RESEARCH ARTICLE

Editorial Process: Submission:06/25/2025 Acceptance:01/30/2026 Published:02/06/2026

Investigation of the Anticancer Effects and Senescence Induction of Hesperetin Combined with Cisplatin in Hepatocellular Carcinoma and Embryonic Fibroblast Cell Lines

Anif Nur Artanti[1,2,3], Riris Istighfari Jenie[3,4]*, Rumiyati Rumiyati[4], Adam Hermawan[4], Kato Jun-ya[5], Mila Hanifa[3]

Abstract

Objective: Hepatocellular carcinoma (HCC), the most common form of liver cancer, often develops in individuals with chronic liver diseases, especially cirrhosis. Cisplatin (Cisp), a chemotherapy agent commonly used in HCC treatment, is effective but is known to damage normal cells, including fibroblasts. Hesperetin (HST), a citrus flavanone found abundantly in citrus fruits, has demonstrated antioxidant, anti-inflammatory, and anticancer properties. This study aimed to investigate the synergistic cytotoxic effects and selective induction of senescence by HST in combination with Cisp in HepG2 cancer cells and NIH-3T3 fibroblast cells. **Methods:** The cytotoxic effects of HST were assessed using the MTT assay to determine cell viability. The antiproliferative properties were evaluated using colony formation assays. Senescence was assessed using SA-β-gal staining, while flow cytometry was used to analyze cell cycle distribution and apoptosis. Protein expression related to proliferation and apoptosis was determined via Western blot analysis. **Results:** MTT assay results indicated that both HST and Cisp reduced HepG2 cell viability in a dose-dependent manner, with IC_{50} values of 258 ± 2.47 μM and 5 ± 1.83 μM, respectively. Their combination (HST: 33–130 μM; Cisp: 0.6–2.5 μM) showed synergistic effects (combination index, CI < 1) co-treatment with HST (65 and 130 μM) significantly enhanced senescence in HepG2 cells. Clonogenic assays showed inhibition of colony formation, supported by reduced expression of p-ERK1/2 and Cyclin D1. Flow cytometry revealed increased apoptosis and G2/M phase arrest, with upregulation of Bax and caspase-3, and downregulation of Bcl-xL. In NIH-3T3 cells, HST showed minimal cytotoxicity (IC50 > 500 μM), and co-treatment with Cisp reduced senescence markers. **Conclusion:** These results suggest that HST and Cisp co-treatment synergistically reduces cancer cell viability while protecting normal fibroblasts from senescence, supporting its potential as a co-chemotherapeutic agent in HCC treatment, while also serving as a protective agent against senescence in healthy tissues.

Keywords: hesperetin- cisplatin- HCC- senescence- fibroblast

Asian Pac J Cancer Prev, **27 (2)**, 557-568

Introduction

Hepatocellular carcinoma (HCC) represents the predominant form of primary liver cancer and ranks among the top global causes of cancer mortality [1]. The Asian Pacific region has an HCC prevalence of approximately 530,000 [2]. Current chemotherapeutic agents, such as cisplatin (Cisp), effectively induce cytotoxicity in cancer cells. Clinical applications extend beyond HCC to include other types of hepatic tumors. However, their lack of selectivity often results in severe side effects and toxicity in healthy tissues [3]. Thus, there is a growing demand for novel therapeutic strategies that enhance the efficacy of existing treatments on HCC while minimizing adverse effects on non-cancerous cells. Hesperetin (HST), a naturally occurring flavonoid found in citrus fruits, has garnered significant attention for its wide-ranging pharmacological properties, including anti-inflammatory, antioxidant, and anticancer activities [4]. Recent studies suggest that HST may potentiate the effects of conventional chemotherapeutics and exhibit selective action on cancer cells. HST exerts anticancer effects through multiple mechanisms, including the induction of apoptosis, cell cycle arrest, and the modulation of

*[1]Doctoral Program in Pharmaceutical Science, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. [2]Department of Pharmacy, Vocational College, Universitas Sebelas Maret, Indonesia. [3]Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. [4]Laboratory of Macromolecular Engineering, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. [5]Laboratory of Tumor Cell Biology, Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, Nara 630-0192, Japan. *For Correspondence: riris_jenie@ugm.ac.id*

oncogenic pathways (p-AMPK, p-Akt, p-mTOR) [5, 6]. It also attenuates oxidative stress, suppresses NF-κB, inhibits EMT/MMP-9, induces ferroptosis, and acts as a chemosensitizer, with co-treatment studies showing synergistic cytotoxicity and selective senescence induction in TNBC cells [7–11]. Multiple studies have reported that HST enhances the efficacy of cisplatin through diverse mechanisms, including the induction of apoptosis (*AIF, Bax,* and *cleaved caspase-9/3* upregulation, and *Bcl-2* downregulation), activation of *PTEN*, and inhibition of the *PI3K/AKT* signaling pathway in gastric cancer models [12]. In triple-negative breast cancer, HST–cisplatin co-delivery enhanced anti-tumor and anti-metastatic effects while reducing toxicity [13]. In 4T1 models, it demonstrated synergistic cytotoxicity and protected kidney cells from cisplatin-induced senescence [11]. In cisplatin-resistant lung cancer, HST restored sensitivity by inhibiting P-glycoprotein-mediated drug efflux [14]. Collectively, these findings highlight HST as a promising co-chemotherapeutic agent that enhances efficacy and reduces adverse effects. This study aims to evaluate the cytotoxic effect and induction of cellular senescence by co-treatment with Cisp and HST in both cancerous HepG2 cells, representing the HCC cell line, and non-cancerous NIH-3T3 cells, representative of non-cancerous fibroblasts. In the study, we analyzed cell proliferation, senescence, apoptosis, cell cycle progression, and the molecular expression of *Bax*, *Caspase-3*, *Bcl-xL*, *p-ERK1/2, ERK1/2,* and *Cyclin D1* in *HepG2* cells. We examined cell viability and senescence in NIH 3T3 cells to assess selective senescence induction. By elucidating the differential responses of these cell lines, this research seeks to provide insights into the potential of HST as a co-treatment for enhancing the selectivity and effectiveness of Cisp in cancer treatment.

Materials and Methods

Cell culture

The HepG2 hepatocellular carcinoma cell line (ATCC® HB-8065) and NIH-3T3 fibroblasts (ATCC® CRL-1658) were obtained from the Cancer Chemoprevention Research Center (Yogyakarta, Indonesia). Cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (Sigma-Aldrich, USA), 1% penicillin/streptomycin (Gibco), and 0.5% Fungizone (Corning) at 37°C in a humidified 5% CO_2 atmosphere. Cultures were monitored every 3–4 days for media changes and subcultured at 80–90% confluence using 0.25% trypsin-EDTA dissociation reagent. Hesperetin (Sigma) and cisplatin (Wako) were the compounds used in the study.

Cell Proliferation Assessment via MTT Assay

The cell viability assay was performed using the MTT tetrazolium reduction method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, USA), a colorimetric microculture technique, as described by Mosmann. In brief, cells at a density of 10,000 per well were treated with varying concentrations of HST or Cisp and incubated for 24 hours. Following treatment, 100 µL of MTT solution (0.5 mg/mL; Biovision) was added to each well and incubated for 2-4 hours. The reaction was terminated with an SDS solution in 0.01N HCl. Absorbance measurements were taken at 595 nm using an ELISA microplate reader, with cell viability expressed as a percentage relative to untreated controls. The half-maximal inhibitory concentration (IC_{50}) was calculated through linear regression analysis of dose-response curves [15].

Colony Formation Assay

Cells were plated at 500 cells per well in 24-well plates and cultured for 24 hours before HST, Cisp, and co-treatment with Cisp and HST. Following 24-hours drug exposure, the medium was replaced with fresh complete medium, and cells were maintained for 8 days to allow colony formation. Colonies were then fixed with 4% paraformaldehyde, stained with 0.5% (w/v) crystal violet, and quantified using ColonyArea image analysis software [16].

