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Effect of Selected Pure Compounds of Plant Origin on the Proliferation, Adhesion, Migration, and Invasion of Human Prostate Cancer Cells

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ABSTRACT

Signalling cascade of autocrine/paracrine growth factors plays an important role in promoting prostate cancer (PCa) cells growth, survival, migration, invasion, and progression. PCa is one of the most important causes of death in men, thus new therapeutic approaches are needed. The objective of this study is to investigate whether α-terpinene, *β*-caryophyllene, linalool, luteolin, and quercetin exert antiproliferative, anti-adhesion, anti-migratory, and anti-invasion activity in PCa cell line models. The results showed that all five compounds exhibited growth inhibitory activities on both PCa cell lines (PC3 and DU145). Luteolin was the most potent antiproliferative compound against PC3 and DU145 cells with IC_{50} values of 70.19 µM and 37.46 µM, respectively. A number of techniques, including cell adhesion, wound healing, Transwell migration and invasion assays were used to investigate the ability of these compounds to inhibit migration/invasion of PCa cells at non-toxic concentrations. Our findings highlight the potential of luteolin and quercetin to inhibit the invasion and migration of PC3 and DU145 cells without causing cytotoxicity. However, further studies are still needed to reveal their molecular mechanisms of action.

Keywords: Antiproliferative activity, Medicinal plants, Natural Products, Prostate cancer.

Introduction

Prostate cancer (PCa) is a leading cause of death in men in the developing countries.[1] It has been estimated that PCa death cases in 2019 will exceed 31,000 cases in the US, where PCa is the second-leading cause of cancer death among men.[2,3] PCa is the third most common cause of death among males in Jordan, accounting for 6.2% of the total cancer cases.[4] The mortality rate of PCa remains high, as prostate cancer cells have the tendency to migrate to lymph glands through the lymphatic and circulatory systems.[5]

The signaling cascade of autocrine/paracrine growth factors plays an important role in promoting prostate cancer cells growth, survival, migration, and progression. In its early stages, PCa is hormone-dependent, and can be controlled using the hormone-ablation therapy that suppresses the rate of PCa growth. However, the cancer overcomes its hormone-dependence in advanced stages leading to castration-resistant PCa that can metastasize to the lungs, liver and bones.[6] Conventional chemotherapy has poor outcome and causes severe side effects. Therefore, there is an urgent need to develop new agents with improved activity and side effect profiles. Testing plant extracts and natural products has been widely practised in the pharmaceutical industry. It has been a valuable source of drug leads for many years.[7] Numerous *in vitro* studies have been conducted to explore the cytotoxic or chemopreventive activity of naturally-occurring compounds and identify their respective molecular mechanisms of action.

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In the current work, we highlight the antiproliferative, anti-adhesion, anti-migratory, and anti-invasion activity of selected pure compounds of plant origin against PCa cell line models, PC3 and DU145.

In an effort to sample different classes of natural products, it was decided to include two monoterpenes that are commonly found in the essential oils of several plants (*α*-terpinene and linalool), a sesquiterpene (*β*-caryophyllene) and two flavonoids that exhibited a wide variety of pharmacological activities (luteolin and quercetin).[8,9] The antiproliferative activities of Jordanian flora rich in these secondary metabolites were previously identified in our laboratory.[10-12]

Materials and Methods

Materials

All material used were either high purity grade or tissue culture grade when needed. The pure compounds under investigation were isolated, purified and identified in our laboratories.

Human cancer cell lines and culture conditions

All cell lines were grown in recommended media and specified additives according to their source, American Type Culture Collection (ATCC™). Fibroblast (HDFa) cells were cultured in a high glucose Dulbecco's minimum essential medium (DMEM, Euro-Clone™, Italy) supplemented with 10% fetal bovine serum (FBS; Biowest, USA), penicillin (100 I.U./mL) and streptomycin (100 µg/mL) (Euro-Clone™, Italy). PC3 and DU145 cells were cultured in RPMI 1640 medium (Euro-Clone™, Italy) supplemented with 10% FBS, penicillin (100 I.U /mL) and streptomycin (100 µg/ mL). Cells were grown and cultured under strict sterile conditions at 37°C in a humidified environment containing 5% CO_2 incubator.

