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Effect of naringin and cisplatin combination on cell viability and cell death in bladder cancer cells

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ABSTRACT: Bladder cancer is a prevalent malignancy characterized by high recurrence rates and limited therapeutic options, particularly due to resistance and toxicity associated with cisplatin therapy. Bladder cancer remains a significant global health concern, and while cisplatin is a cornerstone of treatment, its efficacy is often limited by resistance and toxicity. Therefore, there is a critical need for novel agents that can enhance cisplatin's effects while mitigating its drawbacks. This study investigates the potential of naringin, a natural flavonoid, to exhibit antiproliferative and pro-apoptotic effects in human bladder cancer cell lines (HTB-9 and HT-1376), both as a monotherapy and in combination with cisplatin. Cytotoxicity was assessed via the MTT ((3-(4,5-dimetiltiazol-2-il)-2,5 difeniltetrazoliumbromid) assay, and apoptosis was evaluated using Annexin V/PI staining and caspase 3/7 activation assays. Results demonstrated that naringin reduced cell viability in a dose-dependent manner in both cell lines. When combined with cisplatin, naringin significantly enhanced the antiproliferative and pro-apoptotic effects compared to either treatment alone. Caspase 3/7 activity was markedly elevated in the combination groups, indicating an amplified apoptotic response. These findings suggest that naringin can potentiate cisplatin's efficacy and could serve as a promising adjunctive therapy in bladder cancer treatment. Further studies are warranted to explore the underlying mechanisms and potential clinical applications of naringin in enhancing cisplatin-based chemotherapy.

KEYWORDS: Apoptosis; bladder cancer; caspase 3/7; cisplatin; naringin; HTB-9; HT-1376

1. INTRODUCTION

Bladder cancer is one of the most prevalent malignancies of the urinary system, characterized by high recurrence rates and complex biological behaviors [1,2]. Bladder cancer is the 10th most commonly diagnosed cancer worldwide. In 2020, approximately 573,000 new cases and 213,000 deaths were reported [3]. Despite advancements in diagnostic and therapeutic approaches, the five-year survival rate for advanced-stage bladder cancer remains dismally low, emphasizing the urgent need for more effective and less toxic treatment strategies [3]. Chemotherapy, particularly with cisplatin, a platinum-based chemotherapeutic agent, remains a cornerstone for treating advanced bladder cancer. Cisplatin exerts its effects primarily by inducing apoptosis through DNA crosslinking, which disrupts DNA repair mechanisms and triggers cell cycle arrest. Additionally, cisplatin induces mitochondrial dysfunction by disrupting the mitochondrial membrane potential, leading to the release of cytochrome c and activation of the intrinsic apoptotic pathway. This dual mechanism of action—targeting both nuclear DNA and mitochondria—makes cisplatin a potent chemotherapeutic agent, but it also contributes to its dose-limiting toxicities, such as nephrotoxicity and neurotoxicity [4, 5]. Recent studies have also highlighted cisplatin's role in generating reactive oxygen species (ROS), which further exacerbates cellular damage and promotes apoptosis through oxidative stress pathways [6]. However, its clinical application is often limited by significant side effects and the emergence of chemoresistance, necessitating the exploration of complementary or alternative therapeutic options [5, 7].

In this context, naturally occurring compounds has emerged as a promising candidate due to their therapeutic potential and relatively favorable safety profiles. Naringin, a flavonoid predominantly found in citrus fruits, has shown promise due to its multifaceted pharmacological activities, including anti-inflammatory, antioxidant, and anticancer effects [6-8]. Notably, previous studies have demonstrated that naringin can induce apoptosis and inhibit the proliferation of various cancer cell lines, suggesting its

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potential as an adjunctive agent in cancer therapy. For instance, naringin has been shown to induce apoptosis and inhibit proliferation in liver, breast, and gastric cancer cells, suggesting its potential as an adjunctive agent in cancer therapy [9-12]. Despite these findings in other cancers, there is a lack of comprehensive studies exploring naringin's potential in bladder cancer, particularly its impact on key signaling pathways and cellular processes unique to this malignancy. This gap highlights the need for further research to elucidate naringin's mechanisms and therapeutic potential in bladder cancer. Unlike traditional chemotherapeutics, naringin exhibits unique mechanisms of action that may offer synergistic effects when combined with cisplatin.