Senescence-associated β-galactosidase (SA-βGal) Senescence-based Assay

NIH-3T3 and HepG2 cells were seeded at 2×10^4 cells/well in 24-well plates and allowed to attach for 24 hours. Cells were then treated with HST at 10-500 µM, Cisp at 0.01-10 µM, or co-treatment with HST and Cisp for 24 hours before proceeding to the senescence assay. Following treatment, cells were washed twice with PBS, fixed, and incubated with X-Gal staining solution at 37 °C for 72 hours. Senescent cells were subsequently identified as blue-stained cells under light microscopy (Olympus CKX-41, 200× magnification) [17].

Cell Cycle Profile Analysis

Cell cycle analysis was conducted following a previously published method with some modifications. Briefly, cells were seeded, incubated, and exposed to HST at 130 µM, Cisp at 3 µM, or to a co-treatment of HST and Cisp for 24 hours. After treatment, cells were detached using trypsin to achieve a single-cell suspension. Cells are fixed with cold 70% ethanol, washed, then incubated with staining solution containing 100 µg/mL PI, 50 µg/mL RNase A, and 0.1% Triton X-100 in PBS for 30 min at room temperature in the dark before flow cytometry analysis. Flow cytometry analysis was performed using the CytoFlex system to determine the distribution of cells across sub-G1, G1, S, and G2/M phases of the cell cycle [18].

Apoptosis Profile Analysis

HepG2 cells (2×10^5 cells/well in 6-well plates) were treated with test compounds (single or combination therapy) for 24 hours. Cells were then collected, washed in binding buffer, and dual-stained using Annexin V-FITC/PI from a commercial apoptosis detection kit (Roche, Mannheim). After 5-minute dark incubation at RT, samples were immediately analyzed by Beckman Coulter CytoflexS flow cytometry to quantify apoptotic populations [19].

Western Blot

Western blot analyses were performed using the Protein Simple Abby System®. The lysates were diluted with 0.1× sample buffer and then combined at a 4:1 ratio with 5× fluorescent master mix, which contained 5× sample buffer, 5× fluorescent standard, and 200 mM DTT, followed by the addition of a blocking reagent. Primary antibodies were used at a 1:100 dilution. The analysis began by loading the plate for electrophoresis and immunodetection, with molecular weight standards monitored using a biotinylated ladder [20]. WB analysis of the protein bands was performed using [SW Compass software]. The intensity of each target protein band was normalized against the corresponding loading control (β-actin). Ratios were then calculated relative to the untreated control. The fold changes of the treated groups are presented accordingly.

Statistical analysis

Data represent the mean ± standard error of mean (SEM). After initial analysis in SPSS 21.0, one-way ANOVA with Tukey's HSD post hoc test (GraphPad Prism 9.0) identified statistically significant differences versus untreated controls (*p≤0.05, **p≤0.01, ***p≤0.001).

Results

Effect of HST and Cisp on the Viability of HepG2 and NIH-3T3 Cells

The study aims to investigate the impacts of HST and Cisp on various cancer characteristics and their preference for normal cells in a laboratory setting. Initially, we assessed the harmful effects of HST and Cisp on HepG2 and NIH-3T3 cells over 24 hours. HepG2 cells simulated hepatocellular carcinoma (HCC), while NIH-3T3 cells represented normal fibroblasts. HST, at concentrations between 20 and 500 μM, progressively decreased HepG2 cell viability, with an IC_{50} of 258 μM (Figure 1), indicating its relatively modest cytotoxicity. Conversely, HST did not affect NIH-3T3 cell viability at concentrations up to 500 μM. These findings yielded a selectivity index (SI) greater than 2, indicating that HST exerts preferential cytotoxic effects on malignant cells while exhibiting minimal toxicity toward normal fibroblasts. The SI was defined as the ratio of the IC_{50} value in normal cells (NIH-3T3) to the IC_{50} value in cancer cells (HepG2). The resulting SI value exceeded 2, suggesting that HST exerts selective cytotoxicity against cancer cells while demonstrating minimal toxicity toward normal fibroblasts. We also revised the figure (Figure 1).

Co-treatment Effect of HST with Cisp on Cell Viability of HepG2 cells

In single cytotoxicity assays, HST showed promising potential despite its relatively low cytotoxicity. We then examined its combined cytotoxic effects with Cisp to explore potential synergistic interactions against HCC (Figures 2A and 2B). The findings revealed that co-treatment of HST and Cisp demonstrated strong synergistic effects in inhibiting HepG2 cell growth, with a combination index (CI) value of less than 1 (Figure 2C). Combination therapy is often used in cancer treatment to enhance therapeutic efficacy and minimize systemic toxicity. A key principle underlying this approach is the synergistic effect, where the combined action of two agents produces greater anticancer activity than the sum of their individual effects. In the case of HST and Cisp, co-treatment has been reported to result in synergistic inhibition of various cancer cell growth. Such synergy is typically quantified using models such as the combination index (CI) method proposed by Chou–Talalay, where a CI value < 1 indicates synergy, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism [21]. In this study, we evaluated a range of HST (33–130 μM) and Cisp (0.7–2.8 μM) concentrations in HepG2 cells. The most substantial synergistic effect was observed at HST 130 μM combined with cisplatin 2.8 μM, as evidenced by a combination index (CI) value of less than 1.

HST co-treatment with Cisp suppressed the cell colony in HepG2 cells

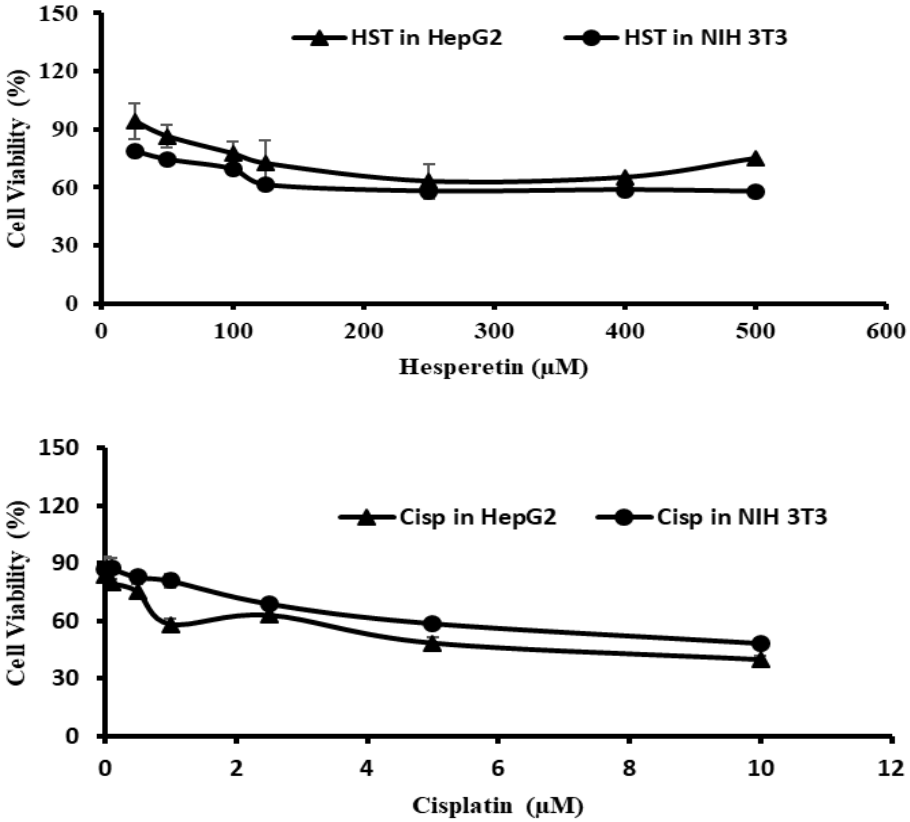
Hepatocytes grown in vitro can retain their ability to replicate and remain differentiated, but only for a limited period. The colony-forming or clonogenic assay is a quantitative laboratory technique used to evaluate a single cell's capacity to grow and form a colony through clonal expansion. This method is widely used to determine the effectiveness of different cytotoxic compounds. Results from the clonogenic assay demonstrated that the combination of HST and Cisp (Figures 3A and 3B) significantly decreased the survival of HepG2 cells compared to untreated controls after 8 days of treatment. The number of colonies in each treatment condition was counted and normalized to the control group (set as 100%). The results are now presented as percentage colony formation relative to the control.