Cell viability using sulforhodamine B colorimetric (SRB) assay and MTT assay

The antiproliferative activity of the pure compounds was investigated using the Sulforhodamine B (SRB) colorimetric assay as previously

described by Skehan *et al*. with slight modifications.[10] Serial dilutions of the five natural compounds were prepared using tissue culture media followed by incubation for 72 h. Cell viability (whenever needed) was done using the MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, USA) over two different incubation periods of 24 h and 48 h.

In vitro cell adhesion assay
The cell adhesion assay was performed as previously described with slight modifications.[13] In brief, 96-well flat-bottomed culture plates were coated with 50 µL of fibronectin (25 µg/mL) (R&D systems, UK) in phosphate buffered saline (PBS) overnight at 4°C. The plates were then blocked with 0.2% FBS and incubated at 37°C for 1 h. Next, the plates were washed three times with serum free media RPMI 1640.
PC3 and DU145 cells were grown to 80% confluency in serum-free RPMI 1640. The plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 overnight, before being treated with the determined IC_{10} value (using the SRB assay) for each compound in the respective cell line. The cells were collected and diluted to 500,000 cell/mL, and 50 µL of the diluted cells were added to each well of the coated 96-well plates in quadruplicate and incubated for 30 min at 37°C. Subsequently, the unbound cells were removed and attached cells were quantified using MTT assay. After subtraction of background cell binding to bovine serum albumin-coated wells, the percentage of adherent cells was calculated by dividing the optical density of the treated adherent cells divided by that of the control cells multiplied by 100.

In vitro wound healing assay
The cell-cell interaction and their migration potential were investigated using the wound healing assay. PC3 and DU145 cells were seeded into 6-well culture plates and grown to confluence. The attached monolayer cells were carefully scratched using a sterile plastic 200 µL pipette tip. The scratched monolayers were washed twice with PBS. Then the compounds under investigation were added to the pre-labeled wells and incubated for 24 h at 37°C, 5% CO_2. IC_{10} concentrations were used for each compound in respective cell line. Negative control which contains 0.3%DMSO was included. The cultures were photographed immediately, incubated at 37°C, CO_2 5%, and monitored for 24 hours. After 24 h, the media was removed, and the cells were washed and fixed with methanol then crystal violet dye was added to the fixed cells for 10 min, then the excess dye was removed and washed. The wells were photographed immediately. Wound closure was monitored with an inverted microscope (Carl *Zeiss*™ *Primo Vert*™ Microscope, Germany).

In vitro cells trans-migration assay
The migration capability of the PCa cell lines, PC3 and DU145 cells was investigated with or without the indicated treatments using a Trans-well inserts system. The BD BioCoat™ Migration Insert (BD Biosciences™) was used. PC3 and DU145 cells were grown to 80% confluency and starved in serum-free RPMI 1640 for 20 h. After hydration, the cells were trypsinized, centrifuged, and suspended at 10^6 cells/mL in serum-free RPMI 1640 media. Approximately 1×10^5 of both cell lines with a final volume 1500 µL of serum-free RPMI 1640 was mixed with either luteolin (at 35.1 µM for PC3 cells and 18.73 µM for DU145 cells) or quercetin (at 50 µM for both PC3 and DU145 cells) in the upper chambers/inserts. Negative controls which contain 0.3% DMSO were included in the assay. The lower chambers contained 10% FBS in RPMI 1640. The plates were incubated for 22 h. At the end of the assay time, the media from the upper inserts were removed and the cells that did not migrate were carefully removed with a cotton swab, and the inner inserts were washed with PBS twice. The cells that migrated through the membrane pores were fixed by 3.7% formaldehyde for 10 min then permeabilized by 100% methanol. Finally, cells on the lower side of the membrane were stained with freshly prepared 1% crystal violet for 10 min at room temperature (RT). The cells that migrated were digitally photographed under 400X magnification using a light microscope. The experiments were performed in duplicates and five random fields were subjected to cell counting and used for photography. The migration index was calculated as the mean ± SD of cells counted per-field. Pure natural compound-treated cells were compared to control cells.

