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Original Article

In vitro study of effects of hesperetin on human oral cancer using KB cell model

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الملخص

أهداف البحث: تبحث هذه الدراسة في التأثيرات المضادة للسرطان للهسبيريتين الأصلي على خلايا سرطان الفم "كي بي" من خلال عدد من الاختبارات في المختبر.

طريقة البحث: تمت زراعة خلايا سرطان الفم "كي بي"، وتم استخدام اختبار "ام تي تي" لتقييم قابلية الخلايا للبقاء، وتم فحص الاختلافات في إمكانات غشاء الميتوكوندريا، وتم استخدام اختبار "كوميت" لتقييم تلف الحمض النووي التأكسدي.

النتائج: توضح الدراسة أن الهسبيريتين الأصلي يمارس تأثيرا ساما قويا على خلايا سرطان الفم "كي بي"، مما يقلل من قابلية الخلايا للبقاء بطريقة تعتمد على الجرعة. مع زيادة تركيز الهسبيريتين، فإنه يعطل إمكانات غشاء الميتوكوندريا، مما يؤدي إلى موت الخلايا المبرمج. يكشف الفحص المجهري الفلوري أن الهسبيريتين يحفز موت الخلايا المبرمج والنخري، فضلا عن تلف الحمض النووي التأكسدي الكبير.

الاستنتاجات: تؤكد هذه النتائج على الإمكانات العلاجية للهسبيريتين في علاج السرطان، وتسلط الضوء على قدرته على إحداث موت الخلايا من خلال آليات متعددة، مما يجعله مرشحا واعدا لمزيد من التحقيق في علاج سرطان الفم.

الكلمات المفتاحية: الهسبيريتين؛ العلاجات؛ سرطان الفم؛ موت الخلايا المبرمج

Abstract

Objective: This study investigated the anticancer effects of native hesperetin on the oral carcinoma KB cell line based on in vitro tests.

Methods: Oral carcinoma KB cells were cultured and the MTT assay was employed to assess cell viability. Variations in the mitochondrial membrane potential were examined and the comet assay was utilized to evaluate oxidative DNA damage.

Results and conclusion: Native hesperetin had a potent cytotoxic effect on oral carcinoma KB cells, reducing cell viability in a dose-dependent manner. As the hesperetin concentration increased, the mitochondrial membrane potential was disrupted, leading to apoptosis. Fluorescence microscopy showed that hesperetin induced apoptotic and necrotic cell death, as well as significant oxidative DNA damage. These findings highlight the therapeutic potential of hesperetin in cancer treatment due to its ability to induce cell death through multiple mechanisms, thereby making it a promising candidate for further investigation in oral carcinoma therapy.

Keywords: Apoptosis; Hesperetin; Oral cancer; Therapeutics

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Introduction

Oral carcinoma is a common type of head and neck cancer that is highly problematic because of its aggressive nature and high recurrence rates. Investigating innovative therapeutic agents is necessary to treat oral carcinoma because the traditional treatment approaches, including surgery, radiation, and chemotherapy, have frequent significant side effects and inconsistent success. In this context, naturally occurring compounds have attracted considerable interest because of their potential anticancer properties. In particular, hesperetin is a flavonoid that is commonly found in citrus fruits, and various studies have demonstrated its promising anticancer activities.

Polyphenolic substances called flavonoids are found in many different types of plants. Flavonoids have diverse biological actions, including antiviral, anti-inflammatory, antioxidant, and anticancer properties. In particular, hesperetin is a flavonoid with strong antioxidant properties that may help to mitigate oxidative stress, which is often linked to cancer progression. The anticancer potential of hesperetin can be attributed to its ability to interfere with multiple cellular processes that are crucial for cancer cell survival and proliferation.

The mechanism associated with the anticancer effects of hesperetin involves several pathways, including the induction of apoptosis, which is a programmed cell death process that is essential for eliminating cancer cells. Both internal (mitochondrial) and extrinsic (death receptor) mechanisms can initiate apoptosis. The intrinsic pathway is of particular interest because it involves disrupting the mitochondrial membrane potential to cause the release of pro-apoptotic proteins and activation of caspases, which control the cell death process. Given the pivotal role of mitochondria in apoptosis, targeting mitochondrial integrity represents a strategic approach to cancer therapy.