Senescence Effect of HST co-treatment with Cisp on HepG2 cells

Recent findings demonstrate that several anticancer agents can induce cellular senescence, highlighting its potential as a novel approach in cancer prevention and treatment. In this study, we investigated the impact of combining HST and Cisp on the induction of senescence in cancer cells. Observations were conducted after 24 hours of treatment. The results revealed that co-treatment with HST and Cisp at concentrations of 130 μM and 3 μM, respectively, led to a significant increase in senescence in HepG2 cells (Figures 4A and 4C). Senescence was quantified by counting β-galactosidase–positive cells and expressing the results as a percentage of the total cell population.

HST co-treatment with Cisplatin induction G2/M arrest in HepG2 cells

The cytotoxic effects of HST, Cisp, and co-treatment of HST with Cisp were further evaluated by analyzing cell cycle profiles. The results indicated an increase in G0/G1 arrest in untreated cells (Figure 5A). Additionally, HST treatment led to an elevated G0/G1 arrest in HepG2 cells compared with Cisp single treatment. HCC cells were divided into four treatment groups: control, 130 μM

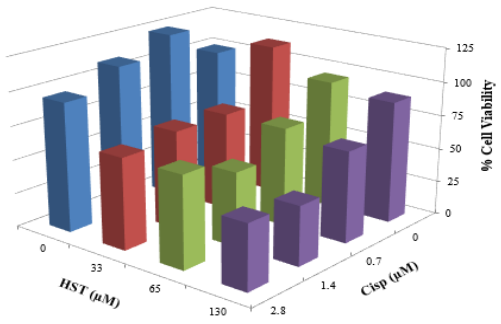


Cell line	IC_{50} value (µM) and selectivity index (SI)			
		HST	Cisp	SI
HepG2	IC_{50} (µM)	258 ± 0.11	5.83 ± 0.34	>2
NIH 3T3	IC_{50} (µM)	>500	6.54 ± 1.04	

Figure 1. Cytotoxicity of HST on HepG2 and NIH 3T3 Cells. HepG2 cells (1×10^4 cells/mL) and NIH 3T3 (1×10^4 cells/mL) were seeded in 96 well-plate and treated with Hesperetin and Cisplatin for 24 hours, and subsequently measured using MTT assay. Data were means from three independent experiments ± standard error (SE).

HST, 3 µM cisplatin, and a combination of 130 µM HST with 3 µM cisplatin. After 24 hours, in untreated cells, an accumulation in the G0/G1 phase was observed (Figure 5A). Treatment with HST (130 µM) markedly increased G0/G1 arrest (65.30 ± 2.63%) compared with Cisp (31.64 ± 0.44%) and the combination group (32.25 ± 1.53%). In

A



B

HST (µM)	Cisp (µM)	CI
33	0.6	0.51
33	1.2	0.60
33	2.5	0.86
65	0.6	0.51
65	1.2	0.17
65	2.5	0.29
130	0.6	0.46
130	1.2	0.06
130	2.5	0.05

Figure 2. Cytotoxicity Effect of HST Co-Treatment with Cisp on HepG2 Cells. (A) HepG2 cells (1×10^4 cells/mL) were seeded in 96 well-plate and treated with HST for 24 hours and subsequently measured using MTT assay. Cell viability of HepG2 with the treatment of HST sub-doses (33, 65, and 130 µM) combined with Cisp sub-doses (0.6, 1.2, and 2.5 µM). The combination treatment showed a decrease in cell viability compared to a single treatment of each compound. (B). A combination Index value of HST and Cisp.

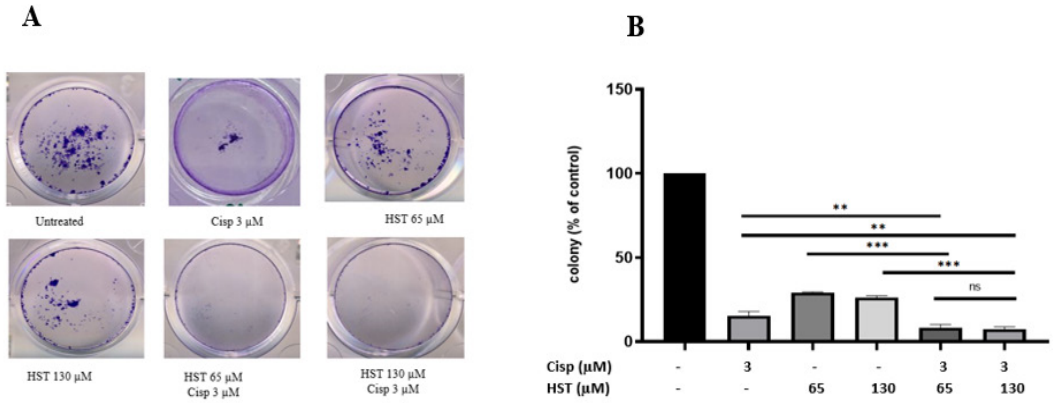


Figure 3. Effects of HST and Cisp Treatment Inhibit Colony Formation on HepG2 Cells. The surviving colonies were counted and analyzed with ImageJ, and the results are shown as mean±SD (n=3). (B) Quantification of HepG2 cells undergoing % colony. *: p ≤0.05 ; ** : p≤0.01; *** : p≤0.001 ; ns : not significant p ≥0.05.

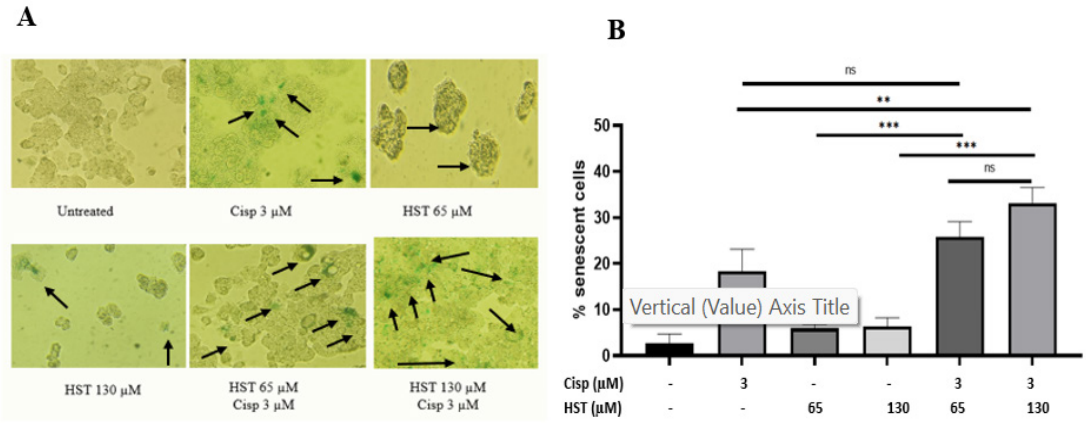


Figure 4. Effects of HST and Cisp Treatment on HepG2 Senescent Cells. (A) Cells are experiencing senescence. The green colour show β-galactosidase-positive cells, which are expressed in senescent cells. (B) Quantification of HepG2 cells undergoing senescence. *: p ≤0.05 ; ** : p≤0.01; *** : p≤0.001 ; ns : not significant p ≥0.05.

contrast, low-dose Cisp (3 µM) induced S-phase arrest (35.77 ± 2.31%). Interestingly, when HST was combined with Cisp, the proportion of cells in S phase was reduced (28.79 ± 1.58%), while a significant increase in G2/M phase arrest was detected (33.21 ± 0.90%). These findings indicate that while HST primarily drives G0/G1 arrest, its combination with Cisp shifts the arrest toward the G2/M phase, reflecting a distinct mechanism of action in

