Advances in Cancer Biology - Metastasis 6 (2022) 100077

Contents lists available at ScienceDirect

Advances in Cancer Biology - Metastasis

journal homepage: www.journals.elsevier.com/advances-in-cancer-biology-metastasis

Hesperetin modulates TGFβ induced metastatic potential of prostate cancer cells by altering histone methylation marks

Nidhi Dalpatraj, Jyoti Tak, Ankit Naik, Noopur Thakur *

Biological and Life Sciences, School of Arts and Sciences, Ahmedabad University, Commerce Six Roads, Navrangpura, Ahmedabad, 380009, Gujarat, India

ARTICLE INFO

Keywords:
EMT
Prostate cancer
Hesperetin
TGFβ
Histone modification

ABSTRACT

Prostate cancer is the most prevalent cancer in males, and usually, death occurs due to bone metastasis. TGFβ has been shown to play an essential role in the metastasis of prostate cancer cells by promoting epithelial-to-mesenchymal transition (EMT). Hesperetin is known to possess anti-microbial, anti-fungal, antioxidant, and anti-cancer properties and good bioavailability. Therefore, this study investigated the effect of hesperetin on TGFβ-induced cell proliferation and EMT in prostate cancer cells (PC3). Interestingly, we found that hesperetin can significantly inhibit the cell proliferation of PC3 cells and arrest the cells in the S and G2M phases of the cell cycle. The invasion and migration assay results decipher the inhibitory effect of hesperetin on TGFβ-induced invasion and migration of prostate cancer cells. Our results confirmed that hesperetin also acts through the canonical signaling pathway, as we observed a significant decrease in the expression of pSmad3. Hesperetin can inhibit the TGFβ induced EMT by increasing E-cadherin expression and decreasing N-cadherin expression. Hesperetin could modulate the TGFβ induced histone methylation marks. Further investigation is required to understand the role of hesperetin in modulating these marks and thus inhibiting TGFβ-induced EMT. Hence, the results of our study identified the potential of hesperetin to modulate TGFβ-induced cell proliferation and invasion and migration of prostate cancer cells which may help inhibit the metastatic growth of prostate cancer cells.

1. Introduction

Phytochemicals have been important chemopreventive and chemotherapeutic agents for the last decades. Researchers around the globe are trying to find the most effective phytochemical against many cancer types because they have good efficacy and lesser side effects compared to presently available chemotherapeutics. In search of the same, many phytochemicals were explored for decades, such as vinblastine, resveratrol, curcumin, hesperidin, silibinin, etc. Out of which the FDA approved very few. Few, such as Vincristine [1], Irinotecan, and Topotecan [2], are FDA-approved phytochemicals against different cancers. The primary reason for the failure of many phytochemicals in clinical studies is their poor bioavailability. To overcome that, one can explore the metabolite of the phytochemicals, which have more extended bioavailability than the pure compound.

Hesperetin is one such metabolite of hesperidin. Hesperidin is a citrus flavanone (3, 5, 7-trihydroxy-4- methoxy-flavanone) in oranges and lemons [3]. Hesperetin is an aglycone of hesperidin released during digestion via hydrolysis by the colonic microbiota [4]. It has been proven to have anti-inflammatory [5], anti-fungal [6], anti-viral [7–9], anti-oxidant [10], and anti-cancer [11,12] properties. As an anti-cancer compound, the efficacy and mode of action of hesperetin were explored by many researchers, specifically against gastric [13], breast [14], cervical [15], glioma [16], thyroid [17], and prostate [18] cancer. Studies exploring the potential of hesperetin against prostate cancer found its effects on apoptosis *in-silico* and *in-vitro*. Still, the effects of hesperetin are yet to be examined against other essential aspects of metastatic prostate cancer. Altogether, the properties of hesperetin gave us insight into its effect on TGFβ-induced invasion and migration in PC3 cells.

Prostate cancer occurs due to malfunctioned cells of the prostate gland in men. Major risk factors for prostate cancer are age, obesity, family history, and ethnicity [19]. Globally 1.4 million new cases have been reported, and 375,000 deaths due to prostate cancer [20]. The vast number of incidents and mortality rates make prostate cancer the second most frequent cancer and the fifth leading cause of cancer deaths worldwide [20]. Though incidence and mortality rates are lesser in India than in the world, data suggests that prostate cancer incidents are consistently increasing yearly. However, consistent research in medicine

* Corresponding author.
E-mail address: noopur.thakur@ahduni.edu.in (N. Thakur).

https://doi.org/10.1016/j.adcanc.2022.100077
Received 23 July 2022; Received in revised form 3 November 2022; Accepted 7 November 2022
Available online 17 November 2022
2667-3940/© 2022 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

and pharmacology found few ways to fight against prostate cancer. Surgery, radiation therapy, hormonal therapy, and chemotherapy are the available treatment options for prostate cancer. With these many treatment options, it is still challenging to combat metastatic types of prostate cancer. So, we were interested in exploring the preventive and therapeutic effectiveness of hesperetin against metastatic prostate cancer cells.

High-grade malignancies in cancer are caused due to a combination of processes; epithelial-to-mesenchymal transition (EMT) is one of them. EMT plays a vast role, from embryonic development to tissue regeneration, organ fibrosis, and adult tumor progression [21]. In tumorigenesis, EMT involves a transition of the tumor cells from the epithelial state to the mesenchymal state, allowing their migration and invasion at different sites [22]. The cells lose the expression of E-cadherin and start expressing mesenchymal proteins like N-cadherin, fibronectin, and vimentin, which are well-known molecular markers of EMT. It is a very complex process due to which multiple transcription factors are involved in the induction of EMT, such as SNAIL, TWIST, ZEB, Wnt/β-catenin, and the TGFβ signalling pathway [23]. The TGFβ signalling pathway is renowned for inducing EMT during cancer progression.

TGFβ is a member of a large family of cytokines, which is involved in various functions such as regulation of cell growth, differentiation, apoptosis, tissue repair, and modulation of the immune system [24]. TGFβ signalling pathway is essential to regulate crucial events like cell growth, apoptosis, invasion, and migration along with cancer metastasis. TGFβ signalling is facilitated through SMAD (canonical) and non-SMAD (non-canonical) pathways which involve SMAD 2/3/4 and Akt, Erk1/2, p38-MAPK, respectively [24]. Therefore, in our study, we used TGFβ to induce invasiveness of aggressive prostate cancer cells, namely PC3 and were interested in exploring the effect of hesperetin on TGFβ induced invasion and migration of PC3 cells.