This study investigates the effect of naringin and its combination with cisplatin on cell death, cell viability, and proliferation in different stages of bladder cancer cells (HTB-9 grade 2, HT-1376 grade 3) [13]. By analyzing the apoptotic and anti-proliferative effects of each treatment individually and in combination, the research aims to uncover potential synergistic interactions and the underlying molecular pathways involved. The findings from this study could provide a foundation for integrating natural compounds like naringin with conventional chemotherapy, contributing to the development of more effective therapeutic strategies with reduced toxicity for bladder cancer.

2. RESULTS

2.1. Naringin inhibits bladder cancer cells growth

HTB-9, and HT-1376 human bladder cancer cells was treated with naringin at 0, 5, 10, 25, 50 and 100 µg/ml doses. The incubation time was 24 and 48 hours. IC50 was found to be 37.66 µg/ml, and 44.58 µg/ml at 24 hours and 32.98 µg/ml and 30.38 µg/ml at 48 hours for HTB-9 and HT-1376 cells, respectively. Cisplatin IC50 value was 11.05 µM, 14.85 µM at 24 h and 8.78 µM, 10.05 µM for HTB-9 and HT-1376 cells, respectively. According to the MTT results, the viability percentages at 0, 5, 10, 25, 50 and 100 µg/ml concentrations for HTB-9 cells treated with naringin were 100%, 98.16%, 72.15%, 65.79%, 41.5%, 24.73% for 24 hours and 100%, 95.9%, 86.7%, 56.78%, 30.4%, 26.78% for 48 hours, respectively. The viability percentages at 0, 5, 10, 25, 50 and 100 µg/ml concentrations for HT-1376 cells treated with naringin were 100%, 92.1%, 73.87%, 57.94%, 48.95%, and 35.62% for 24 hours and 100%, 85.73%, 75.23%, 42.55%, 38.16%, and 36.08% for 48 hours, respectively (Figure 1). The percentage of cell viability in HTB-9 and HT-1376 cell lines under varying concentrations of cisplatin and naringin over 24-hour and 48-hour treatment periods. Cell viability in HTB-9 cells decreased as cisplatin concentration increased. After 24 hours, viability drops significantly at higher concentrations (≥5 µM), with the decline continuing at 48 hours, where the viability at 50 µM is very low, indicating high cytotoxicity over time (Figure 1a). Similar to HTB-9, HT-1376 cells show decreased viability with increasing cisplatin concentration. The reduction is notable at concentrations of 5 µM and higher. At 48 hours, cell viability is lower than at 24 hours, indicating an accumulation of cytotoxic effects over time (Figure 1c).

For HTB-9 cells treated with naringin, cell viability remains relatively high at lower concentrations (5-10 µg/mL) for both 24 and 48 hours. However, at concentrations above 25 µg/mL, cell viability begins to decline, especially at 48 hours, suggesting that naringin has a delayed cytotoxic effect at higher doses (Figure 1a, and 1b). HT-1376 cells demonstrate a slight reduction in viability at higher naringin concentrations (≥25 µg/mL) after 24 hours, with a more pronounced decrease at 48 hours. At concentrations of 50 µg/mL and 100 µg/mL, viability is significantly reduced, indicating increased cytotoxicity over longer exposure (Figure 1c, and 1d). Both cell lines (HTB-9 and HT-1376) exhibit dose- and time-dependent reductions in viability with both cisplatin and naringin treatments. Cisplatin shows stronger immediate cytotoxic effects compared to naringin, while naringin's effects is more gradual, especially noticeable at higher doses and longer treatment durations.

