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Hesperidin as a Potential Candidate for Improving the Response of Detroit 562 cells to Cisplatin—An In Vitro Study

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Abstract

Background: Oropharyngeal squamous cell carcinoma (OPSCC) is one of the most common cancers globally, occurring in all types of populations, but current therapy faces multiple barriers, urgently requiring new alternatives for improvement. Hesperidin (HSP) is a bioflavonoid with pleiotropic properties that has been mentioned in studies for enhancing response to chemotherapy, including in the case of cisplatin (CIS). Methods: Using Detroit 562 cells, HSP, CIS, and the combinatorial treatment of the two were examined by MTT assay, morphological analysis, and mitochondrial and nuclear assessment for 24 h. Results: The results showed that the combination of HSP+CIS produces superior effects, reducing cell viability by up to 16%and producing cell rounding and shrinkage and significant alterations in nuclei and mitochondria compared to individual treatments. Conclusions: HSP may be an important candidate in improving CIS cytotoxicity in Detroit 562 cells and could be considered a promising natural adjuvant. The combined treatment of the phytocompound and CIS led to superior outcomes, enhancing the effects of CIS.

Keywords: *hesperidin; cisplatin; combinatorial treatment; oropharyngeal squamous cell carcinoma*

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1. Introduction

Oropharyngeal squamous cell carcinoma (OPSCC), often referred to as throat or tonsil cancer, is a type of head and neck cancer (HNC) that affects the base and posterior third of the tongue, tonsils, soft palate, and the posterior and lateral pharyngeal walls [1]. OPSCC is ranked as the seventh most common cancer worldwide [2], accounting for approximately 300,000 deaths annually [3]. Its incidence is rising at an alarming rate in both younger and older populations, highlighting its growing public health significance. Conventional approaches such as surgery, chemotherapy, and radiation remain standard. However, each carries significant limitations. Surgery can affect speech and swallowing; radiotherapy and chemoradiation are known to cause adverse effects like mucositis, dental damage, and osteonecrosis. Chemotherapy drugs that are usually used might cause nephrotoxicity, myelosuppression, and neuropathy [4–10]. Current treatment strategies have expanded considerably in recent years. Emerging remedies (i.e., gene therapy, epigenetic modulators, nanomedicine-based approaches, and PROTAC-based strategies) show promise. Despite that, overall survival rates for OPSCC remain around 50% [8]. This underscores the urgent need to explore safer, more effective alternatives, including natural compounds, as potential adjuvant therapies.

Cisplatin (CIS) is a chemotherapeutic compound that is used in a wide range of oncological fields and is also among the most commonly used clinically in OPSCC. From a historical perspective, in 1968, it was discovered that it has the

ability to cause tumour regression in a tumour-bearing mouse model, and its antitumor efficacy was subsequently validated in several solid tumours. However, the use of CIS in OPSCC also produces chemoresistance and numerous side effects, which have led to the development of analogues [11]. Furthermore, in several studies, CIS and other chemotherapeutic agents have been evaluated in combination with phytochemicals with antitumor potential to improve treatment responses [12]. Hesperidin (HSP) is a botanical compound belonging to the flavonoid class; being a flavonone, it has significant activity in cancer, but also in other pathologies such as cardiovascular or neurodegenerative diseases. Its therapeutic effects have been associated with its recognised antioxidant and anti-inflammatory actions. Evidence highlights its potential in tumour inhibition, apoptosis induction, cell cycle arrest, metastasis inhibition, and chemoresistance inhibition [13]. Another noteworthy property, particularly important in the field of antitumor agents, is that HSP has been shown to be non-toxic in normal healthy cells [14]. The combination of chemotherapeutic agents with less toxic phytochemicals has recently become one of the important strategies in tumour management [15]. HSP and CIS have shown in a cell line of osteosarcoma that they have the capacity to improve, through their association, anticancer activity based on the activation of apoptosis [16].

In light of the above, this paper aims to evaluate HSP, CIS, and their combination on Detroit 562 cells (pharyngeal cancer cells), based on the hypothesis that combinatorial treatments involving botanical compounds and conventional chemotherapy may be promising strategies. The study achieves its main objective by determining cell viability 24 h after treatment using the MTT technique, bright-field morphological analysis, evaluation of cell nuclei using Hoechst 33342 staining, and evaluation of the impact on mitochondria using MitoTracker. These investigations have the potential to bring new perspectives on possible strategies for improving current therapies, which face barriers to therapeutic success.

