Hesperetin Modulates TGF-β Induced Invasion and Migration in Prostate Cancer Cells

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Abstract

Hesperetin is known to possess anti-microbial, anti-fungal, antioxidant and anti-cancer properties along with good bioavailability. TGF-β induced cell proliferation and EMT are well known to have pivotal roles in metastatic prostate cancer. Therefore, in this study we investigated the effect of Hesperetin on TGF-β induced cell proliferation and EMT in prostate cancer cells (PC3). To explore the effect of hesperetin on cell viability, MTT assay was carried out. Effect of hesperetin on cell cycle progression of PC3 cells was measured using FACS. Wound healing assay and Invasion assay were done to investigate the effect of hesperetin on TGF-β induced migration and invasion of PC3 cells. Western blot technique was performed to investigate whether the canonical Smad signaling pathway is disrupted by Hesperetin and whether the TGF-β induced EMT markers like N-cadherin were changed. We found that hesperetin significantly inhibited cell proliferation of PC3 cells. Cells were also arrested in the S and G2M phase of cell cycle in the presence of hesperetin. Results of invasion and migration assay decipher the inhibitory effect of hesperetin on TGF-β induced invasion and migration in prostate cancer cells. Western blot analysis confirmed that hesperetin significantly decreased the expression of pSmad3 and N-cadherin which are molecular markers of canonical TGF- β signalling and EMT respectively. Hence, results of our study identified the potential of hesperetin to modulate TGF-β induced cell proliferation as well as invasion and migration of prostate cancer cells which may help to hinder the metastatic growth of prostate cancer cells.

Keywords

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INTRODUCTION

Phytochemicals have been important chemopreventive as well as chemotherapeutic agents since the last decades. Researchers from the globe are trying to find the most effective phytochemical against many cancer types because they have good efficacy and lesser side effects as 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 very few were approved by the FDA. Few such as Vincristine [1], Irinotecan, and Topotecan [2], are the FDA approved phytochemicals against different cancers. The major 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 longer bioavailability than the pure compound.

Hesperetin is one such metabolite of hesperidin. Hesperidin is a citrus flavanone (3, 5, 7-trihydroxy-4-methoxy-flavanone) present in oranges and lemons

[3]. Hesperetin is an aglycone of hesperidin which is released during digestion via hydrolysis by the colonic micro-biota [4]. It has been proven to have anti-inflammatory [5], anti-fungal [6], anti-viral [7][8][9], anti-oxidant [10], and anti-cancer [11][12] properties. As an anti-cancer compound, 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* as well as *in-vitro*, but effects of hesperetin are yet to be explored against other important aspects of metastatic prostate cancer. Altogether, the properties of hesperetin gave us the insight to look at its effect on TGF-β induced invasion and migration in PC3 cells.

Prostate cancer occurs due to malfunctioned cells of the prostate gland in men. Major risk factors of 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 huge numbers of incidents and mortality rates make prostate cancer the second most frequent cancer and the fifth leading cause of cancer deaths worldwide [20]. Though in India incidence and mortality rates are lesser in comparison to the world, data suggests that prostate cancer incidents are consistently increasing year after year [21]. However, consistent research in the field of medicine 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 difficult to combat metastatic types of prostate cancer. So, we are interested in exploring the preventive as well as 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 has a vast role to play from embryonic development to tissue regeneration, organ fibrosis, and tumour progression in adults [22]. In tumorigenesis, EMT involves a transition of the tumour cells from epithelial state to mesenchymal state which allows their migration and invasion at different sites [23]. 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. As 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 TGF-β signalling

pathway [24]. The TGF-β signalling pathway is renowned to induce EMT during cancer progression. TGF-β is a member of a large family of cytokines, which is involved in a variety of functions such as regulation of cell growth, differentiation, apoptosis, tissue repair, and modulation of the immune system [25]. TGF-β signalling pathway is essential to regulate crucial events like cell growth, apoptosis, invasion, and migration along with cancer metastasis. TGF-β 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 [25]. Therefore, in our study, we used TGF-β to induce invasiveness of aggressive prostate cancer cells namely PC3 and were interested to explore the effect of hesperetin on TGF-β induced invasion and migration of PC3 cells. Results of our study suggest that hesperetin inhibits cell proliferation and arrests the cell cycle in the S and G2M phase. Hesperetin also inhibited TGF-β induced migration and invasion of PC3 cells via canonical Smad signaling pathway.