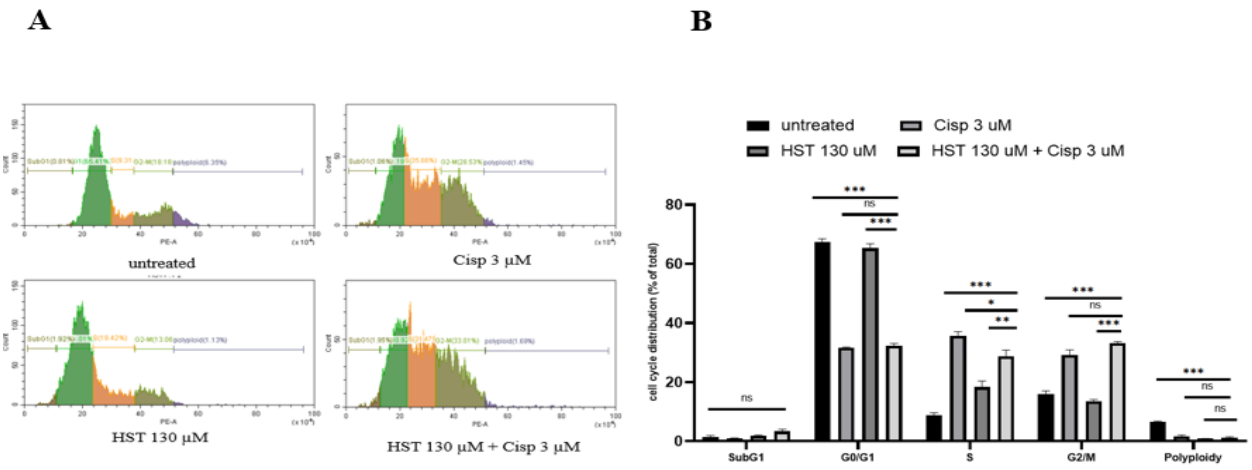


Figure 5. The Cell Cycle Distribution of HepG2 Cells after 24 hours of Treatment. (A) The cell cycle profile of HepG2 cells treated for 24 hours with half the IC50 concentrations of HST, Cisp and their combination. The cells were stained with PI solution and analyzed by flow cytometry. (B) The phase-wise distribution of HepG2 cells within the cell cycle is also shown. *: p ≤0.05 ; ** : p≤0.01; *** : p≤0.001 ; ns : not significant p ≥0.05.

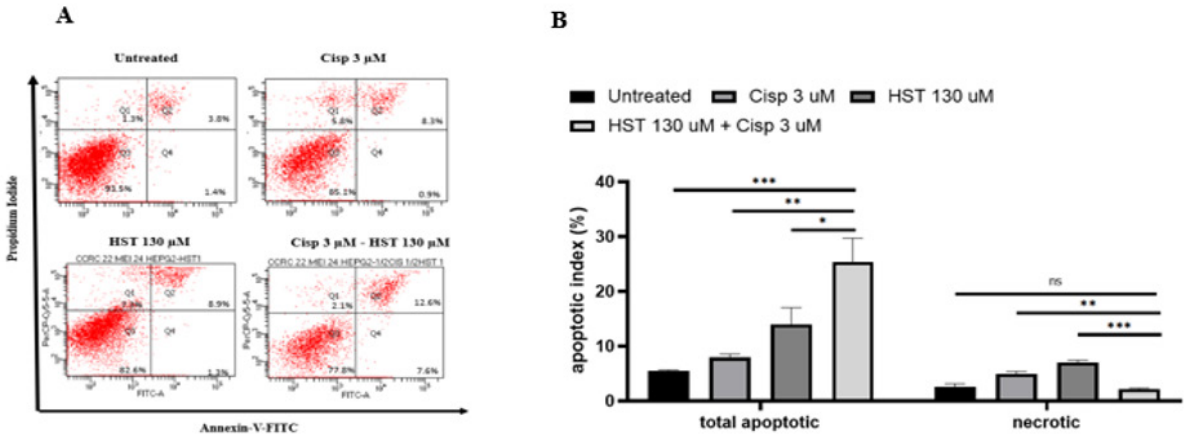


Figure 6. The Apoptosis Profile of HepG2 Cells Following 24 Hours of Treatment. (A) The impact of ½ IC_{50} concentrations of HST, Cisp, and their combination on HepG2 cells after 24 hours. The cells were treated, stained with PI reagent, and analyzed using flow cytometry. (B) The analysis results demonstrate the extent of apoptosis induced by each treatment in HepG2 cells. *: p ≤0.05 ; ** : p≤0.01; *** : p≤0.001 ; ns : not significant p ≥0.05.

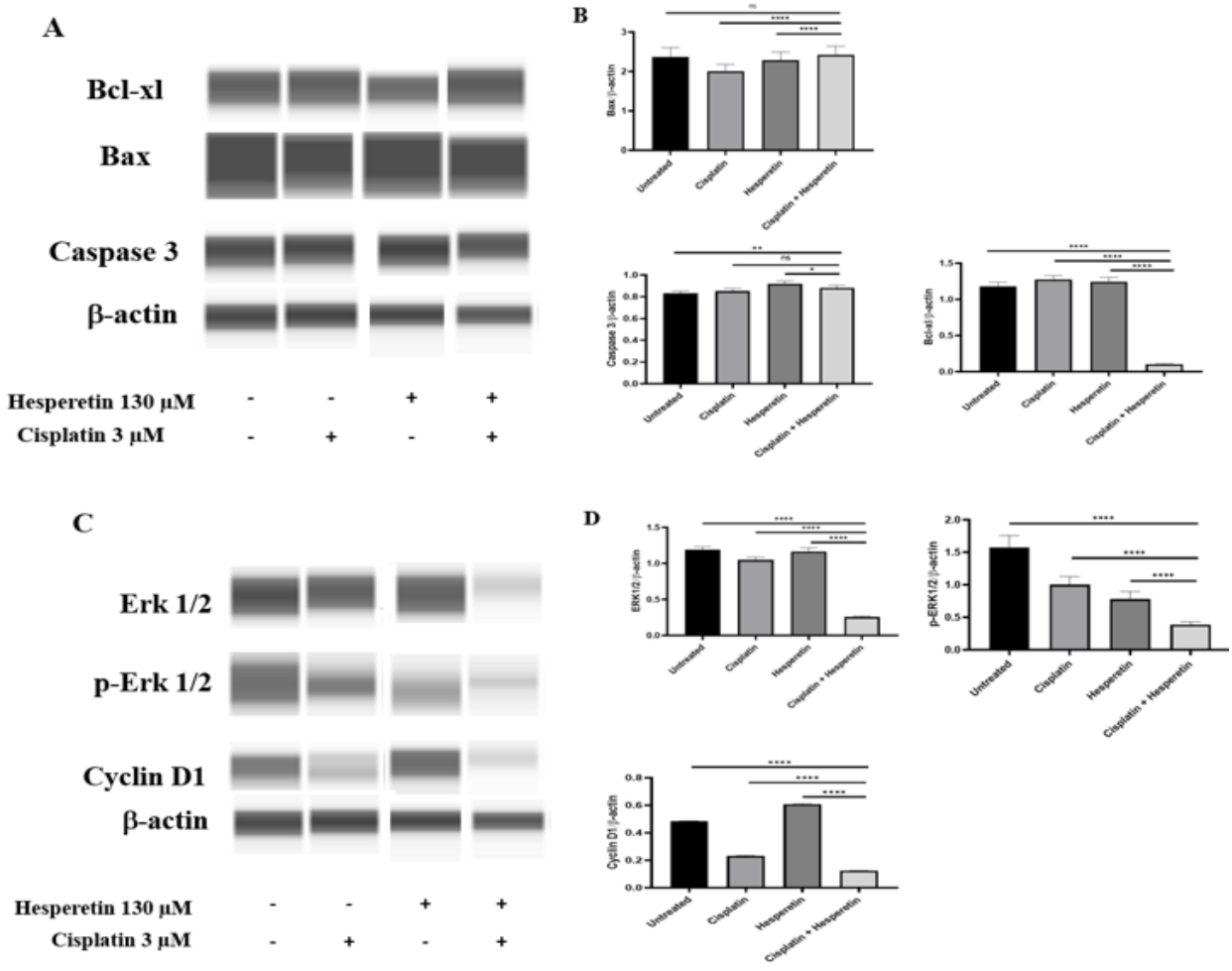
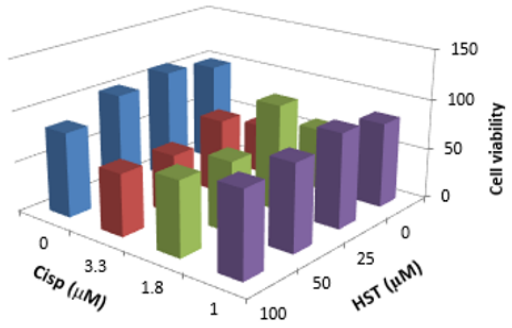