2. Materials and Methods

2.1. Reagents and Instruments

Hesperidin and cisplatin, the MTT cell viability kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), trypsin–EDTA solution, and phosphate-buffered saline (PBS) were sourced from Sigma Aldrich, Merck KGaA (Darmstadt, Germany). Eagle's Minimum Essential Medium (EMEM) and foetal bovine serum (FBS) were obtained from ATCC (American Type Cell Collection, Lomianki, Poland). Paraformaldehyde 4% was supplied by Santa Cruz Biotechnology (Dallas, TX, USA). Hoechst 33342 dye and MitoTracker™ Red CMXRos were procured from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin 100 U/mL–streptomycin 100 µg/mL and dimethyl sulfoxide (DMSO) were purchased from PanBiotech (Aidenbach, Germany). Cytation 5, Lionheart FX, and Gen5™ Microplate Data Collection and Analysis Software (v3.14) were purchased from BioTek Instruments Inc. (Winooski, VT, USA).

2.2. 2D Cell Culture Conditions

The cells used in the study, Detroit-562 (pharyngeal carcinoma), were cultured in EMEM supplemented with 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin. The cell line was kept in a humidified incubator at 37 °C with 5% CO_2 and checked daily under a microscope. HSP was prepared in DMSO, making sure the final DMSO concentration did not go over 0.5%. The CIS stock solution was prepared by dissolving the compound in ultrapure water.

2.3. Cell Viability Assessment Using the MTT Method

As a first step, to evaluate the impact of the samples in vitro, the MTT colorimetric technique was utilised to measure cell viability. Following the 24 h treatment period of Detroit 562 cells with HSP 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, and 100 µM; CIS 1 µM, 2 µM, 5 µM, 10 µM, 15 µM, and 20 µM; and the combinatorial treatment HSP 5 µM + CIS 1 µM, HSP 10 µM + CIS 2 µM, HSP 25 µM + CIS 5µM, HSP 50 µM + CIS 10 µM, HSP 75 µM + CIS 15 µM, and HSP 100 µM + CIS 20 µM, 10 µL/well of MTT kit 1 was administered for 3 h while the plates were incubated. After this period, 100 µL/well of MTT solubilising solution was added for 30 min at room temperature. Utilising Cytation 5, the absorbance at 570 nm and 630 nm was determined.

2.4. Bright-Field Cell Morphology Analysis

To verify changes in cell structure after treating Detroit 562 cells with the samples of interest (HSP 5 µM, 10 µM, 25 µM, 50 µM, 75 µM, 100 µM; CIS 1 µM, 2 µM, 5 µM, 10 µM, 15 µM, 20 µM; and the combinatorial treatment HSP 5 µM + CIS 1 µM, HSP 10 µM + CIS 2 µM, HSP 25 µM + CIS 5 µM, HSP 50 µM + CIS 10 µM, HSP 75 µM+CIS 15 µM, HSP 100 µM+ CIS 20 µM) for 24 h, they were analysed using a Lionheart FX microscope and the Cell Analysis Tool included in the Gen5™ Microplate Data Collection and Analysis software (version 3.14) at 20× magnification in bright field.

2.5. Mitochondria and Nuclei Staining Evaluation via MitoTracker and Hoechst 33342

To analyse mitochondrial and nuclear morphology, Detroit 562 cells were exposed to HSP, CIS, and the HSP+CIS association for 24 h. Following this treatment period, to perform the evaluation, the MitoTracker dye stock solution (1 mM) in DMSO was diluted in the specific culture medium to a final concentration of 300 nM, and after a 30 min incubation with the staining solution under standard culture conditions, the cells were rinsed to remove excess dye with culture medium. Afterwards, following MitoTracker staining, to preserve cellular structures, the cells were fixed for 10 min with 4% paraformaldehyde at room temperature, followed by thorough washing with PBS.

To examine nuclear alteration induced by the test samples, staining with Hoechst 33342 was performed. After the fixation process, Hoechst 33342 solution (diluted 1:2000 in PBS) was added to each well and incubated in the dark for 5–10 min. Then, the staining solution was removed, and the wells were washed three times with PBS.

Representative images were collected using the Lionheart FX automated microscope at 20× magnification. Image analysis was performed using Gen5™ Microplate Data Collection and Analysis software (version 3.14; BioTek Instruments Inc., Winooski, VT, USA). The entire protocol was performed in accordance with Talpos et al. [3].