2.2. The combination of naringin and cisplatin provides stronger apoptotic effects on HTB-9 and HT-1376 cells

Effects of naringin and the naringin + cisplatin combination on apoptosis in HTB-9 and HT-1376 cells were evaluated using flow cytometry after 24 and 48 hours incubation. In HTB-9 cells, the control group showed a high percentage of viable cells (mean value 99.06%), with minimal apoptotic cells, indicating the healthy state of the untreated cells. Treatment with naringin for 24 hours reduced the viable cell rate to 77.81% and significantly increased the percentage of early and late apoptotic cells, suggesting an initiation of apoptosis. After 48 hours of naringin treatment, the viable cell rate further decreased to 34.86%, with a notable increase in the percentage of early apoptotic cell population, indicating that prolonged exposure to

naringin intensifies the apoptotic response (p<0.0001) (Figure 2 a,b). In the combination treatment (naringin + cisplatin), the apoptotic effect was even more pronounced. After 24 hours, the viable cell rate dropped to 40.27%, with substantial increases in both percentage of early and late apoptotic cells. By 48 hours, the viable cell population remained low (19.125%), and the percentage of early apoptotic cells continued to increase, underscoring the enhanced apoptotic effect of combining naringin with cisplatin over time.

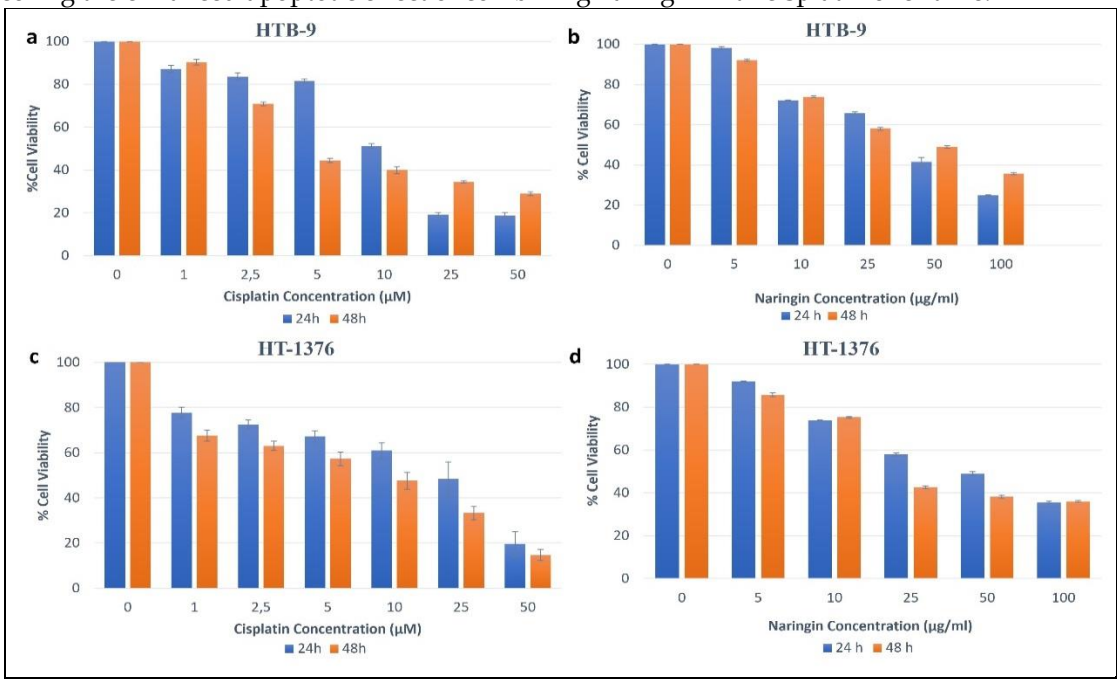

Figure 1. Cell viability of HTB-9 and HT-1376 bladder cancer cells treated with varying concentrations of cisplatin and naringin for 24 and 48 hours. (a) Percentage of viable HTB-9 cells after treatment with cisplatin. (b) Percentage of viable HTB-9 cells after treatment with naringin. (c) Percentage of viable HT-1376 cells after treatment with cisplatin. (d) Percentage of viable HT-1376 cells after treatment with naringin. Cell viability was assessed using the MTT assay. Data are presented as mean ± SD from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to the control group.