MATERIALS AND METHODS
Cells and Culture Conditions
PC3 cells were acquired from Dr. Sanjay Singh's lab at the Biological and Life Sciences, Ahmedabad University (Ahmedabad, India). The cells were grown in RPMI-1640 (HiMedia) supplemented with 10% fetal bovine serum (Gibco) and 1% Antibiotic-Antimycotic solution (HiMedia) at 37ºC in a 5% CO_2 atmosphere. PC3 cells were trypsinized at 80-90% confluency using 0.25% Trypsin-EDTA (HiMedia).

MTT Assay
Cell proliferation, cell cytotoxicity and cell viability were checked using the Meta thiazolyl tetrazolium (MTT) assay. To determine the effect of Hesperetin (Sigma-Aldrich), the PC3 cells were exposed to different concentrations (50μM, 100μM, 200μM, 300μM, 400μM, 500μM) of Hesperetin for 48hrs and 72hrs. Dimethyl Sulfoxide (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 viability of TGF-β 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 hours. This was followed by treatment with 5ng/ml TGF-β and 200μM hesperetin, both individually as well as in combination. MTT assay was carried out as mentioned above. Student's t-test was

carried out for calculating significance using Sigma-plot software v14.5.

Cell Cycle Analysis

To determine the effect of hesperetin on cell cycle progression in TGF-β induced PC3 cells, flow cytometric analysis was done after Propidium Iodide (PI) DNA staining [26]. Briefly, 1 x 10^5 cells were seeded per well in a 6 well plate and allowed to attach overnight. After serum starvation for 24 hours, cells were treated with hesperetin alone, TGF-β alone, and both together. After 48 hours of treatment, cells were trypsinized and washed with ice-cold PBS twice then stored in 70% ethanol at 4ºC overnight. Cells were then 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 Flow-Cytometer (BD FACSCalibur™) and the percentage of cells present in each phase of the cell cycle was calculated.

Wound Healing Assay

Cells were grown on 6-well plates at a seeding density of 2.5 x 10^5 cells per well. These cells were starved in serum-free RPMI-1640 for 24 hours. After starvation, a scratch was made in each well using 2-200µl pipette tips. They were then treated with 5ng/ml TGF-β and 200µM hesperetin in 1% FBS, both individually as well as in combination. DMSO was taken as the solvent control. Images were taken at 0, 4, 12, 24 and 48 hours, and the width of the wound was measured to determine the closure of the wound. The average width from three different points of the same wound was considered for final analysis [26].

Western Blot analysis

Cells were grown on 6-well plates at a seeding density of 2.5 x 10^5 cells per well. These cells were starved in serum-free RPMI-1640 for 24 hours. They were then treated with 5ng/ml TGF-β and 200µM hesperetin, both individually as well as in combination. After 48 hours of treatment, the cells were scraped in RIPA lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Sodium Deoxycholate, 1.0 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide at a pH of 7.4), for protein extraction. 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. The membranes were then blocked in 5% skimmed milk followed by exposure to primary and secondary antibodies as per the manufacturer's instructions. Primary antibodies used were p-SMAD3 (Santa Cruz), N-cadherin (Novus), p-c-Jun (Abcam) and GAPDH (Invitrogen) and 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 ECL reagent (Thermo Scientific SuperSignal West Femto Maximum Sensitivity Substrate).

Invasion Assay

Cells were cultured till 75% confluency and then starved in serum-free media for 24 hours. After starvation cells were trypsinized and washed twice in serum-free media. Approximately 30,000 cells were seeded with 500µl serum-free media in the upper well of invasion transwell chambers (BD BioCoat 8.0 micron). Complete media with 10% FBS (750µl) was filled in lower chambers. After 48 hrs of incubation with 200µM hesperetin alone, 5ng/ml TGF-β alone, and the combination of TGF-β 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 then counted under the light microscope at 100X magnification for each sample (n=3). A minimum of five microscopic fields were counted from each membrane.