2.6. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The differences between the cells treated with HSP, CIS, and the HSP+CIS associative treatment groups, and the control were determined using one-way ANOVA and Dunnett's multiple-comparison test. All statistically significant results were noted with "*": *** $p < 0.001$; **** $p < 0.0001$.

3. Results and Discussions

Even though recent efforts have been made to moderate and reduce tobacco and alcohol consumption, several countries have reported an increase in OPSCC, especially among young people. The significant proportion suggests that a substantial cause could be infection with HPV (human papillomavirus), acquired through sexual contact, and the increase in incidence has also been attributed to changes in sexual behaviour in the Western world, where increases in the disease have been noted [17]. CIS is a chemotherapeutic agent often employed in OPSCC, serving as a first-line treatment for many cancers. Cells treated with this agent undergo suppression or attenuation of tumorigenesis and metastasis. CIS acts by interfering with DNA synthesis and repair in cancer cells, which implicitly leads to cell death. However, CIS presents many barriers that highlight the urgent need for improvement. In addition to nephrotoxicity, cardiovascular problems, and other adverse effects, resistance in malignant tumours is one of the major challenges, as cancer cells may be resistant to CIS from the outset or acquire this resistance [18]. Many studies published in the scientific literature have proven that combining CIS with various natural compounds can lead to therapeutic success in oncology. Therefore, over the last few decades, CIS has been evaluated in combination with saponins, alkaloids, flavonoids, polysaccharides, and many others in order to improve treatment responses [18]. HSP is a bioflavonoid that has attracted attention for its anticancer potential through various pieces of in vivo and in vitro evidence. Together with CIS, as a combination, HSP has been shown to enhance CIS's cytotoxicity in osteosarcoma cells, producing interesting results [16]. Considering all of the aforementioned information, this paper evaluated HSP, CIS, and the potential combination of the two (HSP + CIS) in the Detroit 562 cell line, aiming to determine whether the response to treatment is improved through association. Detroit 562 cells were selected as a 2D experimental model and are frequently used in scientific research [3].

3.1. Cellular Viability

To observe the cytotoxic potential of the samples, the first step was the determination of cell viability after 24 h of treatment using the MTT technique. The MTT test is one of the most widely used methods. It is based on the principle of transforming yellow MTT tetrazolium salt into purple formazan crystals. The colour change from yellow to purple gives a visual signal for cell viability. It is an indispensable tool for assessing metabolic activity and is used in areas such as toxicological research, cancer research, new drug discovery, and many others [19]. Initially, as illustrated in **Figure 1**, individual compounds were tested. Both HSP (5–100 μM) and CIS (1–20 μM) caused a dose-dependent decrease in viable cells. HSP at 5 μM reduced cell viability to 78%, while at the highest concentration tested (100 μM), viability decreased further to 57.29%. CIS, at the highest concentrations tested, 15 and 20 μM, significantly reduced the viability to 25% and 24%, respectively.