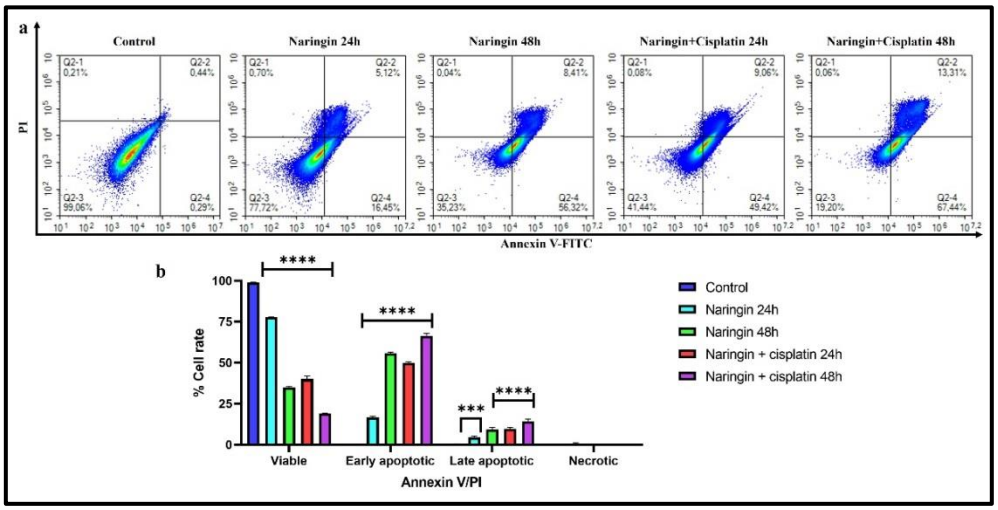

Figure 2. Flow cytometry analysis of apoptosis in HTB-9 bladder cancer cells treated with naringin, cisplatin, or their combination for 24 and 48 hours. (a) Representative flow cytometry plots showing the percentages of viable, early apoptotic, late apoptotic, and necrotic cells. (b) Bar graph summarizing the percentages of viable, early apoptotic, and late apoptotic cells. Data are presented as mean ± SD from three independent experiments. ****p < 0.0001, ***p < 0.001 compared to the control group.

A similar pattern was observed in HT-1376 cells. The control group maintained a high viability rate (95.31%), with only minor apoptotic cell populations. After 24 hours of naringin treatment, the viable cell rate decreased to 70.19%, accompanied by an increase percentage of early apoptotic cells. Extending the naringin treatment to 48 hours led to a further reduction in viability (57.15%) and a higher percentage of late apoptotic cells, indicating a time-dependent apoptotic effect of naringin on HT-1376 cells (Figure 3 a,b, p<0.0001).

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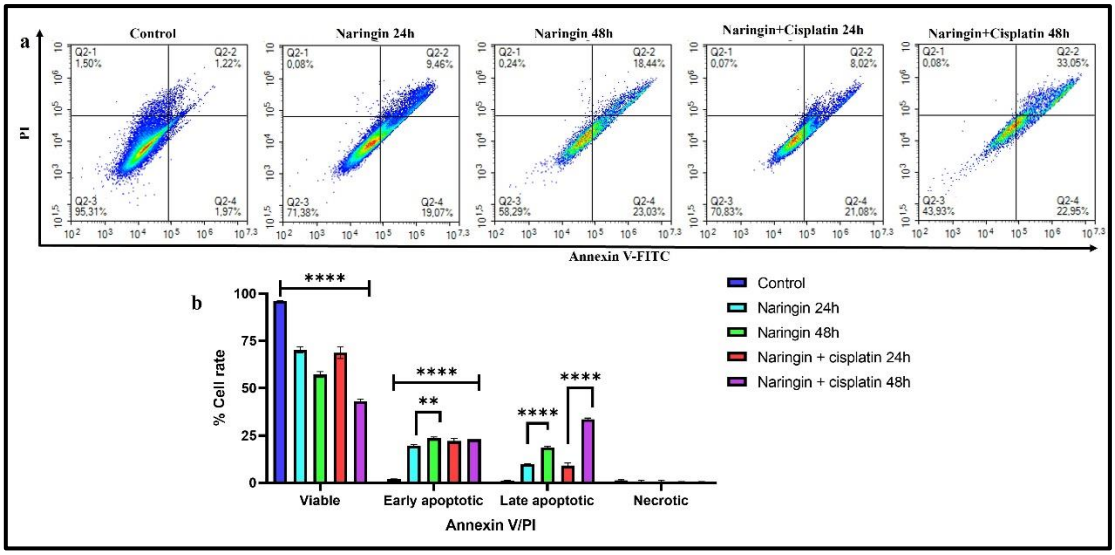

Figure 3. Flow cytometry analysis of apoptosis in HT-1376 bladder cancer cells treated with naringin, cisplatin, or their combination for 24 and 48 hours. (a) Representative flow cytometry plots showing the percentages of viable, early apoptotic, late apoptotic, and necrotic cells. (b) Bar graph summarizing the percentages of viable, early apoptotic, and late apoptotic cells. Data are presented as mean ± SD from three independent experiments. ****p < 0.0001, ***p < 0.001, **p < 0.01 compared to the control group.