In vitro cell invasion assay
The invasion ability of PCa cell lines, PC3 and DU145 cells was investigated with or without the indicated treatment using Cultrex® 24-Well BME Cell Invasion Assay Kit (Catalog #: 3455-024-K, Trevigen™). Briefly, the Transwell porous membrane was coated with the supplied initial stock of the Basement Membrane Extract (BME), then the coated membrane inserts were incubated at 37°C in a CO_2 incubator overnight. PC3 and DU145 cells were grown to 80% confluency and starved in serum-free RPMI 1640 at 37 °C, in a humidified atmosphere of 5% CO_2 for 20 h. The cells then were trypsinized, centrifuged, and suspended at 10^5 cells/mL in serum free RPMI 1640 media. Approximately 1×10^5 of each cell line were mixed without or with each test compound at the IC_{10} of the respective cell line and then were seeded into the upper pre-coated membrane inserts. DMSO (0.3%) was used as negative control. The bottom chambers contained 10% FBS of RPMI 1640, then the plates were transferred and incubated at 37 °C in an atmosphere of 5% CO_2. PC3 cells were allowed to invade for 24 h and DU145 cells for 48 h. At the end of the treatment period, the top surfaces of the membrane inserts were aspirated and dissolution solution that had been premixed with calcien-AM substrate was added to the lower chamber. The plates were incubated at 37°C in a 5% CO_2 incubator for 60 min. Finally, assay wells solutions (lower chamber) were read at 485 nm excitation, 520 nm emission using a multi-mode microplate reader (Synergy™ HTX, BioTek™, USA).

Statistical analysis
All results were expressed as means ± SDs. The concentration resulted in 50% inhibition of the cell grow (IC_{50}) was calculated using the dose-response curve obtained by plotting the percentage of viability versus the concentrations GraphPad Prism (version 7.0 for Windows, USA). Data obtained from viability, adhesion, migration, invasion assays were analyzed using the SPSS™ software using Student's *t*-test ($P < 0.05$) to compare the effect of different treatments and their respective controls. Differences at *p*-value ≤ 0.05 were considered statistically significant.

Results and Discussion

The objective of the present study is to determine the inhibitory effect of α-terpinene, *β*-caryophyllene, linalool, luteolin, and quercetin on the viability, adhesion, migration and invasion of PCa cells. This goes along with our previous efforts aiming to discover new anticancer agents based on naturally-occurring compounds. All five natural compounds exerted antiproliferative activity on the selected PCa cell lines.

Determination of IC_{50} of α-terpinene, β-caryophyllene, linalool, luteolin, and quercetin against PC3 and DU145 cells after 72 h exposure
The antiproliferative activity after incubation of PC3 and DU145 cells with serial dilutions of α-terpinene, *β*-caryophyllene, linalool, luteolin and quercetin were determined and expressed as the concentration that resulted in half inhibition in cell viability (IC_{50}) compared to a negative control as shown in Table 1. The results indicated that cell growth was strongly inhibited in a concentration-dependent pattern by these compounds as shown in Figure 1.
α -terpinene has been previously reported to have anti-inflammatory, antioxidant, antibacterial, and moderate cytotoxic activities against various cancer cells lines and normal fibroblast cells (WS1).[14] Nevertheless, it has not been documented to have antiproliferative activity against prostate cancer cells nor any effects on the invasiveness of these cells. β -caryophyllene is also known to exhibit selective anti-proliferative effects against colorectal cancer cells and induce apoptosis. Furthermore, β-caryophyllene demonstrated

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potent inhibition against clonogenicity, migration, invasion and formation in colon cancer cells.[15] β -caryophyllene exerted cytotoxic spheroid activity on colorectal carcinoma and less cytotoxicity on human breast adenocarcinoma cells.[16] Linalool had shown antiproliferative activity against SW 620, Hep G2, A-549, and T-47D cells via inducing apoptosis and activating antitumor immunity.[17] Linalool showed stronger inhibitory effect on PC-3 cells than on DU145 cells. The PC-3 cells were mainly arrested in the G_2/M phase. In the xenograft model with PC-3 cell transplantation, linalool significantly suppressed tumor growth.[18] Luteolin has been proven to induce apoptosis in prostate DU145 cells through up-regulation of death receptor 5.[19] It also suppresses cell growth and proliferation of DU145 cells, and PC-3 in *in vivo* models by inhibiting insulin like growth factor 1 (IGF-1) and the subsequent activation of IGF-1R, AKT, EGFR, and MAPK/ERK signaling.[20]