In vitro, hesperetin has been shown to cause apoptosis in a variety of cancer cell types by altering the activities of mitochondria. For instance, it was shown that hesperetin dramatically reduced the growth of human cancerous cell lines by causing apoptosis.[1] Similarly, another study[2] found that hesperetin induced apoptosis in human colon cancer cells by activating p53 and inhibiting NF-κB and Bcl-2, thereby highlighting the ability of hesperetin to modulate key apoptotic regulators.

The effectiveness of hesperetin in oral carcinoma has not been studied extensively, although the limited available data support its potential application. Studies have indicated that flavonoids, including hesperetin, can exert selective cytotoxic effects on cancer cells but spare normal cells. This selective cytotoxicity is crucial for reducing the adverse side effects typically associated with conventional chemotherapy.

The present study examined the anticancer properties of hesperetin in human oral carcinoma KB cells, particularly its capacity to trigger apoptosis through mitochondrial pathways. Rhodamine 123 (Rh-123), a lipophilic cationic dye that preferentially accumulates in mitochondria, was used to assess changes in the mitochondrial membrane potential as an early indicator of cell death. The intensity of Rh-123 fluorescence is associated with the integrity of the mitochondrial membrane, so a decrease in fluorescence signifies mitochondrial dysfunction and the onset of apoptosis.[3]

Previous studies have established the use of Rh-123 staining as a reliable method for evaluating mitochondrial health and detecting early apoptotic changes. For example, Kanno et al. (2004) used Rh-123 to evaluate the cytotoxic effects of flavonoids on cancer cells, and the reduction in the intensity of Rh-123 fluorescence indicated apoptosis and changes in the mitochondrial membrane potential. Similarly, Choi (2007) reported that the anti-oxidative effects of hesperetin against oxidative stress in mice were accompanied by changes in mitochondrial function, further supporting the use of Rh-123 in this study.

In the present study, oral carcinoma KB cells were exposed to different doses of natural hesperetin for 48 h, before staining the cells with Rh-123 and incubating to allow the dye to penetrate and stain the mitochondria. Cells were then washed to remove any excess dye, before examination using a fluorescence microscope with a blue filter to observe changes in the mitochondrial membrane potential. The extent of mitochondrial membrane potential alterations and consequent apoptotic effects induced by hesperetin were determined by quantifying the fluorescence intensity.[4,5]

In addition to clarifying the process that allows hesperetin to cause apoptosis in oral cancer cells, the possible uses of hesperetin as a medication were also investigated. Understanding how hesperetin affects the activity of mitochondria and initiates apoptosis may facilitate the development of less harmful and more efficient cancer therapies. Moreover, the results obtained in the present study contribute to broader research into the anticancer properties of flavonoids, thereby supporting the potential use of natural compounds in cancer therapy.[6–9] This study focused on exploring the ability of hesperetin to alter the mitochondrial membrane potential, a key event in the initiation of apoptosis. Hesperetin can trigger programmed cell death in cancer cells by disrupting the function of mitochondrial. The findings obtained in this study could enhance our understanding of the mechanism of action for hesperetin and support its development as a natural, targeted therapeutic agent for oral carcinoma and potentially other cancers.

Methods

Chemicals: Heat-inactivated fetal calf serum (FCS), Dulbecco's modified Eagle medium (DMEM), 3-(4,5)dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), hesperetin, polyvinyl alcohol (MW 25000), and penicillin and streptomycin antibiotics were acquired from Sigma−Aldrich Chemical Pvt Ltd (Bangalore, India) and used as supplied. Phosphate-buffered saline (PBS), Rh-123, ethidium bromide (EB), and acrylidine orange (AO) were obtained from Himedia (Mumbai, India). All other chemicals and solvents were analytical quality.

Cell culture and maintenance: KB human oral carcinoma cell lines were acquired from the National Centre for Cell Science (NCCS) located in Pune, India. The cells were cultivated in DMEM with 10 % FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an incubator with 5 % CO_2 and 95 % humidified air.