When naringin was combined with cisplatin, the apoptotic response was enhanced. The 24-hour combination treatment resulted in a viable cell rate of 68.66% and an elevated percentage of apoptotic cells. After 48 hours, the viable cell rate decreased significantly to 43.22%, with percanteage of late apoptotic cells further increasing, demonstrating the synergistic apoptotic effect of naringin and cisplatin in HT-1376 cells (Figure 3 a,b). Overall, both naringin and the naringin + cisplatin combination induced apoptosis in HTB-9 and HT-1376 cells in a time-dependent manner. However, the combination treatment was more effective in reducing cell viability and increasing apoptotic cell populations than naringin alone, especially after prolonged exposure.

Following the annexin V/PI analysis, we conducted a caspase 3/7 assay to further investigate the specific apoptotic pathway activated by the treatments. By assessing Caspase 3/7 levels, we aimed to determine whether apoptosis was caspase-dependent, shedding light on the mechanisms underlying the observed cellular responses to naringin and cisplatin treatments. When naringin was combined with cisplatin, a significant enhancement in apoptosis was observed at both 24 and 48 hours in HTB-9 and HT-1376 cells. Caspase 3/7 activation is notably higher than with naringin alone, indicating a synergistic or additive effect of this combination in enhancing apoptosis (Figure 4 and Figure 5).

The increase in Caspase 3/7 activity observed, especially in the combined treatment groups, suggests that apoptosis is being mediated through the activation of the caspase cascade. This analysis indicates that both naringin and cisplatin, particularly in combination, likely activate apoptotic signaling through caspase-dependent mechanisms, promoting cell death in cancer cells.

3. DISCUSSION

This study highlights the antiproliferative and pro-apoptotic effects of naringin and its synergistic potential with cisplatin in human bladder cancer cell lines HTB-9 and HT-1376. These findings align with previous literature demonstrating the therapeutic promise of natural compounds, particularly flavonoids, in augmenting the efficacy of conventional chemotherapeutic agents [14,15]

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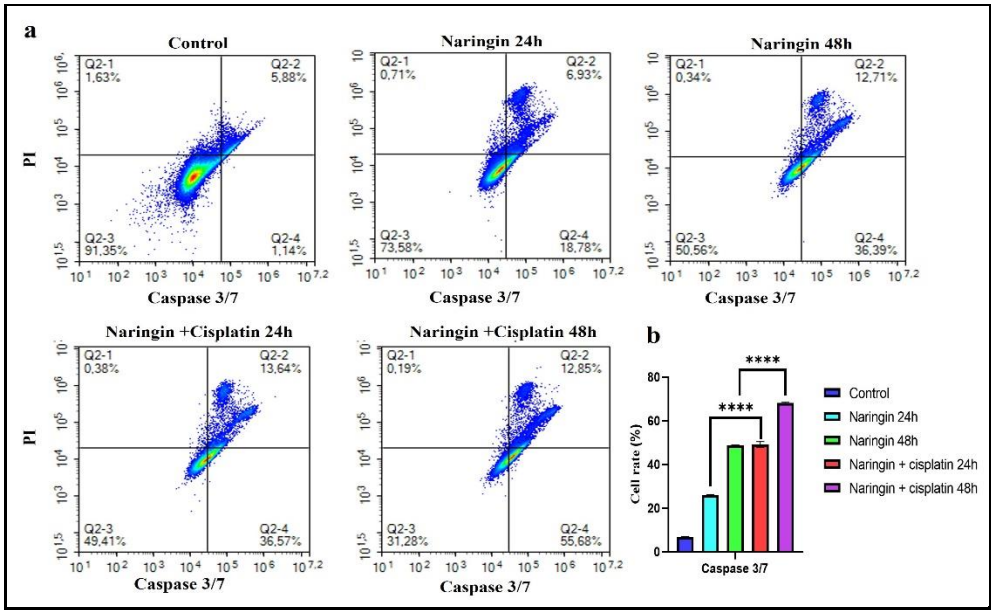