Except for quercetin, all compounds exerted higher potency towards DU145 cells than to PC3 cells. The most potent antiproliferative compound against PC3 and DU145 cell lines was luteolin. In this study, quercetin was used as a positive control as it is a potent sensitizer by down regulating signaling molecules thereby decreasing cell survival, proliferation, migration and invasion of PCa cells. [21]
α -terpinene, β-caryophyllene, linalool, luteolin, and quercetin possessed higher selectivity towards PCa cells. The most selective compound towards PC3 and DU145 cells was α-terpinene with SI values of 4.0 and 6.4, respectively. The molecular mechanism for this selectivity is not yet understood, however, it could be partially induced by selective apoptotic activity of the tested compounds.

Evaluation of the in vitro inhibition activity of α-terpinene, β-caryophyllene, linalool, luteolin and quercetin on PC3 and DU145 cells' adhesion potential after 24 h
The fibronectin cell-adhesion assay was used to investigate the *in vitro* inhibition effect of *α*-terpinene, *β*-caryophyllene, linalool, luteolin and quercetin on PC3 and DU145 PCa cell adhesion potential. After 24 h of treatment, the results demonstrated that cell adhesion of both PCa (PC3 and DU145) cells was only slightly reduced by α-terpinene, luteolin and linalool. On the contrary, β-caryophyllene (87.0% and 91.7% for PC3 and DU145 respectively) and quercetin (81.0% and 92.0% for PC3 and DU145 respectively) exhibited significant reduction on adhesion ($p < 0.05$).

Evaluation of the in vitro inhibition activity of α-terpinene, β-caryophyllene, linalool, luteolin, and quercetin on PC3 and DU145 cells wound healing process after 24 h and transwell migration
The *in vitro* inhibition effect of α-terpinene, β-caryophyllene, linalool, luteolin and quercetin on PC3 and DU145 cancer cell migration and motility potential were studied using the scratch wound migration assay. The migration and motility ability of PC3 and DU145 cells after 24 h of treatment with luteolin and quercetin were significantly reduced in a dose-dependent manner without resulting in any morphological changes. Low concentration of α-terpinene (63.4 and 40.3 μM) did not affect the wound healing process of both of PC3 and DU145 cells, but at high concentrations it led to the detachment and death of the cells. The inhibitory effects of β-caryophyllene and linalool on PC3 and DU145 cells migration were more pronounced at higher concentration (155.7, 47.3 μM), (190.4, 152.0 μM), (190.1, 120.8 μM), respectively. In contrary to these results, Zhao *et al* (2017) reported a significant inhibition of the migration of both DU145 and PC3 cells by luteolin, which were dose- and time-dependent.[18] In another study, wound recovery was inhibited by luteolin in PC3 cells expressing high levels of ANO1 (a transmembrane protein also known as 16A). [22] The results are shown in Figures 2 and 3.
In the Transwell migration experiments, our findings illustrated that the tested cell lines exhibited different migration characteristics; PC3 cells migrated more, as proved by cells counting, than DU145 cells. After 22 h of incubation with luteolin and quercetin, the migration of PC3 and DU145 cells was significantly inhibited with a *p*-value ($p < 0.05$) (Table 2). Earlier, it was reported that quercetin inhibited the migration of PC3 cells through down-regulation of uPA, uPAR and EGF, EGF-R mRNA expressions.[23]

