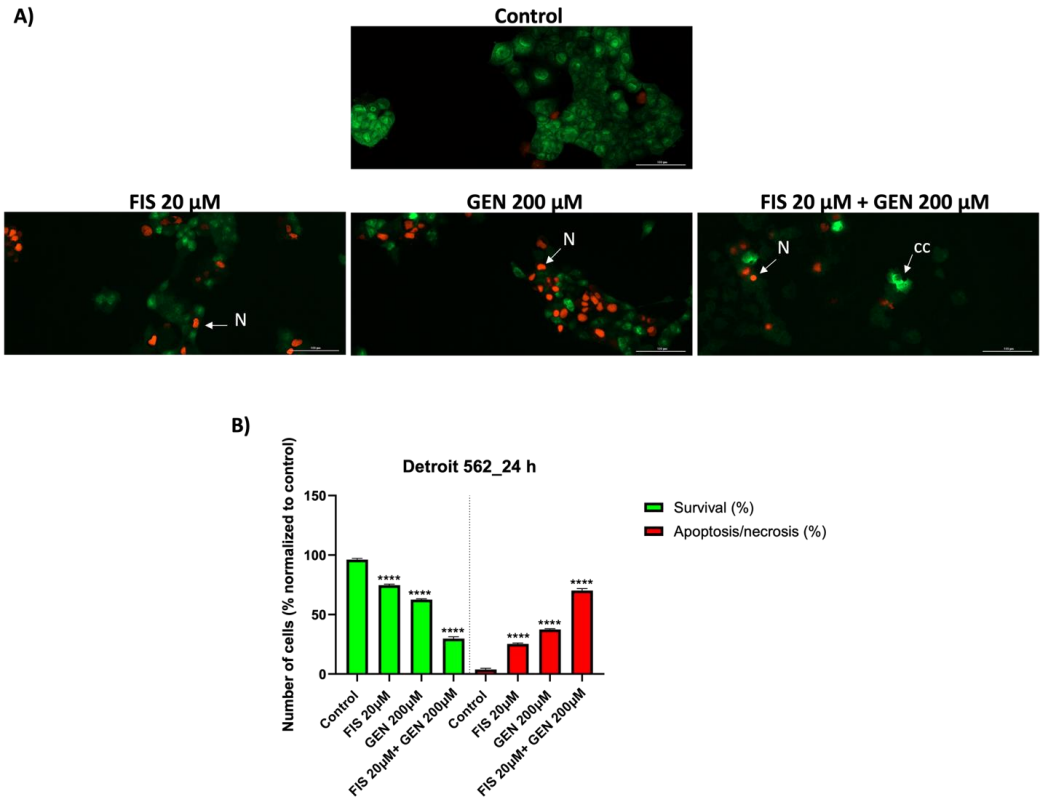

Figure 6.

(A) Representative images of the impact and (B) Quantification of the number of survival and apoptotic cells after 24 h of exposure to FIS (20 µM), GEN (200 µM), and their combination FIS + GEN (20 µM + 200 µM) in Detroit 562 cells stained with AO/PI. The scale bar was 100 µm. CC-chromatin condensation, N-necrosis. The statistical differences between the control and treated groups were analyzed using a one-way ANOVA and Dunnett's multiple-comparison test (****$p < 0.0001$).

The combination of FIS and GEN exhibited no irritant potential in the in ovo model

The combination of FIS and GEN at the highest *in vitro* concentration tested (FIS + GEN at 20 µM + 200 µM) was further evaluated for its potential irritant effect on epithelial tissues using the HET-CAM test (Figure 7). The combination did not induce irritation in the blood vessels and was generally well tolerated. No hemorrhages or coagulations were noted, although minor vascular lysis was detected. In contrast, the positive control, sodium lauryl sulfate (SLS 1%), produced a severe irritant effect on the CAM vasculature, characterized by hemorrhage, vascular lysis, and coagulation, while the negative control (distilled water) did not produce a vascular reaction.

According to Table I, the IS for SLS 1% was 17.93, with hemorrhage at 20 seconds, lysis at 43 seconds, and coagulation at 60 seconds. H_2O was classified as non-irritating (IS = 0.07). Likewise, the FIS + GEN combination exhibited a low irritation score (IS = 0.47).

The chorioallantoic membrane (CAM) of the chick embryo is a thin, highly vascularized extraembryonic structure commonly used as a model for irritation assessment. Due to its low cost, ease of handling, rapid results, and reproducibility, it represents a valuable preliminary alternative to animal testing in fields such as angiogenesis, oncology, toxicology, and immunology. In addition, its use does not require strict ethical approval, since the chick embryo is not considered a living animal before day 17 of development. Moreover, CAM can induce inflammatory responses similar to those observed in conjunctival tissue, making it useful for assessing the irritant potential and tolerability of various substances (19, 51). The HET-CAM assay has been used to evaluate the irritation potential of various natural compounds, including phenolics such as thymol (18) and eugenol (52), as well as flavonoids such as quercetin and myricetin (53), which have been reported as non-irritant.