In a recent study, hesperitin was found to reduce the levels of H3K79me2 and H3K79me3 in gastric cancer cells, while the other methylation marks like H3K9me3, H3K4me3, H3K27me3, H3K36me3 remains unaffected [25]. It was also reported that the decrease in H3K79 methylation levels was due to the downregulation of the DOT1L enzyme, which is known to play an essential role in cancer progression and tumorigenesis [25]. Therefore, we studied the effect of hesperetin on the three most studied methylation marks, H3K4me3, H3K9me3, and H3K27me3, in the PC3 cell line.

2. Materials and methods

2.1. Cells and culture conditions

PC3 cells were acquired from NCCS, Pune (Maharashtra, India). The cell lines were authenticated using STR profiling and tested for mycoplasma using the Hoechst staining/PCR method at NCCS. There was a 100% match between the cell line samples and the ATCC STR profile database. The cell lines tested negative for mycoplasma contamination. The cells were grown in RPMI-1640 (HiMedia) supplemented with 10% fetal bovine serum (Gibco) and 1% Antibiotic-Antimycotic solution (HiMedia) at 37 °C in a 5% CO_2 atmosphere. PC3 cells were trypsinized at 80–90% confluency using 0.25% Trypsin-EDTA (HiMedia).

2.2. MTT assay

Cell proliferation, cytotoxicity, and cell viability were checked using the Metathiazolyl tetrazolium (MTT) assay. To determine the effect of hesperetin (Sigma), the PC3 cells were exposed to different concentrations (50 μM, 100 μM, 200 μM, 300 μM, 400 μM, 500 μM) of hesperetin for 24hrs and 48hrs [26]. DMSO was used as solvent control. The cells treated with hesperetin were exposed to MTT. After 4hrs of incubation, DMSO was added to dissolve the formazan crystals, and absorbance was measured at 570 nm using a microplate reader (Biotek Synergy HT Microplate Reader)[16]. MTT assay was also used to examine the effect of hesperetin on cell proliferation of TGFβ induced PC3 cells. Briefly, the cells were seeded in a 6-well plate with a seeding density of 8000 cells per 96-well plate. The cells were then starved in serum-free media for about 24 h. This was followed by treatment with 5 ng/mL TGFβ and 200 μM hesperetin, both individually and in combination. MTT assay was carried out as mentioned above. Student's t-test was carried out to calculate significance using GraphPad Prism 9 software (v9.4).

2.3. Cell cycle analysis

To determine the effect of hesperetin on cell cycle progression in TGFβ induced PC3 cells, flow cytometric analysis was done after Propidium Iodide (PI) DNA staining [27]. Briefly, 1×10^5 cells were seeded per well in a six-well plate and allowed to attach overnight. After serum starvation for 24 h, cells were treated with hesperetin alone, TGFβ alone, and both together. After 48 h of treatment, cells were trypsinized and washed with ice-cold PBS twice, then stored in 70% ethanol at 4 °C overnight. Cells were treated with RNase A and propidium Iodide in saponin-EDTA solution at RT for 30min. The cells were then analyzed for cell cycle distribution using FACS (BD FACS Calibur), and the percentage of cells present in each phase of the cell cycle was calculated.

2.4. Wound healing assay

Cells were grown on 6-well plates at a seeding density of 2.5×10^5 cells per well. These cells were starved in serum-free RPMI-1640 for 24 h. After starvation, a scratch was made in each well using 2–200 μL pipette tips. They were then treated with 5 ng/mL TGFβ and 200 μM hesperetin in 1% FBS, both individually and in combination. DMSO was taken as the solvent control. Images were taken at 0, 4, 12, 24, and 48 h. The wound area was measured at 0hr and 48hr time points using ImageJ 1.52a software.

2.5. Western blot analysis

Cells were grown on 6-well plates at a seeding density of 2.5×10^5 cells per well. These cells were starved in serum-free RPMI-1640 for 24 h. They were then treated with 5 ng/mL TGFβ and 200 μM hesperetin, both individually and in combination. After 48 h of treatment, the cells were scraped in RIPA lysis buffer for protein extraction. Histones were extracted by suspending cells in TEB buffer to lyse the cells. Once the nuclear fraction was pelleted down, it was resuspended in 0.2 N HCl and incubated overnight at 4 °C to acid extract histones, followed by neutralization with 2 M NaOH. Protein concentration was estimated using the Bradford assay. Equal amounts of protein were then subjected to SDS-PAGE in 12% polyacrylamide gels and then blotted onto PVDF membranes (Bio-Rad). The membranes were then blocked in 5% skimmed milk, followed by exposure to primary and secondary antibodies per the manufacturer's instructions. Primary antibodies used were *p*-SMAD3 (Santa Cruz), N-cadherin (Novus), p-*c*-Jun (Abcam), GAPDH (Invitrogen), H3K4me3 (Invitrogen), H3K9me3 (Santa Cruz), H3K27me3 (Abcam) and Total Histone H3 (Invitrogen). The secondary antibodies were Chicken Anti-Mouse IgG H&L (HRP) (Abcam) and Chicken Anti-Rabbit IgG H&L (HRP) (Abcam). The blot was developed using an ECL reagent (Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate).

2.6. Invasion assay

Cells were cultured till 75% confluency and then starved in serum-free media for 24 h. After starvation, cells were trypsinized and washed twice in serum-free media. Approximately 30,000 cells were seeded with 500 μL serum-free media in the upper well of invasion transwell chambers (BD BioCoat 8.0 μm). Complete media with 10% FBS (750 μL) was filled in lower chambers. After 48 h of incubation with 200