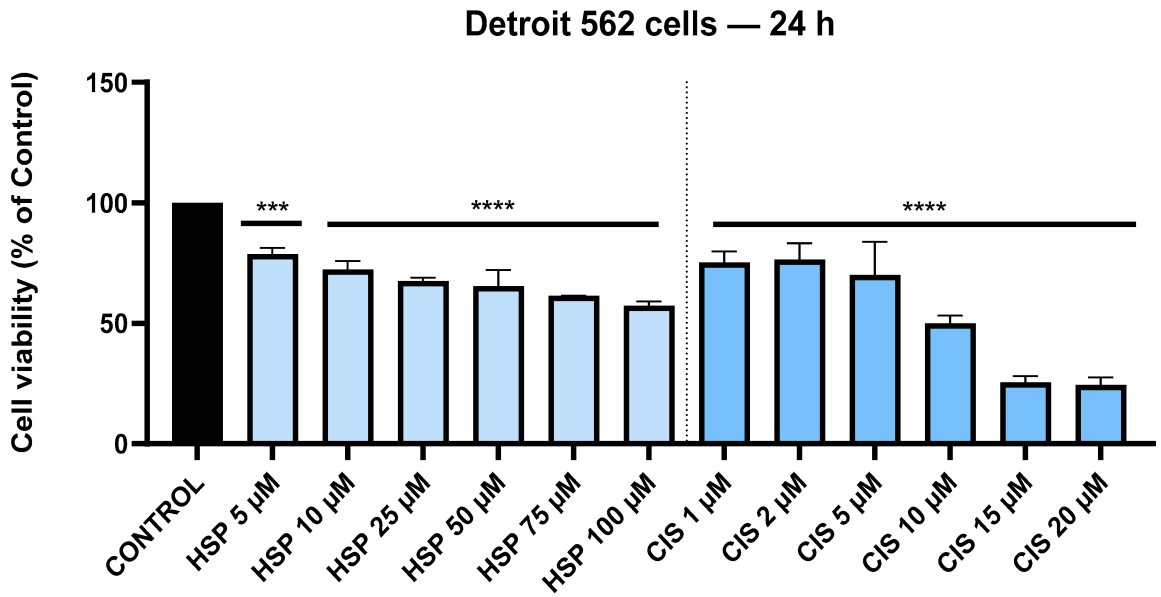

Figure 1. Graphical representations of the effect induced by HSP (5–100 µM) and CIS (1–20 µM) treatment on cell viability after 24 h on Detroit 562 cells. The results are expressed as percentages normalised to the control and are presented as mean values ± standard deviation. Statistical analysis and definitions of symbols are described in the Statistical Analysis Section (*** $p < 0.001$; **** $p < 0.0001$). Experiments were conducted in triplicate.

Subsequently, the combination of the two compounds was evaluated under the same conditions. When HSP 5 µM and CIS 1 µM, the lowest concentrations tested, were combined, cell viability reached almost 63%, followed by percentages such as 56%, 54%, 49%, and 29%, and at the highest concentrations tested in combination, represented by HSP 100 µM and CIS 20 µM, the cell viability percentage was reduced to 16%, as depicted in **Figure 2**.

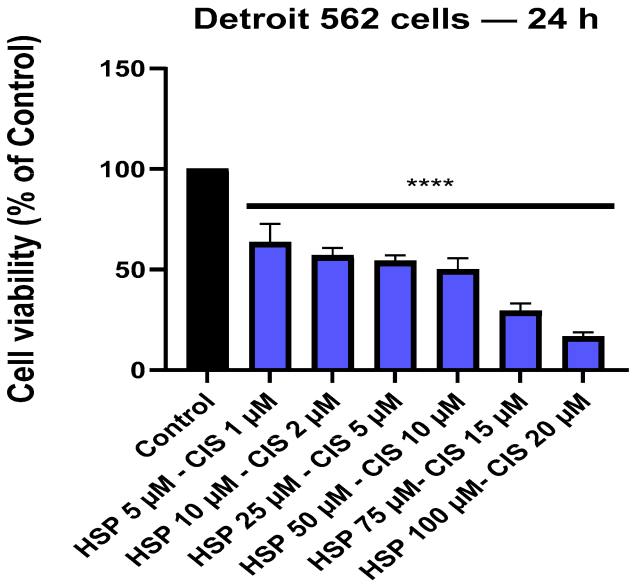

Figure 2. Graphical representations of the effect induced by the combinatorial treatment (HSP 5 µM + CIS 1 µM, HSP 10 µM + CIS 2 µM, HSP 25 µM + CIS 5 µM, HSP 50 µM + CIS 10 µM, HSP 75 µM + CIS 15 µM, HSP 100 µM + CIS 20 µM) on cell viability after 24 h on Detroit 562 cells. The results are expressed as percentages normalised to the control and are presented as mean values ± standard deviation. Statistical analysis and definitions of symbols are described in the Statistical Analysis Section (**** $p < 0.0001$). Experiments were conducted in triplicate.

3.2. Bright-Field Morphological Analysis

The next step was to evaluate the morphology of the cells 24 h after treatment of Detroit 562 cells. Microscopic examination of the cell morphology can collect important information regarding potential mechanisms of action and the specific models of cell death caused by the compounds under investigation [20].

As can be observed in **Figure 3**, treatment of cells with HSP decreases cell confluence in a dose-dependent manner. In the case of CIS, cell confluence also gradually decreases, but cellular dysmorphologies (cell shrinkage, cell rounding) appear upon application of 10 μM CIS, which are accentuated up to a dose of 20 μM. However, the most notable and frequent cellular structure abnormalities can be observed with the application of the combined HSP + CIS treatment, where, in addition to the massive, dose-dependent decrease in confluence, dysmorphologies such as cell rounding, cell shrinkage, and detachment from the plate are evident starting with the lowest concentrations of HSP 5 μM + CIS 1 μM. The combination treatment led to the most significant morphological changes compared to the control.