Cell culture

The oral carcinoma KB cell line culture protocol for maintaining the cells under optimal conditions for growth and experimentation involved several key steps. Initially, oral carcinoma KB cells were thawed from frozen stocks and cultured in T-75 flasks containing complete growth medium, which typically comprised DMEM supplemented with 10 % fetal bovine serum and 1 % penicillin–streptomycin. The cells were then incubated at 37 °C in a humidified atmosphere with 5 % CO_2. After reaching 70–80 % confluence, the cells were detached using trypsin–EDTA solution and sub-cultured at a seeding density of approximately 1×10^5 cells per T-75 flask or equivalent. The medium was changed every 2–3 days to provide fresh nutrients and remove waste products. The cells were routinely monitored to assess their morphology and viability using an inverted microscope and the trypan blue exclusion assay, respectively. In addition, to maintain genetic stability and avoid contamination, the cells were regularly tested to detect mycoplasma contamination using PCR-based methods. For experiments, KB cells were seeded into appropriate culture vessels, such as 96-well plates, at suitable densities and treated with the test compound. Following treatment, cell viability, proliferation, or other endpoints were assessed using various assays, such as the MTT assay, flow cytometry, or immunofluorescence staining, to evaluate the anticancer effects of the test compound on oral carcinoma KB cells. Adhering to this protocol ensured consistent culture conditions and reliable experimental results with oral carcinoma KB cells.

Commercially available hesperetin was utilized and it was added to the KB cell line at several concentrations (10, 20, 40, 60 and 80 μg/mL).

Cell viability assay

The cytotoxic effects of native hesperetin on the oral carcinoma KB cell line were evaluated using the MTT calorimetric assay, which is based on the conversion of a yellow tetrazolium salt to purple formazan crystals by mitochondrial dehydrogenases in viable cells. Initially, KB cell lines were seeded in 96-well plates at a density of 6000 cells per well and incubated for 24 h to acclimate. After this period, the cells were exposed to various concentrations (10, 20, 40, 60, and 80 μg/mL) of native hesperetin, before incubating in a CO_2 atmosphere at 37 °C for 48 h. Following incubation, the medium was replaced with 100 μL of fresh medium containing MTT solution (5 mg/mL), and the cells were incubated for an additional 4 h in a CO_2 atmosphere. The viability of cells was assessed based on the formation of purple formazan crystals from yellow MTT by metabolically active cells. After aspirating the medium, the formazan crystals were dissolved in 200 μL of dimethyl sulfoxide, and the absorbance of the solution was measured at 540 nm using a microplate reader. This absorbance measurement was used to determine cell viability. This experiment provided insights into the effectiveness of native hesperetin in inhibiting the growth of oral carcinoma cells.

Analysis of mitochondrial membrane potential

Early phases of apoptosis are indicated by a change in the mitochondrial membrane potential. Rh-123 is a lipophilic cationic dye that is highly suitable for staining mitochondria, so it was employed to identify changes in cells treated with hesperetin. Cells were treated with different amounts of hesperetin for 48 h, before treating the cells with 10 μg/mL of Rh-123 dye. The cells were then incubated for 30 min to allow the dye to penetrate and stain the mitochondria. After incubation, excess dye was removed by washing the cells with PBS. Changes in the mitochondrial membrane potential were then observed when the cells were examined under a fluorescent microscope with a blue filter. This staining method facilitated observation of early apoptotic changes induced by the treatments. The use of Rh-123 dye was reliable for assessing mitochondrial health and potential disruption caused by hesperetin, thereby providing insights into the mechanisms that allowed apoptosis to be induced by these compounds. The extent of mitochondrial membrane potential alterations was determined by analyzing the fluorescence intensity, thereby indicating the efficacy of native hesperetin at triggering apoptosis in oral carcinoma cells through mitochondrial pathways. This approach was applied to assess the potential therapeutic value of this treatment in promoting cell death in cancerous cells.