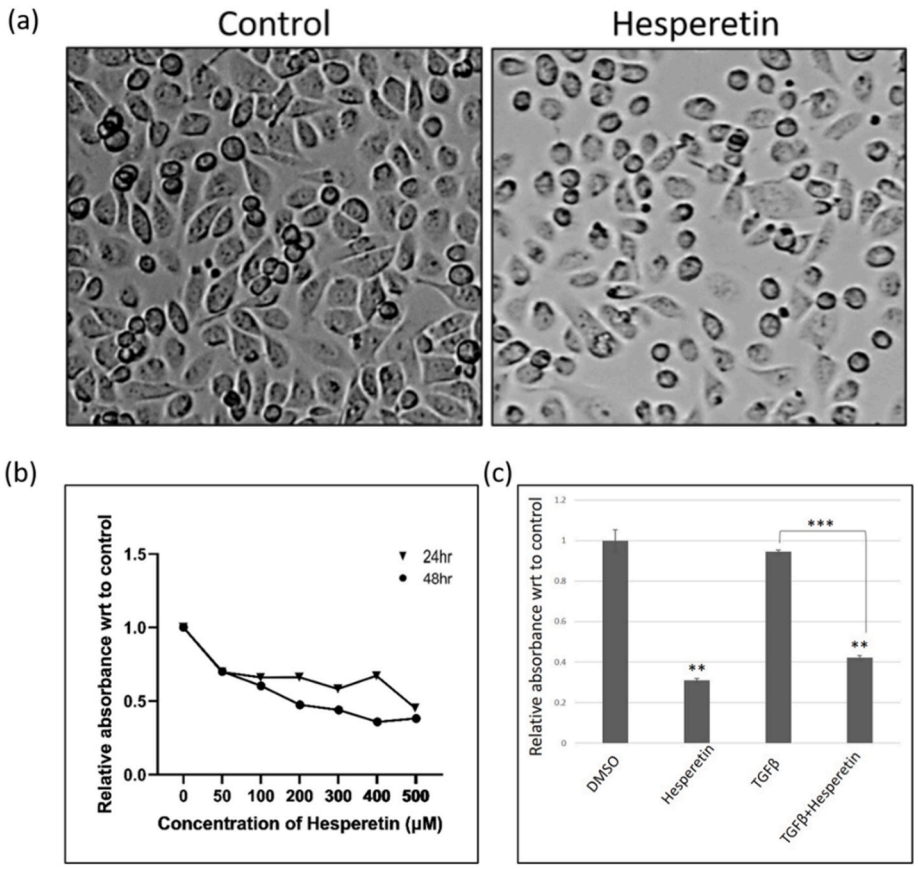


Fig. 1. (a) Microscopic images of PC3 cells with and without treatment of hesperetin, as observed under a light microscope at a total magnification of 100×. (b) MTT assay to determine the effect of hesperetin on cell viability and (c) the combined effect of hesperetin and TGFβ on cell viability. Control represents DMSO-treated cells. **p < 0.01, ***p < 0.001.

μM hesperetin alone, 5 ng/mL TGFβ alone, and the combination of TGFβ and hesperetin, non-invaded cells were removed by cotton swab, and transwell chambers were washed twice with 1xPBS. Invaded cells on the underside of the membrane were fixed and stained with 0.5% crystal violet solution for 30 min at RT. Further cells were de-stained with distilled water till the background was clear. Membranes containing invaded cells were then cut and mounted using DPX mount. The cells were counted under the light microscope at 100× magnification for each sample (n = 3). A minimum of five microscopic fields were counted from each membrane.

2.7. RT PCR

PC3 cells were cultured in a T-75 flask until 70–80% confluency. The cells were then Serum starved for 24 h in a Serum-free Medium. Post serum starvation, TGFβ (Abcam, USA) was added to the cells to a final concentration of 5 ng/mL. TGFβ induction was given for 48hr. The total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. DNA was removed from the samples by the Rnase-free DNase set (Qiagen). The mRNA was then reverse transcribed to cDNA (Eppendorf Mastercycler) with the help of an iScript cDNA Synthesis kit (Bio-Rad, USA). Primers specific for N-cadherin, E-cadherin, and GAPDH with the following sequences were used:

N-cadherin: Forward - 5′-TCTTTGTGCCCATCTCCCAT-3'.
Reverse - 5′-AAGTCCCCAATGTCTCCAGG-3'.
E-cadherin: Forward - 5′- CGGGAATGCAGTTGAGGATC-3'.
Reverse - 5′-AGGATGGTGTAAGCGATGGC-3'.
GAPDH: Forward - 5′-ATGTTCGTCATGGGTGTGAA-3'.
Reverse - 5′-TGTGGTCATGAGTCCTTCCA-3'.

cDNA for N-cadherin, E-cadherin, and GAPDH was amplified (Applied Biosystems Real-Time PCR) using iTaq Universal SYBR Green Supermix (Bio-Rad). The following were the cycling conditions: 30 s at 95 °C, followed by 40 Cycles of 15 s at 95 °C, 1 min at 56 °C, and 30 s at 72 °C. Data was collected and analyzed using QuantStudio Design and Analysis Software v1.5.1.

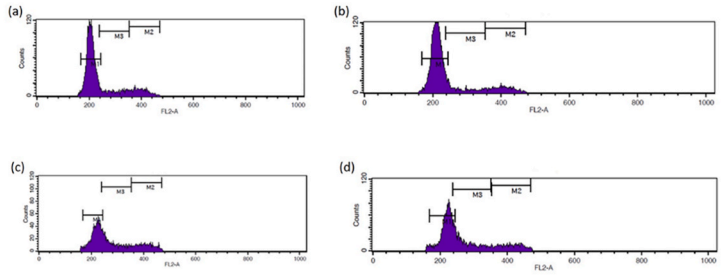
3. Results

3.1. Hesperetin disrupts PC3 cell morphology and decreases the cell viability of PC3 cells in a dose-dependent manner

The PC3 cells, upon treatment with Hesperetin, showed altered morphology. The cells were more spherical than the normal spindle-shaped morphology (Fig. 1a). MTT assay was carried out to determine the effect of hesperetin on the cell viability of PC3 cells. Cells were treated with a range of hesperetin concentrations from 50 μM to 500 μM for 24 h s and 48 h s. Cell viability was significantly decreased by 20–80% ($p < 0.001$) with increasing concentration of hesperetin after 48 h s. IC-50 of hesperetin for PC3 cells was 200 μM (Fig. 1b). We also determined the effect of hesperetin on TGFβ induced PC3 cells and found that hesperetin not only decreases the viability of PC3 cells but also strongly decreases the cell viability even after TGFβ induction. We found that at 200 μM concentration, hesperetin significantly reduces cell viability to almost 50% ($p < 0.001$) in TGFβ induced PC3 cells (Fig. 1c).

3.2. Hesperetin arrests TGFβ induced PC3 cells in the S and G2M phases of the cell cycle

To check the effect of hesperetin on the cell cycle progression of PC3 cells, we performed Flow Cytometry analysis using propidium iodide



	%Cells gated in each phase		
	G1	S	G2M
Control	77.98	11.28	11.4
TGFβ	80.165	12.675	10.83
Hesperetin	50.25	35.595	20.25
TGFβ +Hesperetin	55.44	32.65	17.85

Fig. 2. Cell cycle analysis of PC3 cell line with PI staining using Flow cytometry. (a) Control cells without treatment (b), cells after treatment with TGFβ, (c) treatment with hesperetin, and (d) treatment with TGFβ and hesperetin. Table representing the cell count at different phases of the cell cycle.