Luteolin and Quercetin inhibits PC3 and DU145 cells invasion process
To investigate the anti-invasion effects of luteolin and quercetin on PC3 and DU145 cancer cells, BME coated membrane Transwell invasion assay was used. Luteolin and quercetin, at the concentrations 35.1 μM and 50.0 μM, respectively for PC3, and 18.7 μM and 50.0 μM, respectively for DU145 cells, inhibited the invasion of both cell lines significantly and without significant cytotoxic effects on cells viability (Figure 4).
The result showed that the percentage of PC3 cells invaded to the lower chamber was significantly reduced after 24 h treatment with luteolin and quercetin, and that of DU145 cells after 48 h (Figure 4 a, b). Only the activity of luteolin was significant with a *p*-value < 0.05.
To exclude the gravity effect and the efficiency of BME membrane that should be fit with the tested cancer cell lines, controls included cells without any treatment and chemotaxis gradient effect, i.e. the upper insert contains the cells in serum-free media without treatment, and the lower chamber contains serum-free media.
Zhou *et al*. (2009) reported that luteolin induced the expression of E-cadherin in PCa PC3 cells, leading to inhibition of invasion of PC3 cells.[24] During carcinogenesis, PCa cells acquire mesenchymal characteristics and migratory features concomitant with a loss of epithelial characteristics such as E-cadherin expression.[23,26] Luteolin (10-40 μM) also suppresses angiogenesis and invasion through the down regulation of VEGF-2R in PC3 cells.[27] Similarly, it has been reported that quercetin inhibits invasion of PC3 cells through the down-regulation of uPA, uPAR and EGF, EGF-R mRNA expressions.[21] Our results suggest that luteolin and quercetin inhibit the migration and invasion of PCa cells. Thus, it shows the potential of these compounds in the treatment of invasiveness and metastasis of PCa

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Table 1: Antiproliferative activity of compounds under investigation

Pure natural compound	IC_{50} values (μM) ± S.E			Selectivity Index	
	HDFa	PC3	DU145	PC3	DU145
α-terpinene	512.2 ± 16.1	126.7 ± 8.4	80.5 ± 3.8	4.0	6.4
β-caryophyllene	408.2 ± 11.6	103.8 ± 5.8	94.5 ± 7.7	3.9	4.2
Linalool	408.1 ± 22.3	126.9 ± 3.3	101.3 ± 6.2	3.2	4.0
Luteolin	225.7 ± 19.2	70.19 ± 6.1	37.5 ± 2.4	3.2	6.0
Quercetin	375.3 ± 25.1	99.3 ± 4.7	101.1± 3.9	3.8	3.7

Results present the average of IC_{50} ± SD value of pure compounds on different cell lines after three days of culture. Results present the average and standard error of quadruplicate wells.

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Table 2: The effect of luteolin, and quercetin treatment on the migration potential of PC3 and DU145 cancer cells

Pure natural compound	Number of PC3 cells per five different fields (% of migrated cells compared with control)	Number of DU145 cells per five different fields (% of migrated cells compared with control)
Luteolin	36 ± 3 (67.13)	30 ± 2 (71.43)
Quercetin	41 ± 2 (75.93)	31 ± 3 (73.81)
Control	54 ± 3 (100)	42 ± 2 (100)