Detection of apoptotic morphological changes using AO/EB staining

Apoptotic and necrotic cells were identified and differentiated based on their physical traits using AO and EB dyes, which bind to DNA. Both living and non-living cells absorb the permeable dye AO, which intercalates with double-stranded DNA to produce green fluorescence. By contrast, only non-viable cells can absorb EB, which cannot permeate living cells, and it produces orange-red fluorescence when it intercalates with DNA. The cells were separated from their growth plates following a treatment with hesperetin for 48 h. Remaining medium and separated cells were removed by washing with cold PBS. The cells were then stained for 5 min at room temperature using a solution containing AO (100 μg/mL) and EB (100 μg/mL). This staining procedure allowed the differentiation of live, apoptotic, and necrotic cells. Live cells stained with AO exhibited green fluorescence, whereas apoptotic cells with compromised membrane integrity produced green to orange fluorescence depending on the stage of apoptosis. After taking up EB, necrotic cells exhibited distinct orange-red fluorescence. Furthermore, the stained cells were examined at 40 × magnification using a fluorescence microscope to clearly observe the morphological changes associated with apoptosis and necrosis induced by the treatments. AO/EB staining facilitated detailed analysis of the effects of hesperetin on oral carcinoma cells to provide insights into its potential therapeutic efficacy.

Assessment of oxidative DNA damage

DNA damage was estimated using the comet test, which is an alkaline single-cell gel separation technique that involves subjecting individual cells to electrophoresis in an alkaline environment, thereby making the fragmented DNA strands migrate away from the nucleus, and they resemble comet tails when observed microscopically. A fluorescent microscope with a digital camera was employed to determine the extent

of DNA damage, and image analysis software (CASP) was utilized to interpret the results.

A maximum of 100 comet observations were captured and examined for every sample. Parameters such as the tail moment, tail length, and olive tail moment were measured to determine the degree of DNA damage. The tail moment measures both the length and intensity of the comet tail, reflecting the severity of DNA fragmentation. The tail length represents the distance migrated by the fragmented DNA strands to provide additional information about the extent of damage. The olive tail is a measure that considers the shape of the comet tail to provide insights into the distribution of DNA damage within the cell.

The level of oxidative DNA damage induced by the experimental conditions was effectively quantified by analyzing these parameters. This comprehensive approach provided a thorough assessment of DNA integrity and valuable insights into the potential genotoxic effects of the test compound. The utilization of advanced imaging and analysis techniques ensured that accurate and reliable measurements of DNA damage were obtained, enhancing the precision of the experimental findings and contributing to a better understanding of the mechanisms underlying cellular responses to oxidative stress.

Results

Cell viability assay

The results indicated a clear dose-dependent cytotoxic effect of hesperetin on oral carcinoma KB cells. In the control group with no added hesperetin, the absorbance at 540 nm indicated high cell viability, corresponding to 100 % cell survival. At the lowest hesperetin concentration (10 µg/mL), a modest decrease in cell viability was observed, where approximately 87.1 % of the cells remained viable. As the concentration of hesperetin increased to 20 µg/mL, a more noticeable decrease in viability was recorded, where about 74.6 % of the cells survived. At 40 µg/mL, a significant reduction in cell viability was detected with only 50.9 % of the cells remaining viable. This trend continued with higher concentrations and only 41.8 % of the cells were viable at 60 µg/mL. The reduction in cell viability was greatest at the highest concentration tested (80 µg/mL) and only 22 % of the cells survived (Figure 1).

In the present study, hesperetin had a dose-dependent cytotoxic effect on the oral carcinoma KB cell line. Lower concentrations obtained minimal cytotoxicity, whereas higher concentrations significantly reduced the viability of cells. These findings suggest that native hesperetin is effective in inhibiting the growth of oral carcinoma cells and its effectiveness is greater at higher concentrations.

Mitochondrial membrane potential

The results indicated that native hesperetin induced significant alterations in the mitochondrial membrane potential in oral carcinoma cells according to Rh-123 staining. Fluorescence microscopy showed that the fluorescence intensity was strong in the untreated control cells, indicating healthy mitochondria. By contrast, the fluorescence intensity of cells treated with increasing concentrations of hesperetin (10, 20, 40, 60 and 80 µg/mL) decreased progressively (Figure 2). This reduction in fluorescence correlated with the reduced potential of the mitochondrial membrane, indicating early to advanced stages of apoptosis. Quantitative analysis confirmed that the reduction in the fluorescence intensity of hesperetin-treated cells was statistically significant compared with the control group ($p < 0.05$), where higher hesperetin concentrations caused greater disruption. These findings suggest that hesperetin can effectively induce apoptosis in oral carcinoma cells through mitochondrial pathways, highlighting its potential use as a therapeutic agent in cancer treatment.