RT PCR

PC3 cells were cultured in T-75 flask until 70-80% confluency. The cells were then Serum starved for 24 hours in Serum-free Medium. Post serum starvation, TGF-β (Abcam, USA) was added to the cells to a final concentration of 5ng/mL. TGF-β 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 iScript cDNA Synthesis kit (Biorad, USA). Primers specific for N-cadherin and GAPDH with the following sequences were used:
N-cadherin:
Forward - 5'-TCTTTGTGCCCATCTCCCAT-3'
Reverse - 5'-AAGTCCCCAATGTCTCCAGG-3'
GAPDH: Forward - 5'-ATGTTCGTCATGGGTGTGAA-3'
Reverse - 5'-TGTGGTCATGAGTCCTTCCA-3'
cDNA for N-cadherin and GAPDH was amplified (Applied Biosystems Real Time PCR) using iTaq Universal SYBR Green Supermix (Biorad). The following were the cycling conditions: 30 sec at 95ºC, followed by 40 Cycles of 15 sec at 95ºC, 1 min at 56ºC and 30 sec at 72ºC. Data was collected and analyzed using QuantStudio Design and Analysis Software v1.5.1.

RESULTS

Hesperetin decreases cell viability of PC3 cells in a dose-dependent manner.

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 50µM to 500µM. Cell viability was significantly decreased by 20 to 80% (p<0.001) with increasing concentration of Hesperetin after 48hrs. IC-50 of hesperetin for PC3 cells was 200µM. We also determined the effect of hesperetin on TGF-β induced PC3 cells and found that hesperetin not only decreases the viability of PC3 cells but also strongly decreases the cell viability even after TGF-β induction. We found that at 200µM concentration, hesperetin significantly decreases cell viability to almost 50% (p<0.001) in TGF-β induced PC3 cells (Figure 1).

Figure 1: MTT assay to determine the effect of Hesperetin on cell viability and at the combined effect of Hesperetin and TGF-β on cell viability. Control represents DMSO treated cells. **p<0.01, *** p<0.001.
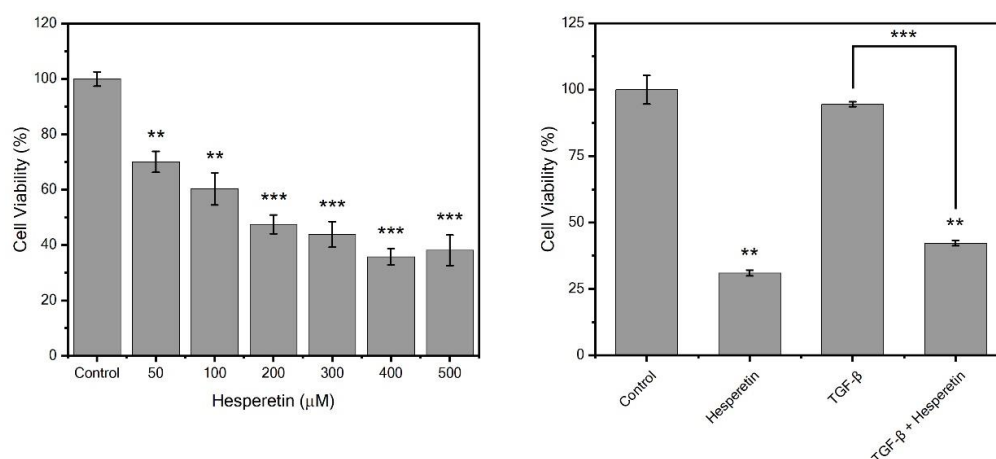

Hesperetin arrests TGF-β induced PC3 cells in the S and G2M phases of the cell cycle.

In order to check the effect of hesperetin on the cell cycle progression of PC3 cells, we carried out Flow Cytometry analysis by using propidium iodide DNA staining. Cell cycle analysis showed that cells treated with Hesperetin were arrested in the S and G2M phase of the cell cycle (Figure 2). Almost about 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% of the cells were arrested in the G2M phase. Cells which were given a combination of TGF 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.