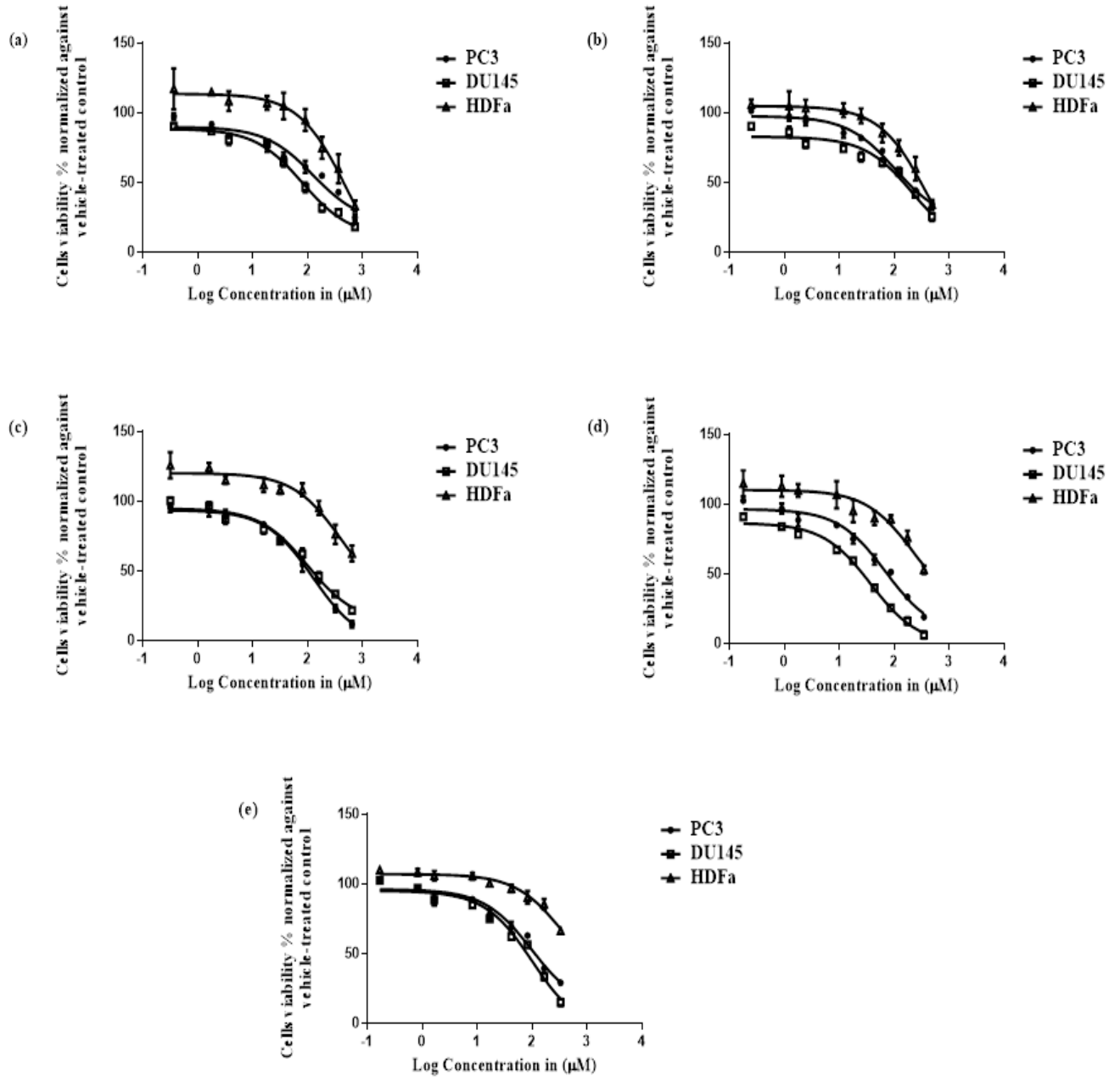

Figure 1: Determination of IC_{50} values for α-terpinene **(a)** *β*-caryophyllene **(b)** linalool **(c)** luteolin and quer**(d)** cetin **(e)**. Each value represents a mean