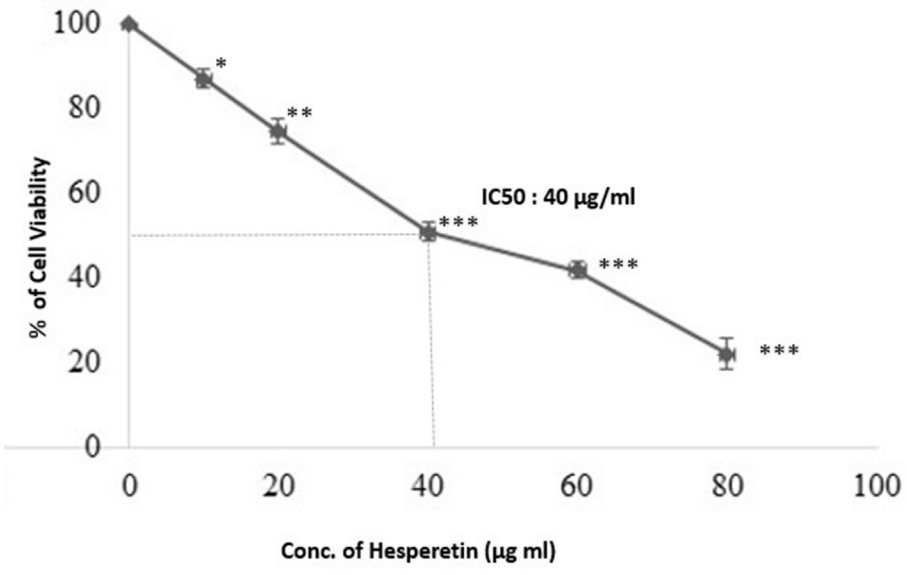

Figure 1: Cell viability using MTT assay ($p \leq 0.05$, regarded as indicating a statistically significant difference; *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0001$).

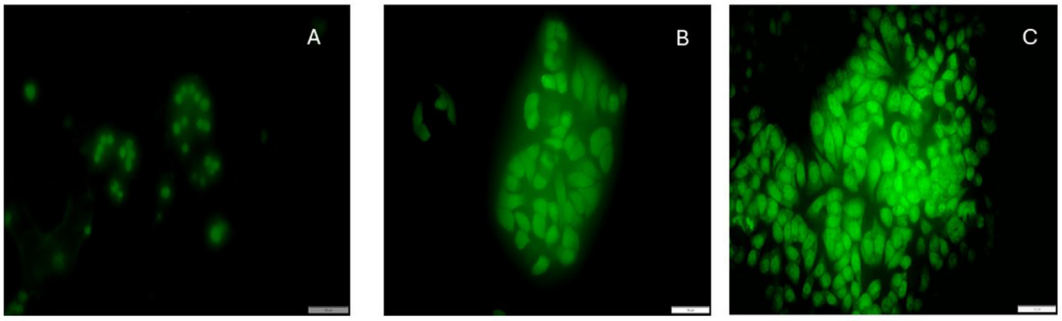

Figure 2: Mitochondrial membrane potential analysis. (A) Control cells; (B) cells after treatment with 60 µg/mL hesperetin; (C) cells after treatment with 80 µg/mL hesperetin.

Detection of apoptotic morphological changes using AO/EB staining

Fluorescence microscopy clearly demonstrated that native hesperetin induced significant apoptotic and necrotic effects in oral carcinoma KB cells. After treatment with hesperetin for 48 h, cells were labeled with EB and AO to distinguish between necrotic, apoptotic, and living cells. Both live and non-viable cells absorbed the permeable dye AO, which then intercalated within double-stranded DNA to produce green fluorescence. By contrast, EB was only taken up by non-viable cells and it produced orange-red fluorescence after intercalating within DNA.