Figure 2: Cell cycle analysis of PC3 cell line with PI staining using Flow cytometry. (a) control cells without treatment (b), cells after treatment with TGF-β (c) treatment with hesperetin (d) treatment with TGF- β and hesperetin. Table representing the cell count at different phases of the cell cycle.
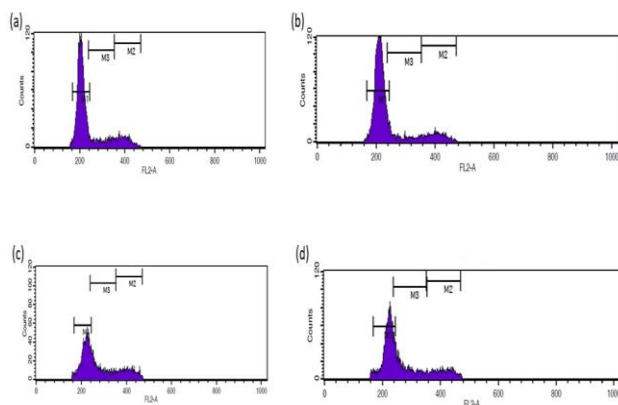

	% Cells gated in each phase		
	G1	S	G2M
Control	77.98	11.28	11.4
TGF-β	80.165	12.675	10.83
Hesperetin	50.25	35.595	20.25
TGF-β +Hesperetin	55.44	32.65	17.85

Hesperetin significantly reduces migration of TGF-β induced PC3 cells.

Scratch assay was carried out to determine the effect of hesperetin on the migration of TGF-β induced PC3 cells. Our data suggest that hesperetin significantly inhibited the migration of TGF-β induced PC3 cells and prevented them from closing the scratch wound as compared to control cells. In Control and TGF-β treated cells the wound was closed in 48hrs, whereas Hesperetin treated cells showed a limited closure of only 20% of the wound even after 48hrs (Figure 3).

Figure 3: Microscopic images of PC3 cells as observed under phase contrast microscope at a magnification of 100x. Graph represents the % of wound width closed with respect to 0 hrs time-point of each sample.
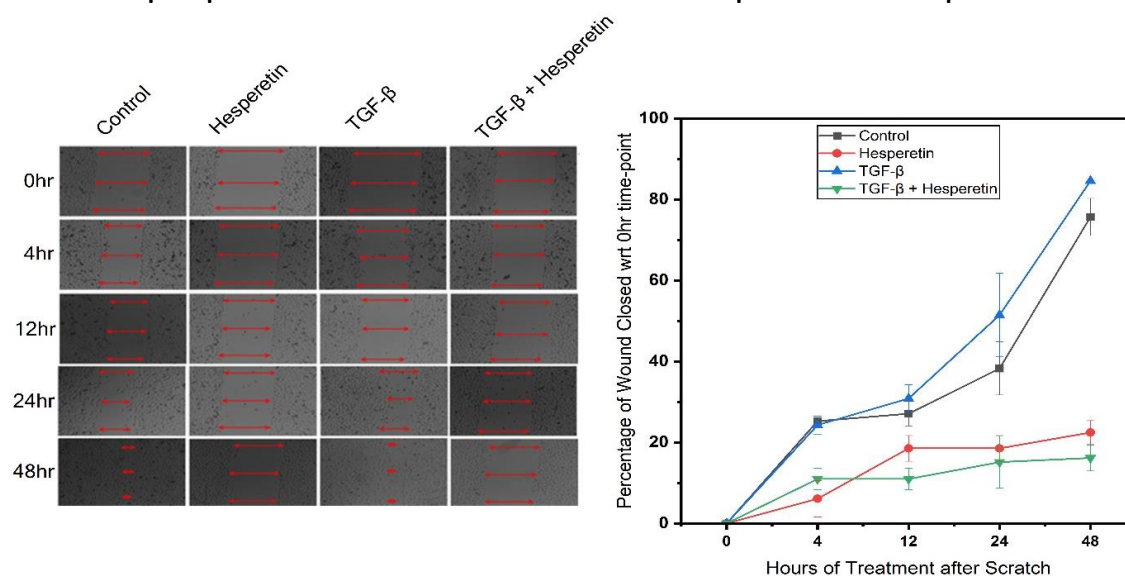

Hesperetin moderately decreases invasion of TGF-β induced PC3 cells.