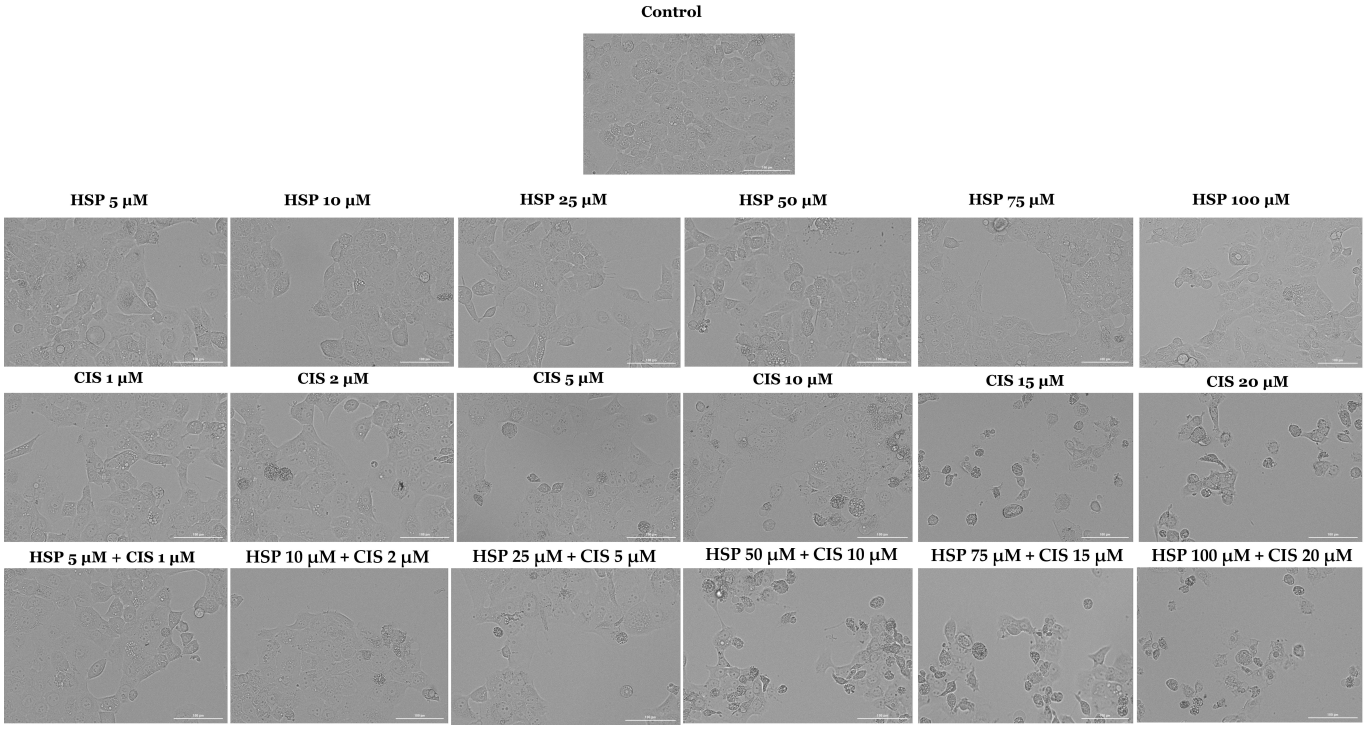

Figure 3. The morphological appearance of Detroit 562 cancer cells after 24 h treatment with HSP 5 μM, 10 μM, 25 μM, 50 μM, 75 μM, 100 μM; CIS 1 μM, 2 μM, 5 μM, 10 μM, 15 μM, 20 μM; and the combinatorial treatment HSP 5 μM + CIS 1 μM, HSP 10 μM + CIS 2 μM, HSP 25 μM + CIS 5 μM, HSP 50 μM + CIS 10 μM, HSP 75 μM + CIS 15 μM, HSP 100 μM + CIS 20 μM. Images were captured at 20× magnification, and the scale bar indicates 100 μm. Experiments were conducted in triplicate.