Figure 7. The Western Blot of HepG2 Cells after 24 Hours of Treatment. (A) The protein expression (Bcl-xl, Bax, caspase-3*)* profile of HepG2 cells treated for 24 hours with half the IC_{50} concentrations of HST, Cisp, and their combination. (B) The analysis results demonstrate the protein expression (Bcl-xl, Bax, caspase-3) induced by each treatment in HepG2 cells, based on experiments performed in triplicate. C) The protein expression profile (ERK 1/2, p-ERK1/2, cyclin-D1) of HepG2 cells treated for 24 hours with half the IC_{50} concentrations of HST, Cisp, and their combination. (B) The analysis results demonstrate the protein expression (ERK 1/2, p-ERK1/2, cyclin-D1) induced by each treatment in HepG2 cells, based on experiments performed in triplicate.*: p ≤0.05;** : p< 0.01;***: p < 0.001 ;**** : p < 0.0001; ns : not significant p ≥0.05. β-actin served as a loading control. The intensity of *Bcl-xl, ERK 1/2, p-ERK1/2*, and *Cyclin D1* was quantified by ImageJ.

A



B

HST (µM)	Cisp (µM)	CI
25	3.3	13.6
25	1.8	4.33
25	1	3.62
50	3.3	10.11
50	1.8	3.13
50	1	14.95
100	3.3	22.16
100	1.8	9.65
100	1	17.08

Figure 8. Cytotoxicity Effect of HST Co-Treatment with Cisp on NIH 3T3 Cells. A. NIH 3T3 cells (8×10^3 cells/mL) were seeded in 96 well-plate and treated with HST for 24 hours and subsequently measured using MTT assay. Cell viability of NIH 3T3 with the treatment of HST sub-doses (25, 50, and 100 µM) combined with Cisp sub-doses (3.3, 1.8, and 1 µM). The combination treatment showed increased cell viability compared to a single treatment of each compound. (B). A combination Index value of HST and Cisp.

co-treated HCC cells.

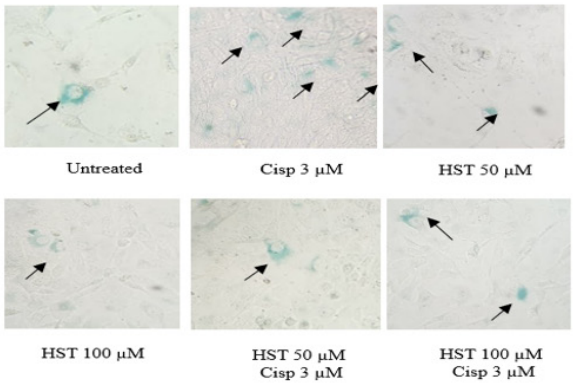
HST co-treatment with Cisp induces apoptosis in HepG2 cells

The impact of co-treatment with HST and Cisp on cell apoptosis was analyzed using flow cytometry following sample preparation and staining with annexin V and PI. This technique enabled the distinction between early apoptotic, late apoptotic, and necrotic cells. Exposure to 3 µM Cisp led to a 4.98% increase in apoptotic cells compared to the untreated group. A single treatment with 130 µM HST caused a 13.07% increase, while the co-treatment of HST and Cisp led to a 19.57% increase in apoptosis (Figure 6B). The co-treatment of HST and Cisp enhanced cell apoptosis compared to the single treatment of HST or Cisp, although the proportion of living cells remained high, above 80%. Based on the cytotoxicity assay, the co-treatment of HST and Cisp effectively induced apoptosis. In brief, the combination caused necrosis in a small number, which was not significantly different from untreated cells, indicating that the combination dominantly induced HepG2 cell death through apoptosis. In addition, we emphasized that HST co-treatment with Cisp markedly enhanced apoptotic cell death in HepG2 cells, which corresponded with a pronounced reduction in cell viability, as shown in Figures 2 and 6.

Co-treatment of HST with cisplatin altered the expression of multiple proteins associated with apoptosis and cell proliferation.

In this study, we investigated the potential interaction between HST, Cisp, and their combination on proteins involved in the apoptotic process using western blot analysis. Notably, co-treatment with HST and Cisp induced apoptosis by downregulating *Bcl-xL* proteins and upregulating *Bax* and *Caspase-3* proteins (Figure 7A, 7B).. The combination of HST and Cisp also inhibited cell survival in HepG2 cells by decreasing ERK1/2, p-ERK1/2, and *Cyclin D1* protein levels (Figure 7C,7D). These findings suggest a molecular mechanism underlying the co-treatment effects of HST and Cisp.

A



B

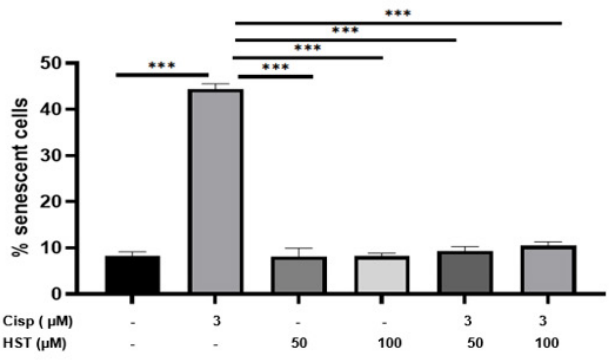


Figure 9. Effects of HST and Cisp Treatment on NIH 3T3 Senescent Cells. (A) Cells are experiencing senescence. The arrows show β-galactosidase-positive cells, which are expressed in senescent cells. (B) Quantification of NIH 3T3 cells undergoing senescence. *: p ≤0.05 ; ** : p≤0.01; *** : p≤0.001 ; ns : not significant p ≥0.05.

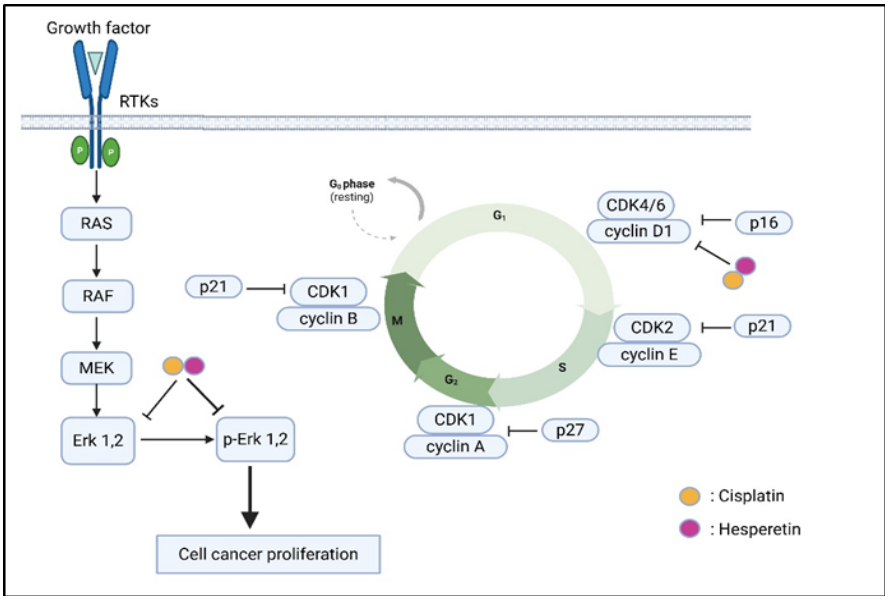


Figure 10. The Illustration Mechanism of HepG2 Cells after Hesperetin (HST) Treatment with Cisplatin and HST is Related to the Proliferation Protein Expression.