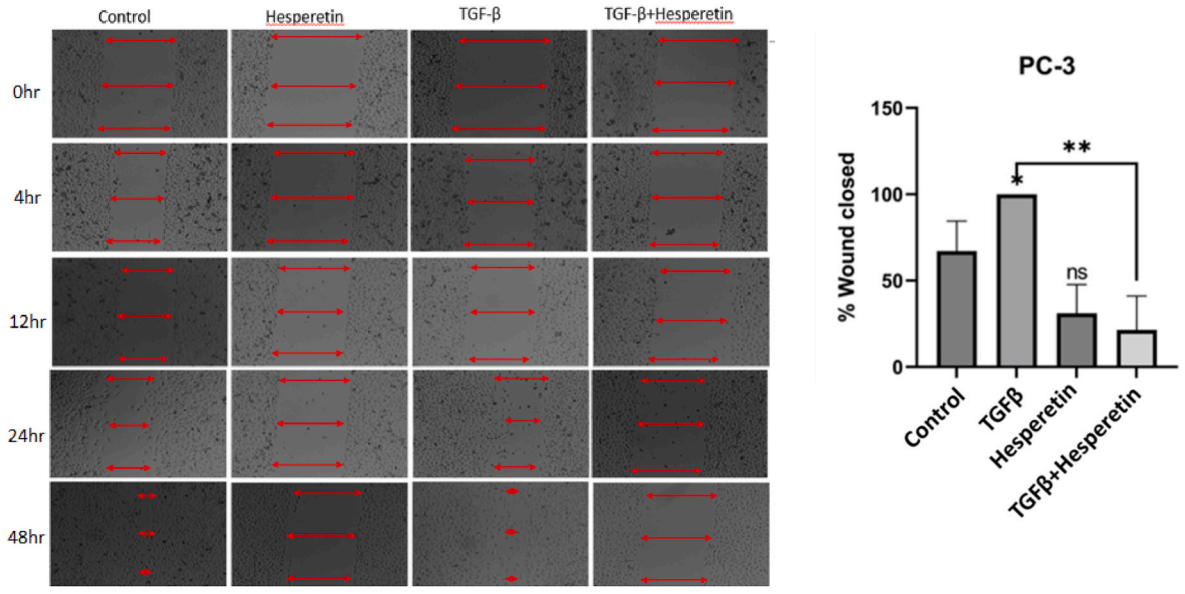


Fig. 3. Microscopic images of PC3 cells as observed under a phase contrast microscope at a magnification of 100×. The graph represents the % of wound area closed with respect to the initial time-point when the wound was created for each sample.

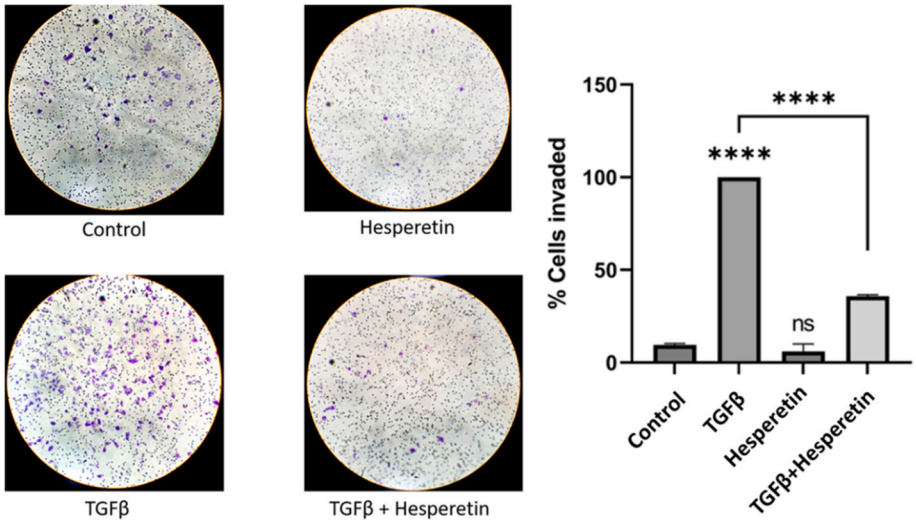


Fig. 4. Invasion assay images were taken under a light microscope at 100× magnification. The bar graph represents the number of cells that invaded the Matrigel. The cells were counted under the light microscope at 100× magnification for each sample (n = 3) using the ImageJ software. Five microscopic fields were counted from each membrane.