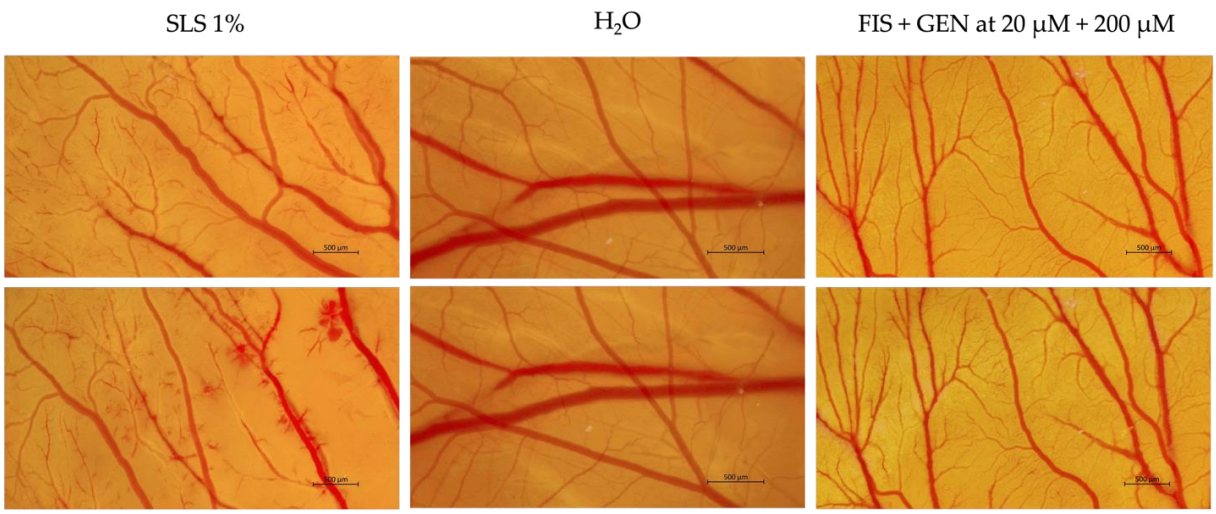

Figure 1.
Stereomicroscope images of the CAMs treated with SLS 1% used as positive control, H_2O used as negative control, and FIS 20 µM + GEN 200 µM, before (T0) and five minutes (T5) post-treatment. Scale bars indicate 500 µm.

Table I
The irritation score for 1% sodium lauryl sulfate (positive control), H_2O (negative control), and FIS + GEN at 20 µM + 200 µM

Sample	IS	Irritation category
SLS 1%	17.93	Severe irritant
H_2O	0.07	Non-irritant
FIS + GEN at 20 µM + 200 µM	0.47	Non-irritant

Taken together, the results of this study indicate promising effects of these two compounds, particularly their combination, as potential candidates for therapy or as adjuvants to other anticancer agents in therapeutic strategies for pharyngeal cancer. Nevertheless, we acknowledge that this study has several limitations, including the fact that the *in vitro* investigation of their effects in pharyngeal cancer was performed on a single cell line and was not extended to other models, such as 3D cell culture systems (*e.g.*, spheroids), tissue models, or *in vivo* animal models. Future research studies aim to investigate the substances at a wider range of concentrations and over longer time periods (48 and 72 hours), as well as to evaluate the safety profile of the combination therapy. Furthermore, future research will focus on a more detailed analysis of the molecular mechanisms of action underlying the treatment.

Conclusions

In conclusion, pharyngeal cancer is a major global health problem associated with high rates of morbidity and mortality. At the same time, conventional treatment options are limited by the development of resistance and adverse reactions. In this context, recent research has focused on identifying alternatives based on natural compounds.

In this study, the cytotoxic potential of FIS, GEN, and their combination was evaluated *in vitro* using the Detroit-562 pharyngeal carcinoma cell line and *in ovo*. The results demonstrated that these compounds induced a dose-dependent decrease in cell viability and a corresponding increase in LDH release, with the effects more pronounced in the combination. Furthermore, cellular and nuclear morphological changes, alterations in the mitochondrial network, as well as apoptosis and necrosis processes were observed, with combined effects observed in the combination treatment. Furthermore, the HET-CAM test confirmed the absence of irritant potential in the FIS and GEN combination, supporting the vascular tolerability of this combination. These findings suggest that FIS and GEN, particularly in combination, may represent promising therapeutic options for the management of pharyngeal cancer. Further studies are needed to investigate their potential mechanisms of action and determine how this combination might be incorporated into future therapeutic strategies for pharyngeal cancer.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

Not applicable.

AI use disclosure

During the preparation of this manuscript, the authors used QuillBot for language improvement, including spelling, grammar, clarity, and readability. The authors reviewed and revised all outputs from these tools and took full responsibility for the final content of the work.

Conflict of interest

The authors declare no conflict of interest.

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