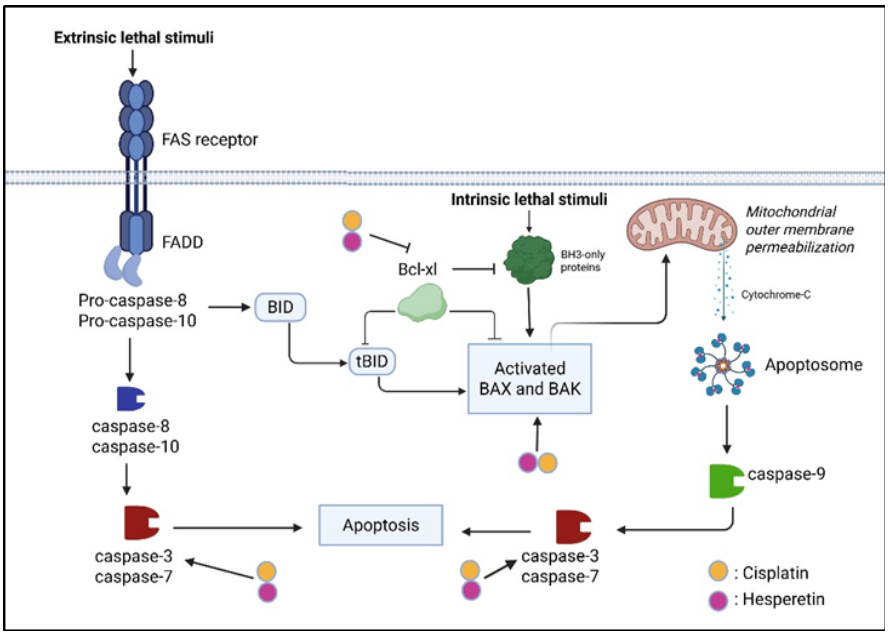


Figure 11. The Illustration Mechanism of HepG2 Cells after Hesperetin (HST) Treatment with Cisplatin and HST is Related to Apoptosis Protein Expression

Co-treatment Effect of HST with Cisp on Cell Viability of NIH-3T3 cells

We also tested the combination of HST with Cisp on normal fibroblast cells, even though HST showed weak cytotoxicity. Unlike their cytotoxic effects on HepG2 cells, HST demonstrated an antagonistic effect when combined with Cisp in NIH-3T3 cells (Figures 8A and 8B). This combination inhibited cell growth, with a combination index (CI) value greater than 1 (Figure 6C).

Senescence effect of HST co-treatment with Cisp on NIH-3T3 cells

Cisplatin (Cisp) is known to produce side effects that can cause cellular damage, potentially affecting fibroblast cells. To explore this, we examined its ability to induce senescence in normal fibroblasts using NIH-3T3 cells. Cisp treatment significantly increased the percentage of β-galactosidase–positive cells, indicating senescence induction ($p<0.01$). However, when HST was combined with Cisp, there was no significant change in the number of β-galactosidase-positive cells compared to the untreated control (Figures 9A and 9B). These findings suggest that HST may offer protective effects against Cisp-induced senescence in normal fibroblasts cells.

Discussion

The HST is recognized for its cytotoxic impact on various cancer cell cultures. Investigating HST as a potential anticancer agent offers promising prospects in cancer therapy due to its minimal effects on normal kidney cells [11] and against UVA-induced photodamage in human dermal fibroblast cells [22]. Therefore, the effectiveness of HST should be optimized through co-treatment with chemotherapy compounds, as it can reduce the side effects of the agent. In the present study, we demonstrated that HST enhances the anticancer activity of cisplatin against HepG2 cells, primarily through synergistic effects that induce apoptosis, cell cycle arrest, and senescence induction. The observed reduction in cisplatin-induced S-phase arrest and the concomitant increase in G2/M arrest following co-treatment suggest that HST modulates cisplatin-mediated DNA damage responses. These findings are consistent with previous reports, such as those by He et al. (2020), who demonstrated that hesperetin enhances cisplatin-induced apoptosis in gastric cancer by upregulating *PTEN* and suppressing the AKT signaling pathway. These comparisons reinforce the translational potential of HST and Cisp as a co-chemotherapeutic strategy, particularly given our observed selectivity for cancer cells over normal cells. Cisplatin, a commonly used drug for HCC treatment, is highly reactive and causes DNA damage by binding to DNA, interfering with the cell cycle, and triggering apoptosis. However, its clinical application is limited by severe side effects, including peripheral neuropathy and nephropathy [23].

Tumor progression in HCC is influenced by genetic and molecular alterations within cancer cells and the tumor microenvironment (TME). Fibroblasts, as a key component of the TME, play significant roles in HCC development and progression [24]. This study demonstrated that a single treatment with HST resulted in an IC_{50} value of 258 ± 0.11 μM, while Cisp had an IC_{50} value of 5.83 ± 0.34 μM in HepG2 cells after 24 hours of treatment (Figure 1). However, although HST exhibits a moderately potent cytotoxic effect compared to Cisp, its combination with Cisp produces a synergistic cytotoxic effect. The combination assay revealed that co-treatment with HST and Cisp exhibited greater toxicity against HepG2 cancer cells compared to single treatments. HST enhanced the effectiveness of Cisp by demonstrating a synergistic effect, as indicated by a Combination Index (CI<1) (Figure 2). Consistent with previous studies, HST has been shown to enhance the anticancer activity of Cisp against 4T1 cells [11], gastric cells, and lung cancer [12]. These findings support the potential of HST as a co-treatment agent with Cisp. Based on this rationale, we selected 3 μM Cisp and 130 μM HST for subsequent experiments. Thus, we used 3 μM Cisp and 130 μM HST for further evaluation. The combination treatment was applied for 24 hours, after which the medium was replaced with a compound-free medium for the remaining 8 days. This treatment regimen resulted in a marked suppression of colony formation in HepG2 cells, with the inhibitory effect being significantly greater than that observed in cells treated with Cisp alone (Figure 3). These results indicate that HST potentiates the long-term cytotoxic effect of Cisp, thereby reducing the clonogenic survival of hepatocellular carcinoma cells. Although several other concentration combinations demonstrated CI < 1, we chose these concentrations because they represent the lowest effective doses that still exhibited a consistent synergistic effect, while minimizing cytotoxicity to normal fibroblast (NIH-3T3) cells. Using sub-toxic concentrations is particularly relevant for translational purposes, as it reflects a clinically desirable strategy to reduce cisplatin-associated adverse effects while preserving or enhancing its anticancer activity.

The possible mechanisms underlying the synergistic anticancer effects were then investigated. Previous reports have shown that low-dose cisplatin can suppress the expression of G0/G1-phase proteins in HCC cells [25], whereas HST has been reported to induce G1-phase arrest in lung cancer cells [26]. In brief, our present study indicated that cell cycle modulation is not the only mechanism underlying the synergistic anticancer effects of HST and cisplatin. The combination also involved other mechanisms, including apoptosis and cell senescence, that orchestrated with the cell cycle arrest, causing the cells to experience apoptosis or be permanently arrested (senescence). The effect of the combination at sub-IC_{50} concentrations is significantly greater than the application of a single treatment, indicating that the combination may increase the efficacy toward the cancer cells and reduce the toxicity in normal cells (fibroblast NIH 3T3 cells).

Apoptosis is intricately linked to cytotoxic effects and the regulation of cell cycles. HST prevents the phosphorylation of ERK1/2, and NF-κB in microglial cells [25]. HST enhances cisplatin-induced apoptosis in gastric cancer by upregulating *PTEN* expression [12] and lung cancer [27]. Hence, HST and its combination may induce apoptosis in HepG2 cells. Our findings showed that co-treatment of HST with cisplatin at concentrations of 130 μM and 3 μM enhanced apoptosis compared to untreated cells and cisplatin single treatment (Figure 6). This indicates that HST acts as a chemosensitizer, amplifying the pro-apoptotic effect of cisplatin in HepG2 cells. However, apoptosis alone may not fully account for the overall cytotoxicity observed. To further investigate additional mechanisms contributing to growth inhibition, we examined cellular senescence. Interestingly, our results confirmed that cisplatin alone can induce senescence in HepG2 cells, and the combination treatment further influenced this effect. Cells that have already undergone senescence may still be detected as viable cells because the apoptosis detection method we used was based on the phosphatidyl serine labelling with Annexin-V.