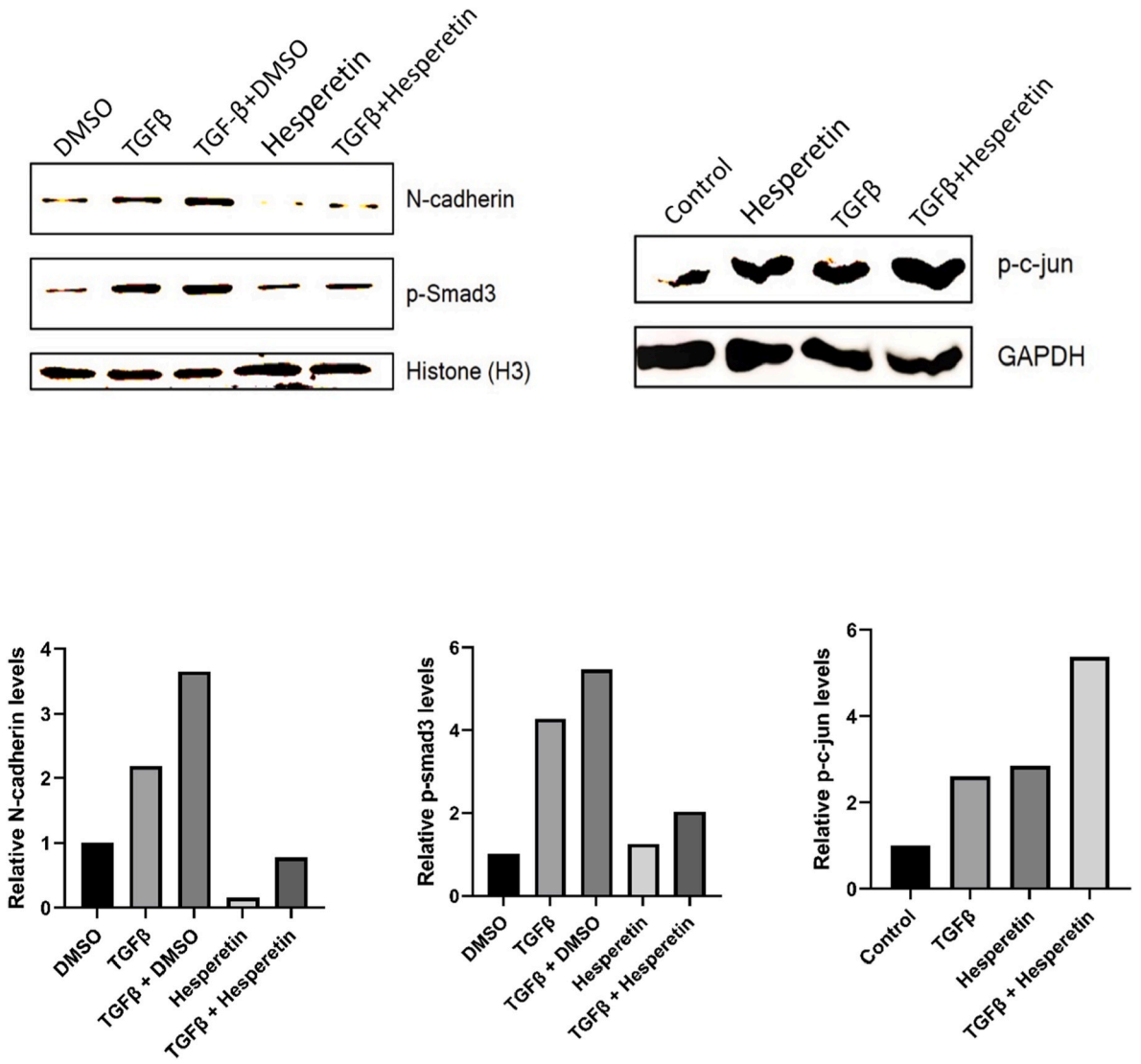


Fig. 5. Western blot images of the levels of (a) *p*-smad3, N-cadherin, and (b) p-*c*-Jun, after treatment with hesperetin in the presence and absence of TGFβ. Histone (H3) and GAPDH were taken as loading controls. The bar graphs indicate relative protein levels measured using ImageJ software.

DNA staining. Cell cycle analysis showed that cells treated with hesperetin were arrested in the cell cycle's S and G2M phases (Fig. 2). Almost 50% of the Hesperetin-treated cells were arrested collectively in both these phases of the cell cycle. In hesperetin-treated cells, 35.6% of the cells were arrested in the S phase and 20.3% in the G2M phase. In cells that were given a combination of TGFβ and hesperetin, 32.6% of the cells were arrested in the S phase, and 17.9% of the cells were arrested in the G2M phase of the cell cycle.

3.3. *Hesperetin significantly reduces migration of TGFβ-induced PC3 cells*

The scratch assay was carried out to determine the effect of hesperetin on the migration of TGFβ-induced PC3 cells. Our data suggest that hesperetin significantly inhibited the migration of TGFβ-induced PC3 cells and prevented them from closing the scratched wound compared to the control cells. In Control and TGFβ treated cells, the wound was closed in 48 h s, whereas Hesperetin-treated cells with and without TGFβ showed a limited closure of only 20% of the wound even after 48 h s (Fig. 3).

3.4. *Hesperetin decreases the invasion of TGFβ induced PC3 cells*

Invasion is an important marker of cancer metastasis which is modulated by many molecular players; one of them is TGFβ. To explore the effect of hesperetin on TGFβ induced invasion of PC3 cells in-vitro, we performed a matrigel invasion assay using Matrigel-coated Boyden chambers. Our findings suggest that TGFβ significantly induced the invasion of PC3 cells. At the same time, hesperetin showed an approximate 50% inhibition in the invasive capacity of PC3 cells when used alone and in combination with TGFβ. Conclusively, our data suggest that hesperetin inhibits the TGFβ induced invasion of PC3 cells (Fig. 4).

3.5. *Hesperetin inhibits canonical TGFβ signalling pathway and EMT*

We carried out a Western blot analysis to check if hesperetin acts through the canonical or non-canonical TGFβ signaling pathway. We checked the levels of *p*-smad3 as well as p-*c*-Jun, respectively. *p*-smad3 levels were reduced to 0.68-fold in the presence of hesperetin as compared to TGFβ treated cells (Fig. 5a). At the same time, the TGFβ induced expression of p-*c*-Jun only increased in the presence of Hesperetin (Fig. 5b). To confirm the effect of hesperetin on EMT, we checked the levels of N-cadherin, which is a molecular marker of EMT.

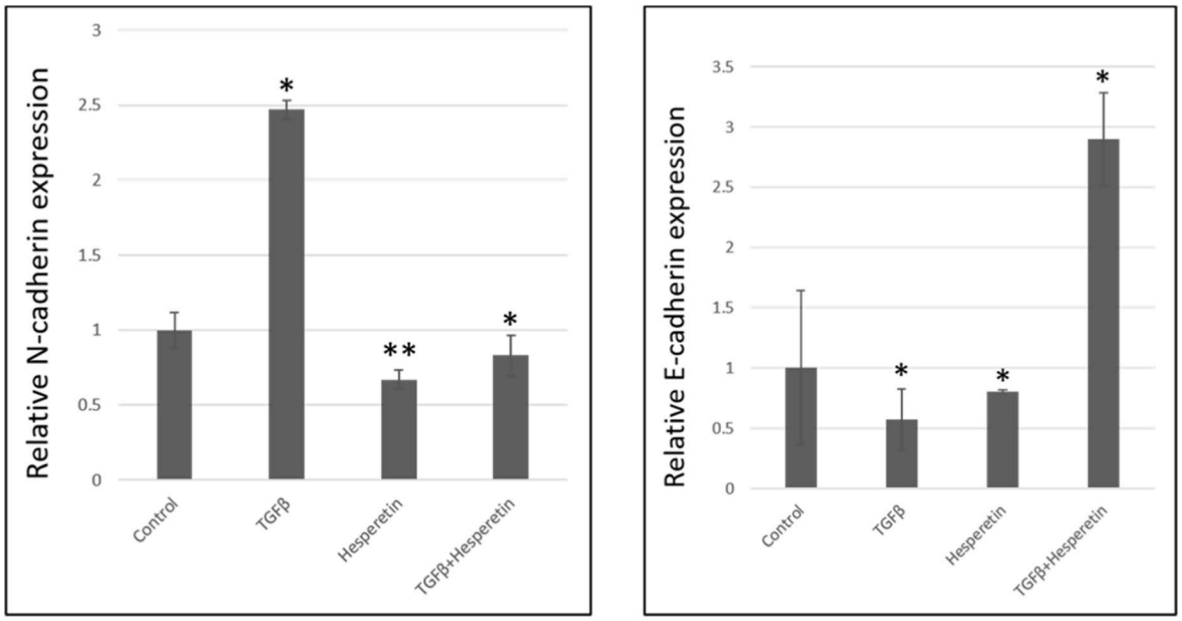


Fig. 6. RT-PCR analysis of N-cadherin and E-cadherin. The graph represents the relative quantification of N-cad and E-cad mRNA in PC3 cells upon treatment with hesperetin in the absence and presence of TGFβ. GAPDH was taken as the endogenous gene control, and the gene expression was normalized with respect to the control. *$p < 0.05$, **$p < 0.01$, $n = 3$.

N-cadherin levels decreased drastically in Hesperetin-treated cells, both in the presence and absence of TGFβ (Fig. 5a). This was further confirmed by RT-PCR results, which again show that the transcript levels of N-cadherin decreased and that of E-cadherin increased upon hesperetin treatment in the presence TGFβ (Fig. 6). This means that hesperetin affects TGFβ signalling mainly via the canonical SMAD signalling pathway.