Figure 4. Caspase 3/7 activity in HTB-9 bladder cancer cells treated with naringin, cisplatin, or their combination for 24 and 48 hours. (a) Representative flow cytometry plots showing caspase 3/7 activation. (b) Bar graph summarizing caspase 3/7 activity. Data are presented as mean ± SD from three independent experiments. ****p < 0.0001 compared to the control group.

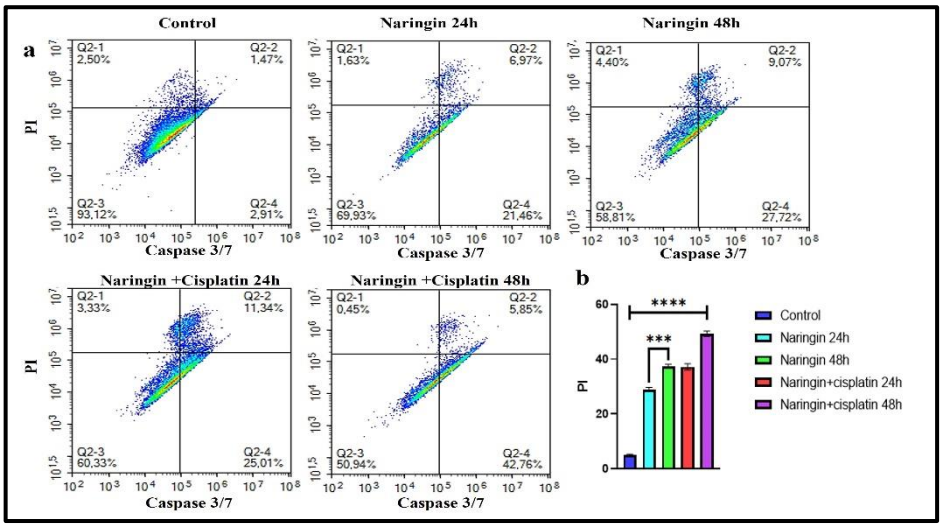

Figure 5. Caspase 3/7 activity in HT-1376 bladder cancer cells treated with naringin, cisplatin, or their combination for 24 and 48 hours. (a) Representative flow cytometry plots showing caspase 3/7 activation. (b) Bar graph summarizing caspase 3/7 activity. Data are presented as mean ± SD from three independent experiments. ****p < 0.0001, ***p < 0.001 compared to the control group.

The IC50 values of naringin for HTB-9 and HT-1376 cells were determined to be 32.98 µg/ml and 30.38 µg/ml, respectively, after 48 hours. These values are consistent with prior studies on naringin's cytotoxic effects in other cancer models. For example, Alji et al. reported that naringin exhibited IC50 values in HepG2 liver cancer cells, highlighting its comparable efficacy across different cancer types. The time-dependent decrease in cell viability observed in this study further supports the notion that naringin's cytotoxic effects may intensify with prolonged exposure, a characteristic that has been observed in various *in vitro* cancer studies [16-18].

Flow cytometry analysis revealed that naringin induces apoptosis in both HTB-9 and HT-1376 cells, with the combination of naringin and cisplatin significantly amplifying apoptotic effects. For instance, after 48 hours of treatment, the percentage of viable cells in HTB-9 was reduced to 19.12% with the combination therapy, compared to 34.86% with naringin alone. A similar trend was observed in HT-1376 cells, where the combination reduced viability to 43.22%, compared to 57.15% with naringin alone. The percentage of early apoptotic cells increased significantly in both cell lines, indicating that naringin and cisplatin synergistically enhance apoptosis.These findings align with those of Effat et al., who demonstrated enhanced apoptosis in

breast cancer cells treated with naringin combined with doxorubicin [18]. The synergistic effects observed in this study suggest that naringin may sensitize bladder cancer cells to cisplatin, potentially overcoming chemoresistance—a significant clinical challenge in bladder cancer treatment. It was also demonstrated that naringin inhibits the growth of SNU-1 gastric cancer cells, arrests cell division at the G0/G1 phase, and induces apoptosis in another study conducted by Duan X. et al. [19].