On the other hand, we also showed that the combination significantly increases cell senescence and cell cycle arrest. Therefore, we suggest that the viable cells in the apoptosis assay are not entirely healthy. Still, some of the cell populations were arrested and/or senescent, as shown in other figures. Our findings showed that co-treatment of HST with cisplatin at concentrations of 130 μM and 3 μM significantly enhanced apoptosis compared to untreated controls and cisplatin monotherapy (Figure 6). This indicates that HST acts as a chemosensitizer, amplifying

the pro-apoptotic effect of cisplatin in HepG2 cells. However, apoptosis alone may not fully account for the overall cytotoxicity observed. To further investigate additional mechanisms contributing to growth inhibition, we examined cellular senescence. Interestingly, our results confirmed that cisplatin alone can induce senescence in HepG2 cells, and the combination treatment further influenced this effect. The co-treatment of HST and cisplatin demonstrated a synergistic effect in promoting senescent cell formation in HepG2 cells. The ability of certain cancer chemotherapies to trigger stress-induced senescence in cancer cells is a relatively new yet crucial approach for enhancing treatment efficacy [28].

Furthermore, the selective elimination of senescent cells, known as 'senolysis,' is gaining interest as an adjunct therapeutic strategy. While inducing senescence can suppress tumor growth, the prolonged presence of senescent cells may contribute to a pro-tumorigenic environment over time due to chronic production of the senescence-associated secretory phenotype (SASP). Therefore, targeting and removing these cells after senescence induction could enhance therapeutic outcomes by preventing cancer recurrence and reducing side effects. Studies have shown that therapy-induced senescence can sensitize cancer cells to other treatments, making senescence-based strategies a promising avenue for advancing anticancer therapies [29].

Hepatocellular carcinoma (HCC) arises from transformed hepatocytes in the progression of chronic liver diseases. As transformed hepatocytes proliferate in vivo, they are exposed to various microenvironmental stresses, including apoptosis. Therefore, apoptosis resistance may confer a selective advantage for the growth and progression of HCC. The molecular mechanisms underlying the synergistic effect of HST and cisplatin represent one of the strongest points of our study. Our findings indicate that the combination promotes apoptosis, modulates cell cycle progression, and enhances senescence induction in HepG2 cells. These effects suggest that HST not only sensitizes cells to cisplatin but also shifts the balance of cellular responses from survival toward programmed cell death and irreversible growth arrest. Previous studies have reported similar mechanisms for flavonoid–cisplatin co-treatments. For example, hesperetin enhanced cisplatin-induced apoptosis in gastric cancer cells by upregulating *PTEN* and suppressing the *PI3K/AKT* pathway [12]. In lung cancer, HST was shown to modulate autophagy and sensitize cells to cisplatin [27]. Comparable synergistic effects have also been described for quercetin with cisplatin in hepatocellular carcinoma, where the combination promoted DNA damage and apoptotic signaling [30].

By integrating our data with these earlier findings, it becomes evident that HST acts through multiple complementary mechanisms, including the modulation of survival pathways (*PI3K/AKT*), mitochondrial apoptosis, and possibly autophagy, to enhance cisplatin efficacy. Therefore, our study extends current knowledge by demonstrating that HST and cisplatin co-treatment exerts multifaceted molecular effects in hepatocellular carcinoma cells, which may explain its pronounced anticancer potential. The *Bcl-xL* protein was highly expressed in HepG2 cell lines, whereas *Bcl-2* was not expressed in any of the cells [31]. We examined the expression and function of *Bcl-xL*, which shares antiapoptotic properties similar to those of *Bcl-2*. Our data showed that *Bcl-xL* expression was significantly decreased in 70.6% of HCC cells following co-treatment with Cisp and HST, compared to both the untreated group and the Cisp single treatment. *Bcl-xL* is well known for its role in regulating apoptosis, so a reduction in its expression could significantly affect HCC cells (Figure 11). The combination of HST and Cisp significantly increased the expression of *Bax* and *Caspase-3* proteins, indicating the activation of apoptotic pathways. *Bax*, a pro-apoptotic member of the *Bcl-2* family, plays a crucial role in mitochondrial-mediated apoptosis, while *Caspase-3* serves as a primary executioner caspase in the apoptotic cascade. The enhanced expression of these proteins suggests that the combination therapy may potentiate apoptosis in HepG2 cells more effectively than either treatment alone. However, it is also possible that other apoptosis-related regulators, such as caspase-8 and caspase-9, are involved in the observed effects, acting as initiator caspases in the extrinsic and intrinsic pathways, respectively. Further investigation is needed to elucidate the complete network of apoptotic signaling triggered by this combination. The chemopreventive properties of HST against different types of cancer are widely recognized, although the molecular mechanisms behind its effects remain unidentified. *Cyclin D1* is a crucial regulatory factor in the transition from G1 to S phase during cell cycle progression. It is well-established that *Cyclin D1* is overexpressed in liver cancers [23]. Therefore, we investigated the chemopreventive effects of HST and its co-treatment with Cisp on *Cyclin D1* expression, as well as the signaling pathways that regulate *Cyclin D1* in HepG2 cells. Western blot analysis revealed that co-treatment with HST and Cisp reduced *Cyclin D1* expression and activity in HepG2 cells, indicating that the growth inhibitory effect of this co-treatment is linked to the downregulation of cell proliferation pathways (Figure 10).

Furthermore, it is well-established that *Cyclin D1* expression is typically regulated by various pathways, such as the Ras-mediated mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) pathway [24]. Our data showed that cells treated with a single HST exhibited an increase in ERK activity, indicating a potential sensitization to apoptosis. In this study, we report that HST inhibits the growth of HepG2 cells by reducing *Cyclin D1* expression and decreasing the expression and activity of the p-ERK1/2 pathways, which results in degenerative changes (Figure 7). These findings suggest that HST holds significant potential as a co-chemopreventive agent against liver cancer, and further studies are warranted. In contrast, NIH-3T3 fibroblast cells displayed lower sensitivity to the HST and Cisp combination. While a certain level of stress response was observed, including mild senescence markers (Figure 8), the overall viability of NIH-3T3 cells remained higher compared to that of HepG2 cells. The recent study reported that HST exhibits protective effects against oxidative stress and demonstrates no cytotoxicity in HK-2 cells [32-34].

This differential effect suggests that HST may provide a protective buffer for non-cancerous cells against the toxicity of Cisp; such selectivity is crucial in reducing the side effects commonly associated with chemotherapy.

In summary, the combination of HST and Cisp demonstrates significant anticancer potential against HepG2 cells while showing reduced cytotoxicity in normal fibroblasts. This selective induction of senescence could pave the way for more targeted and less toxic chemotherapeutic approaches. Further studies are essential to elucidate the underlying mechanisms and translate these findings into in vivo studies.

Author Contribution Statement

All authors contributed equally in this study.

Acknowledgements

General

We appreciate the support of the BPI (Indonesian Education Scholarship), Center for Higher Education Funding and Assessment, Ministry of Higher Education, Science, and Technology of the Republic of Indonesia, and LPDP (Indonesia Endowment Fund for Education). We also thank Professor Yoshitaka Hippo, MD, PhD, director of the Chiba Cancer Center Research Institute, Japan, for providing laboratory facilities for the Western blot analysis.

Ethical Declaration

This study does not involve human participants or animals. Therefore, it does not require approval from an institutional review board or ethics committee.

Data Availability

All data supporting the findings of this study are included within the manuscript and its supplementary materials.