3.6. *Effect of hesperetin on common histone methylation marks*

We looked at the levels of the commonly studied histone modifications, H3K27me3, H3K4me3, and H3K9me3, upon treatment with hesperetin in response to TGFβ. All the histone methylation marks investigated seem to have an increased methylation level in response to TGFβ. We found that there was an accumulation of both H3K4me3 and H3K27me3 levels after treatment with hesperetin. There was a decrease in the H3K9me3 levels when hesperetin was given in the presence of TGFβ, whereas hesperetin alone significantly increased the H3K9me3 levels compared to the control (Fig. 7).

4. Discussion

Most of the anticancer drugs available have serious side effects because of their non-specificity. Many plant metabolites are explored to overcome this problem and are found to be effective with no known severe side effects. Hesperetin, a citrus bioflavonoid, is a metabolite with many properties to combat cancer [5,11]. Therefore, in our study, we aimed to look at the effect of hesperetin on the viability of PC3 cells. Since most cancer deaths are associated with metastasis, we mainly looked at the ability of hesperetin to inhibit the invasive and migratory capabilities of PC3 cells. For metastasis to occur, the cells must transition from epithelial to mesenchymal state, and TGFβ is a known inducer of EMT.

Different studies have demonstrated that varying concentrations of hesperetin can have different cellular effects on prostate cancer cells. Cell proliferation and viability were reduced when PC3 cells were exposed to hesperetin (0–700 μM). Hesperetin (12.5–200 μM) was associated with cell cycle arrest at the G1/S phase and induced apoptosis of PC3 cells [26]. In PC3 cells, hesperetin (400–500 μM) has been shown to increase the expression of IL-6, thus increasing phosphorylation of STAT3, ERK, and AKT, leading to the arrest of cells in the G0/G1 phase. We found that hesperetin inhibited cell proliferation mainly by arresting the cell cycle at the S and G2M phases in prostate cancer cells. Similar results were observed in breast cancer cells wherein hesperetin inhibited cell viability by arresting them at the G2/M phase of the cell cycle [28]. We observed inhibition of the canonical TGFβ signalling pathway in the presence of hesperetin as the levels of TGFβ-induced psmad3 were decreased in the presence of hesperetin. The non-canonical signalling pathway remains unaffected as the levels of p-*c*-Jun were not affected in the presence of hesperetin. A study published by Yang Y et al. showed hesperetin to inhibit TGFβ signalling in HeLa cells by inhibiting phosphorylation of Smad3, thereby confirming our results [15]. N-cadherin and E-cadherin are the key molecular markers of EMT. Epithelial marker E-cadherin expression decreases with TGFβ while the mesenchymal marker N-cadherin increases with TGFβ suggesting an essential role of TGFβ in the process of EMT. Our data indicate that hesperetin can drastically decrease the expression of TGFβ induced N-cadherin and increase the TGFβ inhibited E-cadherin expression. For confirmation, we carried out functional assays such as migration assay and invasion assay where hesperetin inhibited both invasive and migratory abilities of PC3 cells both in the presence and absence of TGFβ. So far, similar observations have been made only in A549 cells [29] and podocytes [30]. Studies in breast cancer showed that hesperetin reduces cell viability by inducing apoptosis [15], accumulation of ROS, and activation of the ASK1/JNK pathway [28]. In the human pancreatic cell line, it has been shown that hesperetin triggered apoptosis by inhibiting NF-κB expression.

A recent study suggested that hesperetin can reduce the methylation of histone H3 at the K79 position while keeping the methylation at other lysines (K4, K9, K27) intact, thus inhibiting the expression of genes related to the metastatic and invasive capability of gastric cancer cells [25]. We looked for the histone K9, K4, and K27 trimethylation in PC3 cells in response to TGFβ and hesperetin. Unlike gastric cancer cells, hesperetin showed an effect on the trimethylation of histone H3 K9, K4, and K27 in the PC3 cells. The trimethylation of all the lysines (K9, K4, K27) increases with TGFβ, and hesperetin can affect the trimethylation individually or in combination with TGFβ. Further studies are required to understand the role of these histone methylation marks in TGFβ