Live cells exhibited green fluorescence under fluorescence microscopy at a magnification of 40 ×, indicating their intact membrane integrity. Apoptotic cells produced a spectrum from green to orange fluorescence, reflecting various stages of apoptosis, where early apoptotic cells still partially excluded EB, whereas those in later stages were characterized by increased permeability. Necrotic cells were identified based on their distinct orange-red fluorescence due to EB uptake, indicating the loss of membrane integrity.

The presence of orange-red fluorescing cells confirmed necrosis, and the green to orange spectrum in apoptotic cells indicated different stages of apoptosis. The detailed visualization of morphological changes under AO/EB staining confirmed the cytotoxic effects of hesperetin on KB cells. The results showed that hesperetin effectively induced cell death through both apoptotic and necrotic pathways, highlighting its potential therapeutic efficacy in treating oral carcinoma. These findings suggest that hesperetin may serve as a potent agent in cancer therapy by promoting significant cell death in oral carcinoma cells (Figure 3).

Assessment of oxidative DNA damage

The results showed that cells treated with native hesperetin exhibited significantly higher values for all three parameters compared with the control cells, indicating increased DNA damage. In particular, the tail moment and olive tail moment values were substantially higher, suggesting extensive DNA fragmentation and distribution of damage. The increased tail length in treated cells further corroborated the presence of significant DNA damage.

This comprehensive analysis confirmed that native hesperetin induced oxidative DNA damage in oral carcinoma KB cells (Figure 4). The advanced imaging and analysis techniques employed ensured that precise and reliable measurements of DNA damage were obtained, highlighting the potential genotoxic effects of hesperetin. These findings provide valuable insights into the mechanisms that allow hesperetin to exert its cytotoxic effects, demonstrating how it damages DNA and advancing knowledge of its potential therapeutic use in the treatment of cancer.

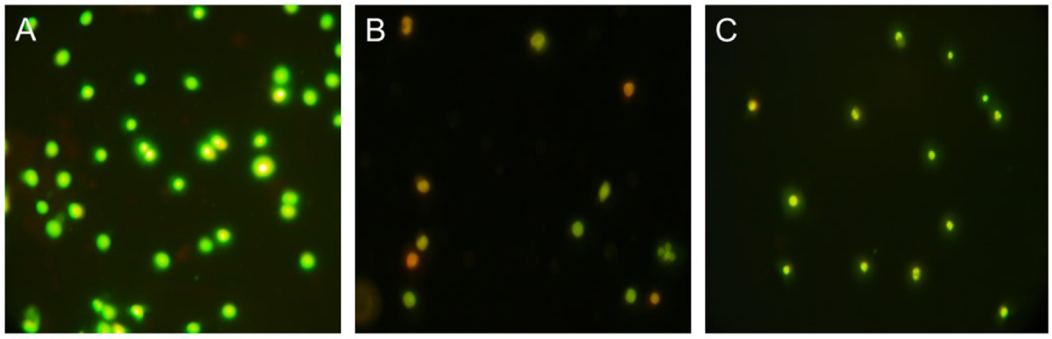

Figure 3: Acrylidine orange (AO)/ethidium bromide (EB) staining results. (A) Control cells; (B) cells after treatment with 60 µg/mL hesperetin; (C) cells after treatment with 80 µg/mL hesperetin.

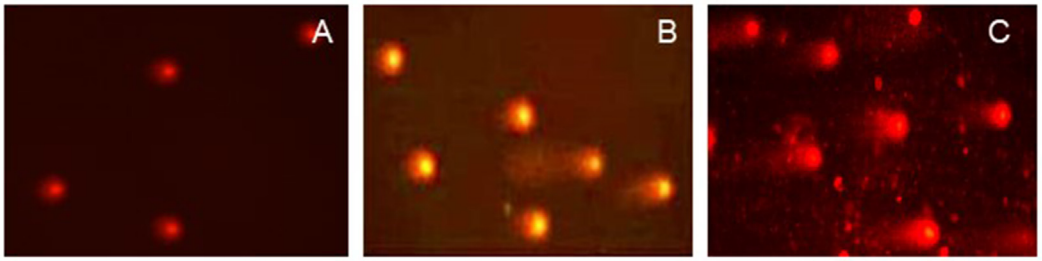

Figure 4: Oxidative DNA damage analysis. (A) Control cells; (B) cells after treatment with 60 μg/mL hesperetin; (C) cells after treatment with 80 μg/mL hesperetin.