Conflict of Interest

The Authors declare that there is no conflict of interest

References

1. Wen N, Cai Y, Li F, Ye H, Tang W, Song P, et al. The clinical management of hepatocellular carcinoma worldwide: A concise review and comparison of current guidelines: 2022 update. Biosci Trends. 2022;16(1):20-30. https://doi.org/10.5582/bst.2022.01061.
2. Toh MR, Wong EYT, Wong SH, Ng AWT, Loo LH, Chow PK, et al. Global epidemiology and genetics of hepatocellular carcinoma. Gastroenterology. 2023;164(5):766-82. https://doi.org/10.1053/j.gastro.2023.01.033.
3. Hamaya S, Oura K, Morishita A, Masaki T. Cisplatin in liver cancer therapy. Int J Mol Sci. 2023;24(13). https://doi.org/10.3390/ijms241310858.
4. Yang AY, Choi HJ, Kim K, Leem J. Antioxidant, antiapoptotic, and anti-inflammatory effects of hesperetin in a mouse model of lipopolysaccharide-induced acute kidney injury. Molecules. 2023;28(6):2759. https://doi.org/10.3390/molecules28062759.
5. Roohbakhsh A, Parhiz H, Soltani F, Rezaee R, Iranshahi M. Molecular mechanisms behind the biological effects of hesperidin and hesperetin for the prevention of cancer and cardiovascular diseases. Life Sci. 2015;124:64-74. https://doi.org/10.1016/j.lfs.2014.12.030.
6. Lin CY, Chen YH, Huang YC. Hesperetin induces autophagy and delayed apoptosis by modulating the ampk/akt/mtor pathway in human leukemia cells in vitro. Curr Issues Mol Biol. 2023;45(2):1587-600. https://doi.org/10.3390/cimb45020102.
7. Sohel M, Sultana H, Sultana T, Al Amin M, Aktar S, Ali MC, et al. Chemotherapeutic potential of hesperetin for cancer treatment, with mechanistic insights: A comprehensive review. Heliyon. 2022;8(1):e08815. https://doi.org/10.1016/j.heliyon.2022.e08815.
8. Wang J, Chen H, Hu Z, Ma K, Wang H. Hesperetin regulates transforming growth factor-β1/Smads pathway to suppress epithelial-mesenchymal transition-mediated invasion and migration in cervical cancer cell. Anti-Cancer Drugs. 2021 Oct 1;32(9):930-8.
9. Lv Y, Liu Z, Deng L, Xia S, Mu Q, Xiao B, et al. Hesperetin promotes bladder cancer cells death via the *PI3K/AKT* pathway by network pharmacology and molecular docking. Sci Rep. 2024;14(1):1009. https://doi.org/10.1038/s41598-023-50476-8.
10. Wang Y.S, Chen WJ, Mo ZY., He YJ, Nie K. Therapeutic Potential of the Citrus Flavonoid Hesperidin and Its Aglycone Hesperetin against Chemotherapy-Induced Toxicity; 2025.
11. Artanti A, Jenie R, Rumiyati R, Meiyanto E. Cytotoxic activity and senescence modulatory effect of hesperetin on triple-negative breast cancer cells and kidney cells co-treatment with cisplatin. Indonesian Journal of Cancer Chemoprevention. 2024;14:181. https://doi.org/10.14499/indonesianjcanchemoprev14iss3pp181-188.
12. He P, Ma J, Liu Y, Deng H, Dong W. Hesperetin promotes cisplatin-induced apoptosis of gastric cancer in vitro and in vivo by upregulating *PTEN* expression. Front Pharmacol. 2020;11:1326. https://doi.org/10.3389/fphar.2020.01326.
13. Wang X, Song Y, Yu L, Xue X, Pang M, Li Y, et al. Co-delivery of hesperetin and cisplatin via hyaluronic acid-modified liposome for targeted inhibition of aggression and metastasis of triple-negative breast cancer. ACS Appl Mater Interfaces. 2023;15(29):34360-77. https://doi.org/10.1021/acsami.3c03233.
14. Kong W, Ling X, Chen Y, Wu X, Zhao Z, Wang W, et al. Hesperetin reverses p-glycoprotein-mediated cisplatin resistance in ddp-resistant human lung cancer cells via modulation of the nuclear factor-κb signaling pathway. Int J Mol Med. 2020;45(4):1213-24. https://doi.org/10.3892/ijmm.2020.4485.
15. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1-2):55-63. https://doi.org/10.1016/0022-1759(83)90303-4.
16. Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. Nat Protoc. 2006;1(5):2315-9. https://doi.org/10.1038/nprot.2006.339.
17. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (sa-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nat Protoc. 2009;4(12):1798-806. https://doi.org/10.1038/nprot.2009.191.
18. Pozarowski P, Darzynkiewicz Z. Cell cycle analysis in cancer research: Recent advances and future directions. Cancers. 2023;15(2), 456.
19. Koopman G, Reutelingsperger CP, Van Heerde W.L. Annexin

V-based assays for detection of apoptosis in flow cytometry. In J. R. W. Masters (Ed.), Methods in Molecular Biology. 2021; Vol. 2255.
20. Zhang L, Wang X, Chen Y, Li H. High-throughput protein quantification in cancer cells using the Abby system. Journal of Cancer Research Methods. 2022; 15(3).
21. Chou TC. Drug combination studies and their synergy quantification using the chou-talalay method. Cancer Res. 2010;70(2):440-6. https://doi.org/10.1158/0008-5472.Can-09-1947.
22. Lu Z, Xia Q, Cheng Y, Lu Q, Li Y, Zeng N, et al. Hesperetin attenuates uva-induced photodamage in human dermal fibroblast cells. J Cosmet Dermatol. 2022;21(11):6261-9. https://doi.org/10.1111/jocd.15230.
23. Acklin S, Zhang M, Du W, Zhao X, Plotkin M, Chang J, et al. Depletion of senescent-like neuronal cells alleviates cisplatin-induced peripheral neuropathy in mice. Scientific Reports. 2020;10(1):14170. https://doi.org/10.1038/s41598-020-71042-6.
24. Tahmasebi Birgani M, Carloni V. Tumor microenvironment, a paradigm in hepatocellular carcinoma progression and therapy. Int J Mol Sci. 2017;18(2). https://doi.org/10.3390/ijms18020405.
25. Liu FY, Wu YH, Zhou SJ, Deng YL, Zhang ZY, Zhang EL, et al. Minocycline and cisplatin exert synergistic growth suppression on hepatocellular carcinoma by inducing s phase arrest and apoptosis. Oncol Rep. 2014;32(2):835-44. https://doi.org/10.3892/or.2014.3248.
26. Ferreira de Oliveira JMP, Santos C, Fernandes E. Therapeutic potential of hesperidin and its aglycone hesperetin: Cell cycle regulation and apoptosis induction in cancer models. Phytomedicine. 2020;73:152887. https://doi.org/10.1016/j.phymed.2019.152887.
27. Liu FY, Wu YH, Zhou SJ, Deng YL, Zhang ZY, Zhang EL, Huang ZY. Minocycline and cisplatin exert synergistic growth suppression on hepatocellular carcinoma by inducing S phase arrest and apoptosis. Oncology reports. 2014 Aug 1;32(2):835-44.
28. Cai X, Guillot A, Liu H. Cellular senescence in hepatocellular carcinoma: The passenger or the driver? Cells. 2022;12(1):132. https://doi.org/10.3390/cells12010132.
29. Saleh T, Bloukh S, Carpenter VJ, Alwohoush E, Bakeer J, Darwish S, et al. Therapy-induced senescence: An "old" friend becomes the enemy. Cancers (Basel). 2020;12(4). https://doi.org/10.3390/cancers12040822.
30. Zhao JL, Zhao J, Jiao HJ. Synergistic growth-suppressive effects of quercetin and cisplatin on hepg2 human hepatocellular carcinoma cells. Appl Biochem Biotechnol. 2014;172(2):784-91. https://doi.org/10.1007/s12010-013-0561-z.
31. Hu W, Fu J, Lu SX, Liu LL, Luo RZ, Yun JP, et al. Decrease of *Bcl-xL*/*Bcl-2*-associated death promoter in hepatocellular carcinoma indicates poor prognosis. Am J Cancer Res. 2015;5(5):1805-13.
32. Parekh P, Motiwale L, Naik N, Rao KV. Downregulation of *Cyclin D1* is associated with decreased levels of p38 map kinases, akt/pkb and pak1 during chemopreventive effects of resveratrol in liver cancer cells. Exp Toxicol Pathol. 2011;63(1-2):167-73. https://doi.org/10.1016/j.etp.2009.11.005.
33. Wu Y, Tang W, Cao Y, Jiang D, Zhao L, Zhao J, et al. A *Cyclin D1*-specific single-chain variable fragment antibody that inhibits hepg2 cell growth and proliferation. Biotechnol J. 2020;15(8):e1900430. https://doi.org/10.1002/biot.201900430.
34. Chen X, Wei W, Li Y, Huang J, Ci X. Hesperetin relieves cisplatin-induced acute kidney injury by mitigating oxidative stress, inflammation and apoptosis. Chem Biol Interact. 2019;308:269-78. https://doi.org/10.1016/j.cbi.2019.05.040.