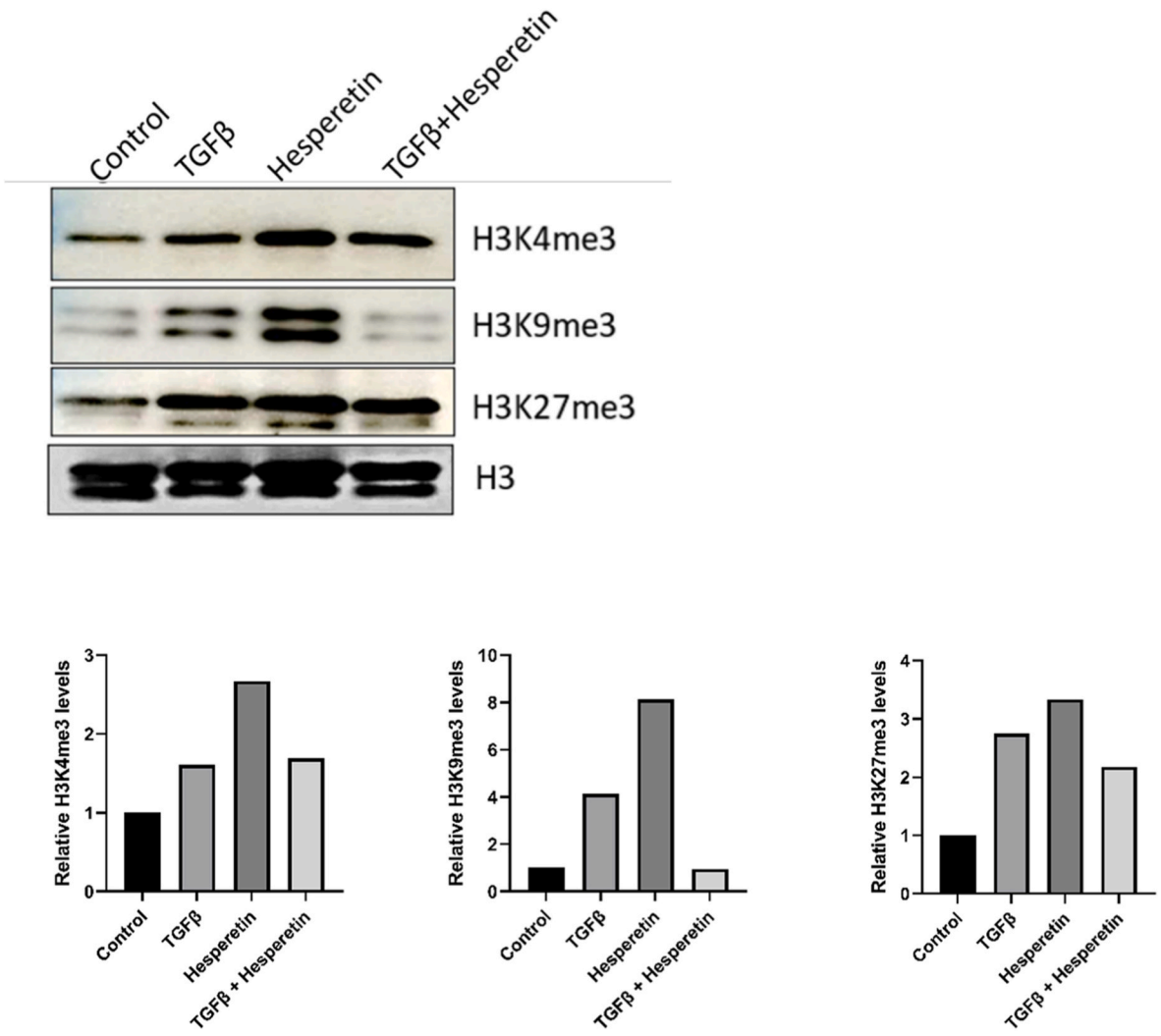


Fig. 7. Western blot of H3K4me3, H3K9me3, and H3K27me3, after treatment with hesperetin in the presence and absence of TGFβ in PC3 cells. Total histone (H3) was taken as the loading control. The bar graphs indicate relative protein levels measured using ImageJ software.

induced EMT, and the mechanism hesperetin can modulate these marks to prevent the invasion and migration of cancer cells.

It would be interesting to investigate the molecular mechanism by which hesperetin inhibits TGFβ induced invasion and migration of PC3 cells. In future studies, we aim to investigate the molecular targets of hesperetin involved in cell cycle arrest and TGFβ signalling. Plant-derived compounds are known to increase the efficacy of marketed drugs. Therefore, we also plan to do a combinatorial study of hesperetin with an approved anticancer drug against prostate cancer to see if it enhances its efficacy and reduces side effects.

5. Conclusion

In our study, hesperetin was found to inhibit cell proliferation mainly by arresting cells at the S and G2M phases of the cell cycle. Western blot data also showed that hesperetin inhibited canonical TGFβ signalling via decreasing *p*-smad3 levels. Reduced N-cadherin and increased E-cadherin expression indicate that hesperetin also inhibits TGFβ induced EMT in PC3 cells. These results were confirmed by migration and invasion assay where hesperetin inhibited both invasive and migratory abilities of PC3 cells in the presence and absence of TGFβ. In all the experiments, it was consistently seen that hesperetin alone significantly reduces cell proliferation, migration, and invasion of PC3 cells. Hence, our study shows that hesperetin is potent in inhibiting TGFβ mediated cell proliferation and EMT in PC3 cells.

Hesperetin also altered the epigenetic landscape by altering the trimethylation levels of H3K4, H3K9, and H3K27. The trimethylation of H3K4 and H3K27 was increased as compared to the control. The H3K9me3 levels were increased in the presence of hesperetin alone but were decreased when hesperetin was given after TGFβ induction. The mechanistic outcome needs to be studied further to get a deeper understanding of these histone modifications in regulating TGFβ induced EMT.

For further studies, we are keen to investigate the molecular targets of hesperetin involved in the modulation of cell cycle progression and TGFβ signalling. It would be interesting to investigate which pathways are involved in hesperetin-mediated modulation of TGFβ induced cell proliferation and EMT in PC3 cells. We are also interested in exploring the effect of hesperetin in combination with an approved anticancer drug against prostate cancer. It might be more potent in eradicating cancer with minimum side effects.

Conclusively our study found hesperetin as a promising future candidate against prostate cancer. More vigorous research is required to better understand the mechanism and effects of hesperetin in to fight against prostate cancer.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by funding from the Science and Engineering Research Board (SERB), Government of India (EMR/2017/001987) and from Ahmedabad University Start-Up Grant (URB-SG7518) to Dr. Noopur Thakur.

Declaration of competing 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.

Data availability

No data was used for the research described in the article.

Acknowledgements

Authors acknowledge Human Resource Development Group, Council of Scientific and Industrial Research (CSIR-HRDG), Government of India for Nidhi Dalpatraj's fellowship and Department of Science and Technology (DST), Government of India for Ankit Naik's INSPIRE-fellowship.