of quadruplicate wells. Experiments were performed twice at different passage number. Error bars are within the symbols

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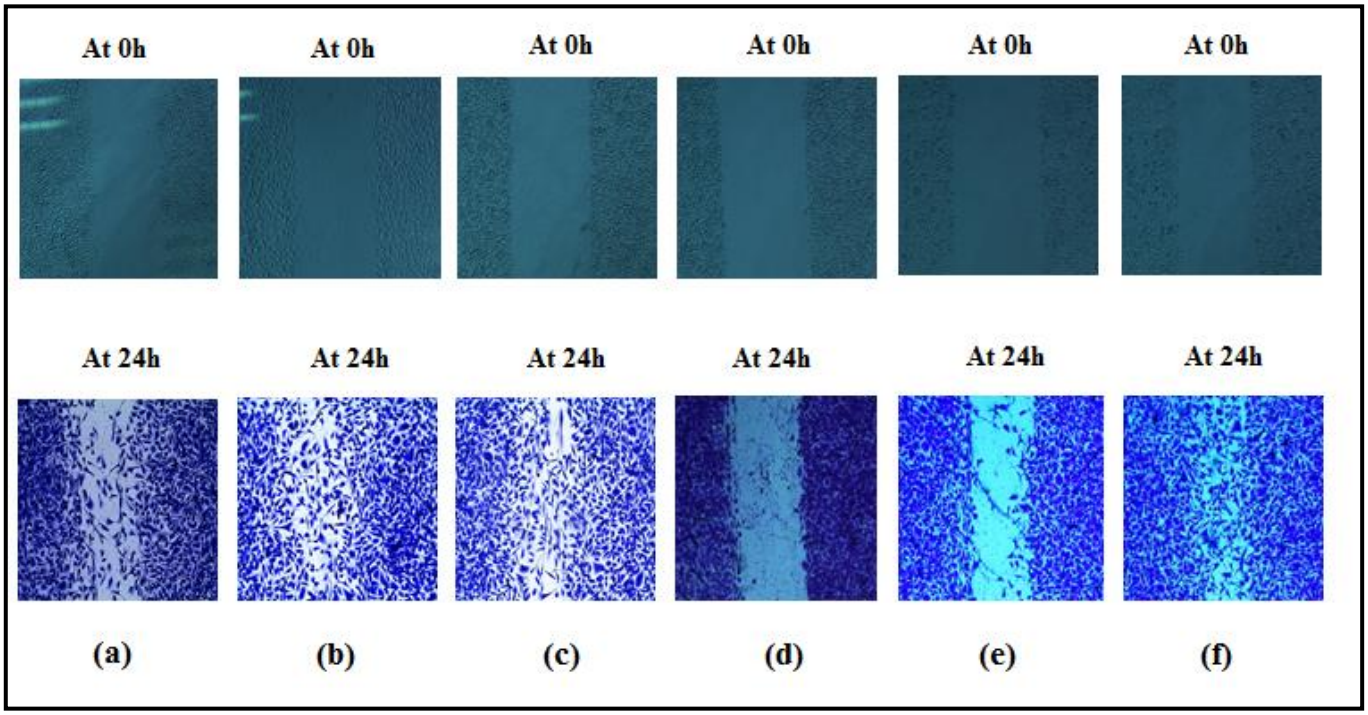