The activation of caspase 3/7, a hallmark of caspase-dependent apoptosis, was prominently increased in both cell lines treated with naringin and its combination with cisplatin. This result is in line with studies such as Dai et al., which reported that naringin-induced apoptosis in leukemia cells was mediated through caspase 3/7 activation [20]. Our data confirm that the apoptotic pathways triggered by naringin involve caspase signaling, further strengthening its role as a pro-apoptotic agent. Moreover, the enhanced caspase activation observed in the combination treatment underscores the potential for naringin to augment the apoptotic efficacy of cisplatin. In addition, Alshatwi et al. found that administration of naringin increased the expression of caspases, p53 and Bax, Fas death receptor and its adaptor protein FADD in servical cancer cells via mitochondria-mediated apoptosis [21].

The dose-dependent and time-dependent reductions in cell viability observed in this study also echo findings from Ansari et al., who demonstrated that naringin suppressed proliferation and induced cell cycle arrest in colon cancer cells [22]. Similarly, the gradual cytotoxic effects of naringin at concentrations above 25 µg/ml suggest that higher doses are more effective, particularly over longer treatment periods. This characteristic may allow for tailored dosing regimens in clinical settings, minimizing potential off-target effects while maximizing therapeutic benefits.

The combination therapy showed marked advantages over monotherapy, as reflected by the pronounced reduction in cell viability and increased rates of early and late apoptosis. These findings resonate with Jan et al., who emphasized the role of flavonoids in reducing chemotherapy-associated toxicity while enhancing efficacy [23]. By increasing the sensitivity of cancer cells to cisplatin, naringin may allow for the use of lower doses of cisplatin, potentially reducing its dose-limiting toxicities such as nephrotoxicity and neurotoxicity. This synergistic relationship between naringin and cisplatin not only supports their combined use in therapeutic protocols but also highlights the potential of natural compounds as adjuncts in cancer therapy [24].

Despite these promising findings, certain limitations of this study should be acknowledged. First, the *in vitro* nature of the experiments may not fully replicate the complex tumor microenvironment observed *in vivo*. For example, factors such as immune system interactions and tumor stroma dynamics are absent in cell culture models, which may influence the therapeutic efficacy of naringin and cisplatin in clinical settings. Second, while this study demonstrates caspase 3/7 activation, the broader apoptotic signaling pathways, including mitochondrial and extrinsic pathways, remain unexplored. Future studies should employ comprehensive molecular analyses to elucidate these mechanisms further. Another limitation is the lack of *in vivo* validation. While naringin and cisplatin demonstrated synergistic effects *in vitro*, animal models or patient-derived xenografts would be essential to confirm these results in a physiologically relevant context. Moreover, potential pharmacokinetic and pharmacodynamic interactions between naringin and cisplatin should be investigated to determine their combined bioavailability, metabolism, and toxicity profiles.

4. CONCLUSION

In conclusion, this study demonstrates that naringin exhibits significant antiproliferative and pro-apoptotic effects in bladder cancer cells, with its combination with cisplatin producing synergistic effects. The observed enhancement in apoptosis, particularly through caspase 3/7 activation, highlights the therapeutic potential of this combination. These findings align with existing literature and provide a strong rationale for further preclinical and clinical investigations into the use of naringin as an adjunct to conventional chemotherapy. Future research should address the identified limitations, including in vivo validation and a deeper exploration of underlying molecular mechanisms, to pave the way for integrating naringin into effective and less toxic therapeutic strategies for bladder cancer.