Discussion

In this study, the anti-tumor activity of hesperetin was assessed in vitro in human oral carcinoma KB cells, and the results indicated its therapeutic potential. The flavonoid hesperetin is derived from citrus fruits and it has been studied extensively due to its anti-inflammatory, anti-cancer, and antioxidant qualities. The capacity of hesperetin to cause apoptosis in KB cells was evaluated this study by focusing on changes in the mitochondrial membrane potential as initial signs of apoptosis.

Flavonoids such as hesperetin can affect various mechanisms, including cell death induction, cellular cycle arrest, angiogenesis suppression, and cancer-promoting signaling pathway modification.[10,11] Disturbance of the mitochondrial membrane potential is a critical event in the intrinsic pathway of apoptosis. Rh-123 staining effectively demonstrated that hesperetin induced a dose-dependent reduction in the mitochondrial membrane potential in KB cells. These results are consistent with comparable mechanisms of action for flavonoids in previous studies.

The dose-dependent effect of hesperetin on the mitochondrial membrane potential was one of the key findings obtained in this study. At lower concentrations (10 μg/mL), hesperetin moderately reduced the fluorescence intensity, indicating early apoptotic changes. The reduction in fluorescence was more pronounced as the concentration increased (60 and 80 μg/mL), suggesting a higher degree of mitochondrial dysfunction and progression toward apoptosis, which is consistent with the general understanding that higher doses of chemotherapeutic agents can induce more significant cellular responses, including apoptosis.

Rh-123 was used as a reliable and quantifiable marker of the mitochondrial membrane potential to assess the extent of mitochondrial disruption. This technique is well-established in apoptosis research and has been used extensively to study the effects of various anticancer agents.[5,12,13] The significant decrease in fluorescence intensity observed in hesperetin-treated cells demonstrated its efficacy at targeting mitochondria, thereby triggering apoptotic pathways.

Moreover, the findings obtained in this study are supported by previous research indicating that hesperetin can modulate several molecular targets involved in apoptosis. For instance, hesperetin has been shown to downregulate anti-apoptotic proteins, such as Bcl-2, but to upregulate pro-apoptotic proteins, including Bax and caspases. These molecular changes facilitate the release of cytochrome *c* from mitochondria and the activation of downstream apoptotic pathways, further corroborating the observed alterations in the mitochondrial membrane potential.

The implications of these findings are significant for the development of hesperetin as a potential anticancer agent. Oral carcinoma remains a challenging cancer to treat, with high recurrence rates and resistance to conventional therapies. The ability of hesperetin to induce apoptosis through mitochondrial pathways provides a promising alternative or adjunctive approach to existing treatments.[3,14] However, a limitation of the present study is that several marker genes were not analyzed that are involved in the apoptotic cascade. Therefore, it is necessary to conduct further studies to understand the full range of the molecular effects of hesperetin and to evaluate its efficacy and safety in both in vivo models and clinical trials.

Conclusion

This in vitro evaluation of hesperetin in oral carcinoma KB cells highlights its capacity as an effective anticancer agent through the induction of apoptosis via mitochondrial membrane potential disruption. These results strengthen the mounting evidence regarding the anticancer effects of flavonoids and highlight the need for more research to fully realize their therapeutic potential in the treatment of cancer.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

Obtained from Research Ethics Committee, Ajman University [D-F-H-13-May].

Authors contributions

ABK conceptualized the study, performed investigation, provided funding and analyzed data. AML Validated the study, provided funding, performed investigation, analyzed and interpreted the data. STK collected organized data and wrote the initial manuscript. NS validated, collected data and wrote the final draft of manuscript. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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Data availability statement

Research data can be made available on request from the corresponding author if required.

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