Figure 2: Effects of *α*-terpinene, *β*-caryophyllene, linalool, luteolin and quercetin treatment on the migration potential of PC3 cells in the scratch wound migration assay. (a) 63.4 µM *α*-terpinene treatment group. b) 51.9 µM *β*-caryophyllene treatment group. c) 63.4 µM linalool treatment group. d) 35.1 µM luteolin treatment group. e) 50.0 µM quercetin treatment group. f) Control group.

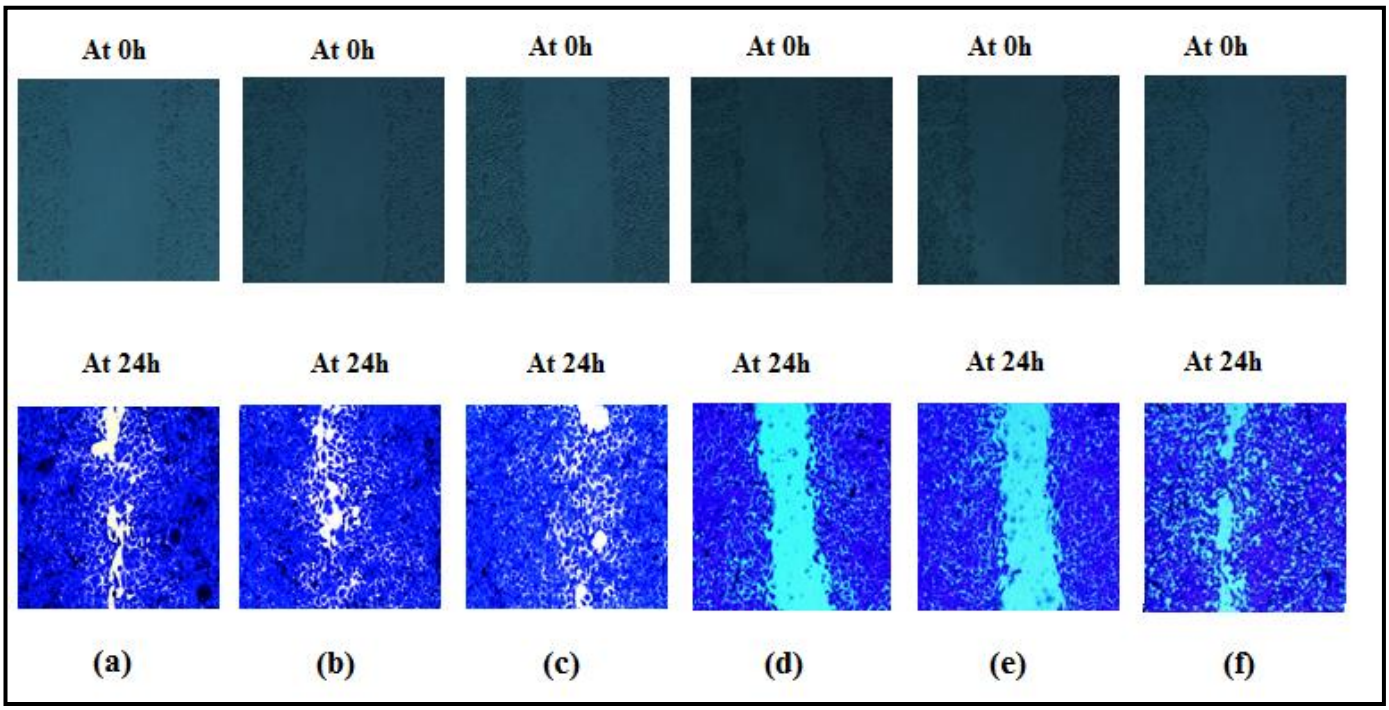

Figure 3: Effects of α-terpinene, β-caryophyllene, linalool, luteolin and quercetin treatment on the migration potential of DU145 cells in the scratch wound migration assay. a) 40.3 µM α-terpinene treatment group. b) 47.3 µM β-caryophyllene treatment group. c) 50.6 µM linalool treatment group. d) 18.7 µM luteolin treatment group. e) 50.0 µM quercetin treatment group. f) Control group.

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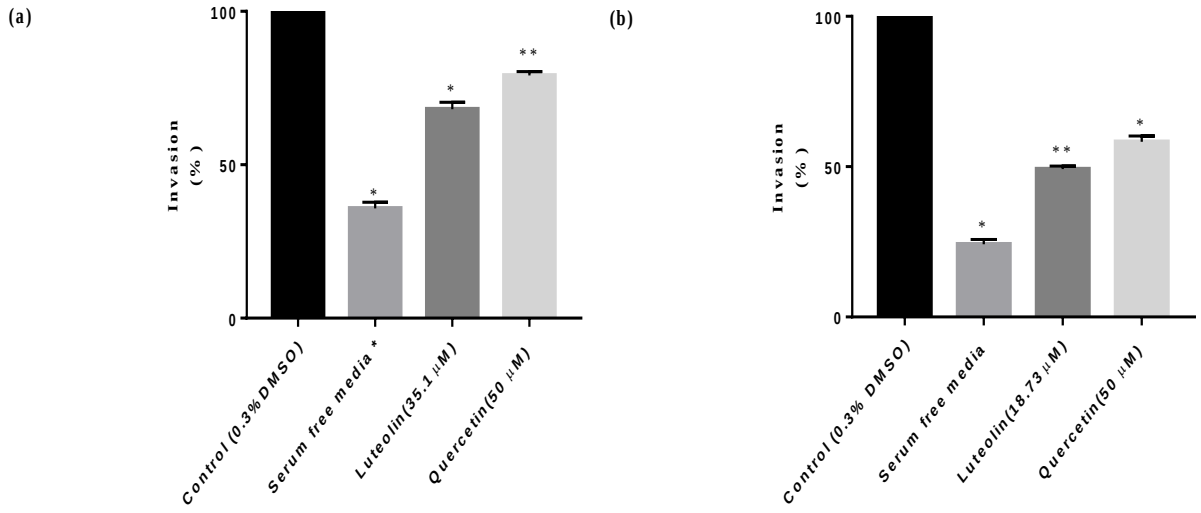

Figure 4: *In vitro* BME coated trans-well invasion assay, and the effect of luteolin and quercetin treatment on the invasion of the human prostate cancer cell lines (a) PC3 cells and (b) DU145 cells. Experiments were performed in duplicates. Data are expressed as the mean ± SD of the invading cells. (*) statistically significant values, with $p < 0.05$. (**) statistically significant values, with $p < 0.01$.

Conclusion

The findings of the present study demonstrated that luteolin, and quercetin potently prevented PC3 and DU145 cell proliferation, adhesion, migration and invasion. The underlying mechanisms governing these effects still need to be revealed. However, the available evidence collectively indicates that both compounds may be of therapeutic benefit in clinical settings. Hence, we can suggest the possible potential use of these compounds as adjuncts to current cancer therapies. Although further work is still needed to elucidate the mechanisms of the antitumor activity, these data provide preliminary evidence for their beneficial utilization in the treatment of human androgen-independent PCa.

Conflict of Interest

The authors declare no conflict of interest

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Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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