5. MATERIALS AND METHODS

5.1. Cell culture and chemicals

Human bladder cancer cell lines HTB-9 (5637) and HT-1376 (CRL-1472) were provided by the from American Type Culture Collection (ATCC). The HTB-9 and HT-1376 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Capricorn Scientific, Germany) and RPMI-1640 medium (Capricorn Scientific, Germany), respectively. The culture media were then supplemented with 10% fetal bovine serum

and 1% penicillin (Capricorn Scientific, Germany) and the cells kept in an incubator at 37°C with 5% CO_2. Following trypsinization with 0.25% trypsin at 80–90% confluence, the cells were cultivated in six-well plates for flow cytometry analysis and ninety-six-well plates for cell viability analysis. Naringin(Sigma) was dissolved in dimethyl sulfoxide (DMSO, Merck, Saint Louis, MO, USA) to have a 100 mM stock solution. Cisplatin (European Pharmacopoeia) was prepared with DMSO to obtain a 500 µM stock solution.

5.2. *In vitro* cytotoxicity assay

The MTT assay was used in this study to assess the cytotoxicity of naringin and cisplatin in two distinct grade bladder cancer cell lines (HTB-9 and HT-1376). The cells were cultivated at 37°C in a humidified incubator with 5% CO_2. Following confluence, the cells were harvested, pipetted to 96-well plates at a density of 1x105 cells/mL, and incubated for the whole night. Cisplatin was made in accordance with our earlier study, and naringin was diluted at 0, 5, 10, 25, 50, and 100 µg/mL. Cells were treated with naringin and cisplatin, and they were incubated for 24 and 48 hours. Following incubation, 200 µl of culture medium containing 13 µl of MTT solution (5 mg/mL, diluted with medium) was added to each well, and the medium containing naringin and cisplatin was withdrawn. 100 mL of isopropanol–HCl was added to the cells after they had been incubated for 4 hours at 37°C in the dark. MTT is converted by living cells into purple formazan crystals, which are then dissolved by isopropanol–HCl. To calculate the % viability, the plate was read at 570 nm using a Varioskan Thermo Scientific microplate reader. The tests were run three times. Naringin and cisplatin's %50 inhibitory concentrations (IC_{50}) were determined using the GraphPad Prism 9.1.0 software. The cytotoxicity rate was calculated by comparing the average absorbance from the wells containing nanorods with the absorbance of the wells without naringin and cisplatin (control), which was accepted as 100%. The cell viability formula is utilized for this: Cell Viability (%)=(SampleOD570/ControlOD570)×100.

5.3. Annexin V/PI Apoptosis assay

HTB-9 and HT-1376 bladder cancer cells were planted into 6-well plates in order to perform the apoptotic test as our previous study [25]. Naringin and cisplatin were applied to the cell lines at IC50 values for HTB-9 and HT-1376 bladder cancer cells. The dead cells were gathered following the incubation period. Trypsin-EDTA was used to separate the cells that had adhered to the plate. Following this, every cell was gathered, cleaned with PBS, and the concentration was set at 1 x 105 cells per 100 µl. The resultant cell solution was then put into 12 x 75 mm polystyrene tubes, and 1X Annexin Binding Buffer (500 µl), 5 µl of Annexin V-FITC, and propidium iodide (PI) were added. Following a 15-minute incubation period at room temperature, the cells were examined using an Agilent ACEA NovoCyte flow cytometry instrument.

5.4. Caspase 3/7Activity Assay

Cells were initially plated at a density of 5 x 105 per well in 6-well plates and incubated overnight. Following incubation, the cells were exposed to naringin at specified concentrations: 37.66 µg/ml, and 32.98 µg/ml for HTB-9 cells, 44.58 µg/ml, and 30.38 µg/ml for HT-1376 bladder cancer cell lines at 24h and 48h respectively. The treated cells were incubated at 37°C for 24h and 48h. Post-incubation, the cells were collected in 0.5 ml of pre-warmed media and subsequently incubated with a caspase 3/7 detection reagent (Cell Meter™ Live Cell Caspase 3/7 Binding Assay Kit, AAT Bioquest) at 37°C for one hour [26]. Following this step, cells were washed and resuspended in 0.5 ml of assay buffer. Caspase 3/7 activity was then assessed via the NovoCyte D3000 flow cytometer, providing insights into the activation levels of these apoptosis-specific caspases in treated cells.

5.5 Statistical Analysis

Statistical evaluations were carried out using GraphPad Prism version 8.4.2. The Two-way ANOVA test was employed for intergroup comparisons, with differences in means compared to the control group assessed using the Tukey test. Results are expressed as mean ± standard deviation, and a significance level of $p < 0.05$ was established.

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