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

Figure 4: Invasion assay images taken under a light microscope at 100x magnification. Bar graph represents the number of cells that invaded across the Matrigel. The cells were counted under the light microscope at 100X magnification for each sample (n=3) using the ImageJ software. Five microscopic fields were counted from each membrane.
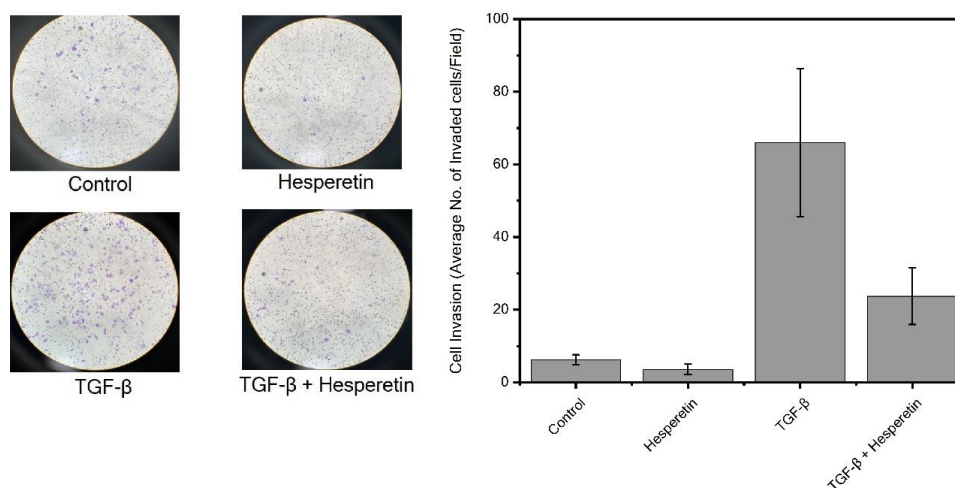
Hesperetin inhibits TGF-β signaling and EMT.
In order to find out if hesperetin inhibits TGF-β signalling, we carried out western blot analysis and checked the levels of smad3 as it is the downstream molecule in the TGF-β pathway. p-smad3 levels were reduced to 0.68-fold in the presence of hesperetin as compared to TGF-β treated cells (Figure 5a). In order to confirm the effect of hesperetin on EMT, we checked the levels of N-cadherin, which is a molecular marker of EMT. N-cadherin levels decreased drastically in hesperetin treated cells, both in the presence and absence of TGF-β (Figure 5a). This was further confirmed by RT-PCR results which again show that the transcript levels of N-cadherin decreased upon hesperetin treatment, both in the presence and absence of TGF-β (Figure 6). In order to check if the non-canonical pathway is affected or not, we checked the levels of p-c-Jun, a non-canonical downstream molecule of TGF-β. We found that there was no significant difference in the levels of p-c-Jun (Figure 5b). This indicated that hesperetin affects TGF-β signaling mainly via the canonical SMAD signaling pathway.