3.3. Nuclear and Mitochondrial Evaluation

The final step in the evaluation of the cytotoxic impact of HSP, CIS, and the combination HSP + CIS in Detroit 526 cells consisted of nuclear and mitochondrial assessments. The Hoechst method provides information on nuclear structure and behaviour, offering economic advantages, while MitoTracker tracks mitochondria [21]. Being the essential source of intracellular reactive oxygen species, mitochondria provide an important function in energy metabolism and control of stress responses. According to its multiple roles, altered mitochondrial function is associated with metabolic diseases, cancer, and other disorders associated with dysregulated cell death [20, 21]. The nucleus provides a necessary regulatory and control centre within the cell. Due to its capacity to guide many aspects of cellular functions, including DNA replication, transcription, and RNA processing, any deviation in nuclear morphology and structure can significantly affect cellular activities [22].

Thus, the mitochondrial and nuclear appearance after 24 h of treatment of Detroit 562 cells is illustrated in **Figure 4**. As can be seen, HSP did not induce notable signs of cytotoxicity at the nuclear level up to a concentration of 25 μM. Once this concentration was exceeded, the 50–100 μM range caused nuclear condensation and slight mitochondrial condensation. In the case of CIS, condensation can be observed in the nuclei and mitochondria starting at 2 μM, with a gradual decrease in confluence. The strongest signs of cytotoxicity are observed with the combined treatment of HSP + CIS, where alterations can be observed starting at the lowest dose applied and gradually progressing. Finally, at the highest concentrations, the nuclei are small and condensed, and the mitochondria have an altered appearance.