References

[1] M. Dhupal, D. Chowdhury, Phytochemical-based nanomedicine for advanced cancer theranostics: perspectives on clinical trials to clinical use, Int. J. Nanomed. 15 (2020) 9125–9157, https://doi.org/10.2147/IJN.S259628.
[2] A.S. Choudhari, P.C. Mandave, M. Deshpande, P. Ranjekar, O. Prakash, Corrigendum: phytochemicals in cancer treatment: from preclinical studies to clinical practice, Front. Pharmacol. 11 (2020) 175, https://doi.org/10.3389/fphar.2020.00175.
[3] C. Manach, A. Scalbert, C. Morand, C. Rémésy, L. Jiménez, Polyphenols: food sources and bioavailability, Am. J. Clin. Nutr. 79 (5) (2004) 727–747, https://doi.org/10.1093/ajcn/79.5.727.
[4] J.K. Aschoff, K.M. Riedl, J.L. Cooperstone, J. Högel, A. Bosy-Westphal, S. J. Schwartz, et al., Urinary excretion of citrus flavanones and their major catabolites after consumption of fresh oranges and pasteurized orange juice: a randomized cross-over study, Mol. Nutr. Food Res. 60 (12) (2016) 2602–2610, https://doi.org/10.1002/mnfr.201600315.
[5] H. Parhiz, A. Roohbakhsh, F. Soltani, R. Rezaee, M. Iranshahi, Antioxidant and anti-inflammatory properties of the citrus flavonoids hesperidin and hesperetin: an updated review of their molecular mechanisms and experimental models, Phytother Res. 29 (3) (2015) 323–331, https://doi.org/10.1002/ptr.5256.
[6] M.S.A. Aboody, M.S. Al Aboody, S. Mickymaray, Anti-fungal efficacy and mechanisms of flavonoids, Antibiotics 9 (2) (2020) 45, https://doi.org/10.3390/antibiotics9020045.
[7] A. Paredes, M. Alzuru, J. Mendez, M. Rodríguez-Ortega, Anti-Sindbis activity of flavanones hesperetin and naringenin, Biol. Pharm. Bull. 26 (1) (2003) 108–109, https://doi.org/10.1248/bpb.26.108.
[8] H. Kim, W. Jeon, B. Ko, Flavanone glycosides from citrus junos and their anti-influenza virus activity, Planta Med. 67 (6) (2001) 548–549, https://doi.org/10.1055/s-2001-16484.
[9] E. De Clercq, Potential antivirals and antiviral strategies against SARS coronavirus infections, Expert Rev. Anti Infect. Ther. 4 (2) (2006) 291–302, https://doi.org/10.1586/14787210.4.2.291.
[10] A. Hirata, Y. Murakami, M. Shoji, Y. Kadoma, S. Fujisawa, Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression, Anticancer Res. 25 (5) (2005) 3367–3374.
[11] J.M.P. Ferreira de Oliveira, C. Santos, E. Fernandes, Therapeutic potential of hesperidin and its aglycone hesperetin: cell cycle regulation and apoptosis induction in cancer models, Phytomedicine 73 (2020), 152887, https://doi.org/10.1016/j.phymed.2019.152887.
[12] S. Sambantham, M. Radha, A. Paramasivam, B. Anandan, R. Malathi, S.R. Chandra, et al., Molecular mechanism underlying hesperetin-induced apoptosis by in silico analysis and in prostate cancer PC-3 cells, Asian Pac. J. Cancer Prev. APJCP 14 (7) (2013) 4347–4352, https://doi.org/10.7314/apjcp.2013.14.7.4347.
[13] P. He, J. Ma, Y. Liu, H. Deng, W. Dong, Hesperetin promotes Cisplatin−Induced apoptosis of gastric cancer in vitro and in vivo by upregulating PTEN expression, Front. Pharmacol. 11 (2020), https://doi.org/10.3389/fphar.2020.01326.
[14] Y. Yang, J. Wolfram, K. Boom, X. Fang, H. Shen, M. Ferrari, Hesperetin impairs glucose uptake and inhibits proliferation of breast cancer cells, Cell Biochem. Funct. 31 (5) (2013) 374–379, https://doi.org/10.1002/cbf.2905.
[15] Y. Yang, J. Wolfram, H. Shen, X. Fang, M. Ferrari, Hesperetin: an inhibitor of the transforming growth factor-β (TGF-β) signaling pathway, Eur. J. Med. Chem. 58 (2012) 390–395, https://doi.org/10.1016/j.ejmech.2012.10.028.
[16] M. Ersoz, A. Erdemir, D. Duranoglu, D. Uzunoglu, T. Arasoglu, S. Derman, et al., Comparative evaluation of hesperetin loaded nanoparticles for anticancer activity against C6 glioma cancer cells, Artif. Cell Nanomed. Biotechnol. 47 (1) (2019) 319–329, https://doi.org/10.1080/21691401.2018.1556213.
[17] P.N. Patel, X.-M. Yu, R. Jaskula-Sztul, H. Chen, Hesperetin activates the Notch1 signaling cascade, causes apoptosis, and induces cellular differentiation in anaplastic thyroid cancer, Ann. Surg Oncol. 21 (Suppl 4) (2014) S497–S504, https://doi.org/10.1245/s10434-013-3459-7.
[18] M. Shirzad, E. Heidarian, P. Beshkar, M. Gholami-Arjenaki, Biological effects of hesperetin on interleukin-6/phosphorylated signal transducer and activator of transcription 3 pathway signaling in prostate cancer PC3 cells, Pharmacogn. Res. 9 (2) (2017) 188–194, https://doi.org/10.4103/0974-8490.204655.
[19] S.W. Leslie, T.L. Soon-Sutton, H. Sajjad, L.E. Siref, Prostate Cancer. StatPearls. Treasure Island (FL), StatPearls Publishing, 2021.
[20] H. Sung, J. Ferlay, R.L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, et al., Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA A Cancer J. Clin. 71 (3) (2021) 209–249, https://doi.org/10.3322/caac.21660.
[21] M.A. Nieto, Epithelial-Mesenchymal Transitions in development and disease: old views and new perspectives, Int. J. Dev. Biol. 53 (8–10) (2009) 1541–1547, https://doi.org/10.1387/ijdb.072410mn.
[22] H. Škovierovï¿½, Okajčekovï¿½ Tz, Jn Strnï¿½del, E. Vidomanovï¿½, E. Halašovï ¿½, Molecular regulation of epithelial-to-mesenchymal transition in tumorigenesis (Review), Int. J. Mol. Med. (2017), https://doi.org/10.3892/ijmm.2017.3320.
[23] R. Kalluri, R.A. Weinberg, The basics of epithelial-mesenchymal transition, J. Clin. Invest. 119 (6) (2009) 1420–1428, https://doi.org/10.1172/JCI39104.
[24] S. Liu, S. Chen, J. Zeng, TGF-β signaling: a complex role in tumorigenesis (Review), Mol. Med. Rep. 17 (1) (2018) 699–704, https://doi.org/10.3892/mmr.2017.7970.
[25] S.W. Wang, H. Sheng, F. Zheng, F. Zhang, Hesperetin promotes DOT1L degradation and reduces histone H3K79 methylation to inhibit gastric cancer metastasis, Phytomedicine 84 (2021), 153499, https://doi.org/10.1016/j.phymed.2021.153499.
[26] M. Sohel, H. Sultana, T. Sultana, M. Al Amin, S. Aktar, M.C. Ali, et al., Chemotherapeutic potential of hesperetin for cancer treatment, with mechanistic insights: a comprehensive review, Heliyon 8 (1) (2022), e08815, https://doi.org/10.1016/j.heliyon.2022.e08815.
[27] Effects of IL-1 and cortisol on β-adrenergic receptors, cell proliferation, and differentiation in cultured human A549 lung tumor cells, Lung Cancer 7 (3) (1991) 186–187, https://doi.org/10.1016/0169-5002(91)90123-n.
[28] I.P. Nurhayati, A. Khumaira, G.P.N. Ilmawati, E. Meiyanto, A. Hermawan, Cytotoxic and antimetastatic activity of hesperetin and doxorubicin combination toward Her2 expressing breast cancer cells, Asian Pac. J. Cancer Prev. APJCP 21 (5) (2020) 1259–1267, https://doi.org/10.31557/APJCP.2020.21.5.1259.
[29] Z. Ren, J. Shen, X. Mei, H. Dong, J. Li, H. Yu, Hesperidin inhibits the epithelial to mesenchymal transition induced by transforming growth factor-β1 in A549 cells through Smad signaling in the cytoplasm, Brazilian Journal of Pharmaceutical Sciences 55 (2019), https://doi.org/10.1590/s2175-97902019000218172.
[30] D. Choi, C.-L. Kim, J.E. Kim, J.-S. Mo, H.-S. Jeong, Hesperetin inhibit EMT in TGF-β treated podocyte by regulation of mTOR pathway, Biochem. Biophys. Res. Commun. 528 (1) (2020) 154–159, https://doi.org/10.1016/j.bbrc.2020.05.087.