Figure 5: Western blot images of the levels of (a) p-smad3, N-cadherin and (b) p-c-Jun, after treatment with TGF-β in the presence and absence of TGF-β. Histone (H3) and GAPDH were taken as loading controls.
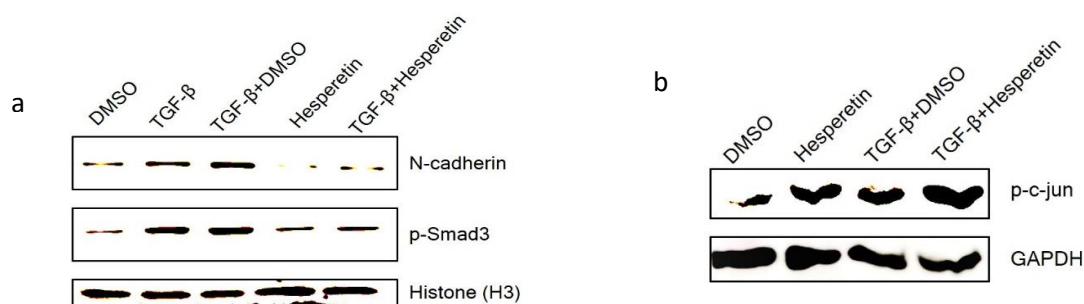
Figure 6: RT-PCR analysis of N-cadherin. The graph represents the relative quantification of N-cad mRNA in PC3 cells upon treatment with hesperetin in the absence and presence of TGF-β. GAPDH was taken as the endogenous gene control and the gene expression is normalized with respect to control.
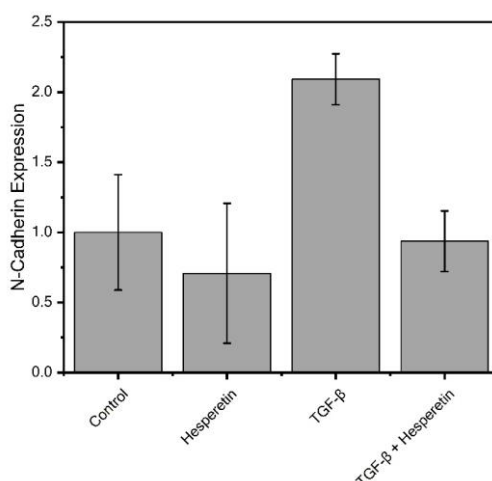
DISCUSSION
Majority of the anticancer drugs that are available have serious side effects because of their non-specificity. Many plant metabolites are explored in order to overcome this problem and are found to be effective with no known serious side effects. Hesperetin, a citrus bioflavonoid, is one such metabolite which possesses 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 the majority of 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 have to undergo a transition from epithelial to mesenchymal state and TGF-β is a known inducer of EMT.

We found that hesperetin inhibited cell proliferation mainly by arresting cell cycle at the S and G2M phases. Similar results were shown in one study wherein hesperetin inhibited cell viability of breast cancer cells by arresting them at the G2/M phase of

the cell cycle [27]. Western blot data showed that hesperetin strongly inhibited TGF-β signaling by decreasing the TGF-β induced p-smad3 levels and thus affecting the canonical signaling pathway and not the non-canonical signaling pathway as the level of p-c-Jun was not affected in presence of hesperetin. Study published by Yang Y, et al. shown to inhibit TGF-β signaling in HeLa cells by inhibiting phosphorylation of Smad3 thereby confirming our results [15]. N-cadherin is a key molecular marker of EMT. Our data suggest that hesperetin drastically decreased the expression of N-cadherin which may lead to inhibition of TGF-β induced EMT in PC3 cells. For confirmation, we carried out functional assays such as migration assay and invasion assay where hesperetin inhibited both invasive as well as migratory abilities of PC3 cells both in the presence and absence of TGF-β. So far similar observations have been made only in A549 cells [28], and podocytes [29].

Studies in breast cancer showed that hesperetin reduces cell viability by inducing apoptosis [15], accumulation of ROS and activation of ASK1/JNK pathway [27]. It would be interesting to investigate the molecular mechanism in detail by which hesperetin inhibits TGF-β induced invasion and migration of PC3 cells. As future studies, we aim to investigate the molecular targets of hesperetin involved in cell cycle arrest and TGF-β signaling. Plant derived compounds are known to increase 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/or reduces side effects.

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 TGF-β signalling via decreasing p-smad3 levels. Decreased expression of N-cadherin indicates that hesperetin also inhibits TGF-β induced EMT in PC3 cells. These results were confirmed by migration and invasion assay where hesperetin inhibited both invasive as well as migratory abilities of PC3 cells in the presence and absence of TGF-β. In all the experiments it was consistently seen that hesperetin alone significantly reduces cell proliferation as well as migration and invasion of PC3 cells. Hence our study gives an insight that hesperetin is potent to inhibit TGF-β mediated cell proliferation and EMT in PC3 cells.

For further studies, we are keen to investigate the molecular targets of hesperetin involved in modulation of cell cycle progression and TGF-β signalling. It would be interesting to investigate which pathways are involved in hesperetin mediated modulation of TGF-β 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, as 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 to fight against prostate cancer.

Declaration of conflicting interests:
The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions:
ND and JT performed the experiments. NT designed the experiment. AN performed and analyzed the qPCR experiment. ND, JT, AN and NT prepared the manuscript. NT supervised the study. All authors read and approved the manuscript.

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