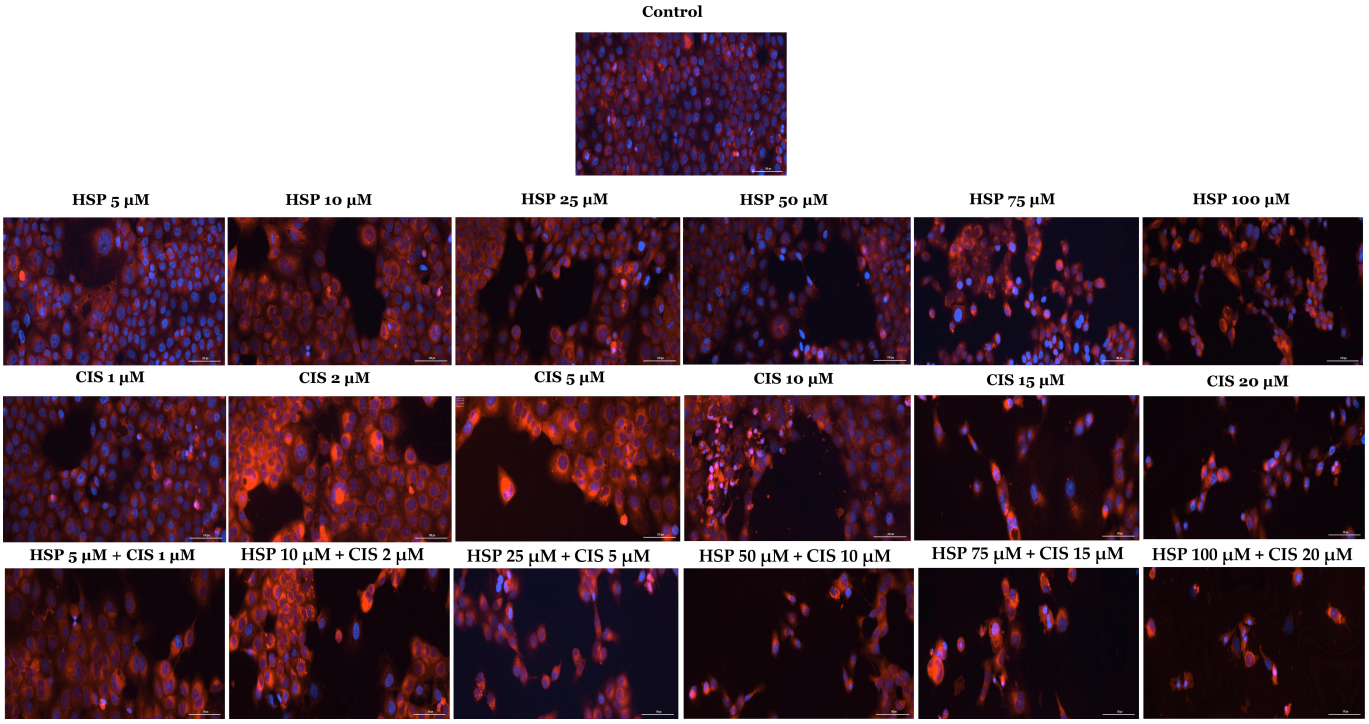

Figure 4. Representative images of the impact of HSP 5 -100 μM, CIS 1 -20 μM, and the combinatorial treatment HSP 5 μM + CIS 1 μM, HSP 10 μM + CIS 2 μM, HSP 25 μM + CIS 5 μM, HSP 50 μM + CIS 10 μM, HSP 75 μM + CIS 15 μM, and HSP 100 μM+ CIS 20 μM on mitochondria and nuclei morphology in Detroit 562 cells after 24 h. Images were captured at 20× magnification, and the scale bar was 100 μm. Experiments were conducted in triplicate.

Chemotherapy is currently the first line of treatment for cancer. Nevertheless, a significant challenge is posed by cancer resistance to chemotherapy, which affects patient well-being and treatment success. Given these considerations, identifying agents that sensitise tumour cells to chemotherapy is a developing and active area of research. Plant-based compounds have emerged as notable alternatives in this direction, helping to reduce side effects and sensitise tumours [23].

HSP is a botanical constituent that has repeatedly been shown to have anticancer properties and to improve results when combined with various chemotherapeutic agents. Along with CIS, through a delivery system, HSP improved the anti-cancer effect, increased apoptosis, and limited adverse effects in MDA-MB-231 cancer cells [24]. Korga et al. showed that both HSP and apigenin have the ability to enhance the toxic effects of doxorubicin, another chemotherapy agent, in HepG2 [25]. Dziedzic et al. investigated several flavonoids, including HSP (5–100 μM), in Detroit 562 cells. The results indicated that cell viability decreases with the applied dose. Furthermore, the same study showed that HSP stimulates cell death [26]. Another study identified that HSP suppresses the growth of oral cancer cells through apoptosis and mechanisms mediated by inflammatory signalling. It was found that treatment with HSP led to significantly lower levels of TNF-α, interleukin-1 beta (IL-1-β), IL-6, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and B-cell lymphoma 2 (Bcl-2), emphasising its inhibitory function in cell proliferation, migration, and inflammation processes. In parallel, hesperidin promoted BAX mRNA expression, suggesting an increase in cell death in the KB cell line [27]. Fatima Bijani and co-workers evaluated CIS together with crocin, a natural carotenoid, to examine the response to treatment in the HN5 cell line of human oral squamous cell carcinoma. According to the group's research, it was found that smaller doses of crocin, used in combination with cisplatin, along with the observed anticancer effect, can reduce the toxicity of cisplatin in healthy tissue [28]. Similarly, in epidermoid cancer cells, A431 HSP, CIS, and the combination of the two were examined. According to cell viability results, after 48 h of treatment, the combination of the two compounds produced the most notable effects, with the lowest percentage of viable cells. It was also observed that the combination improved and promoted apoptosis through mitochondrial-mediated intrinsic pathways, which suggested that HSP acts as a potential adjuvant for CIS by amplifying its pro-apoptotic effects and reducing its resistance in vitro [29].

Overall, in the present study, HSP and CIS individually showed dose-dependent cytotoxic effects on the Detroit 562 cell line. Notably, when the two compounds were combined, the results indicated that the cellular response was enhanced, with cytotoxicity being much more prominent. However, future research directions should include the elucidation of the exact molecular mechanism of action of the combination treatment, as well as examination in other experimental models

specific to OPSCC. Furthermore, this treatment option requires an assessment of biosafety in normal cell lines, as well as its potential irritant effect on tissues. By achieving these objectives in future lines of research, this type of combinatorial strategy may have the chance to be translated into clinical studies, based on proven preclinical results.

4. Conclusions

In conclusion, given that OPSCC is a global health problem, the current study highlighted a possible alternative for improving current treatments. Using a multimodal approach, the results indicated that the combined use of hesperidin and cisplatin improves therapeutic response, as indicated by reduced cancer cell viability through strategic association, greater cellular alteration, and more notable nuclear and mitochondrial changes compared to that of the compounds applied individually. However, all these data together support the need for further studies to elucidate the specific mechanisms of action of the combination between the natural and chemotherapeutic compounds, with the intention to translate the treatment strategy into the management of head and neck cancers.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

Data supporting these findings are available within the article or upon request.

Institutional review board statement

Not applicable.

Informed consent statement

Not applicable.

Additional information

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