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EXPERIMENTAL STUDY

New perspectives of quercetin and vitamin C effects on fibronectin-binding integrins and chemokine receptors in prostate cancer cell lines

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ABSTRACT
OBJECTIVES: The aim of this study is to investigate the effect of two abundant dietary supplements, quercetin and vitamin C on some factors involved in metastasis and proliferation of prostate cancer, which are resistant to conventional chemotherapies in late stages.
BACKGROUND: Bone and brain are two common sites of metastases in prostate cancer, nevertheless the factors involved in their metastatic pathways are not well understood.
METHODS: The effect of quercetin (75μM) and vitamin C (100 μM) on CXCR4, CXCR7 chemokine receptors, α4, α5 and β1 integrins, ki-67 proliferation marker and Vascular endothelial growth factor, VEGF was evaluated using Quantitative Reverse Transcription PCR (RT-qPCR).
RESULTS: The effect of quercetin and vitamin C alone was different on PC3 and DU145 prostate cancer cell lines, but sequential combination reduced significantly the expression of CXCR and CXCR7 chemokine receptors, α4, α5 and β1 integrin subunits, VEGF and Ki-67 proliferation markers in PC3 and DU145 cell lines.
CONCLUSION: Our results indicated the beneficial effect of quercetin and vitamin C on prostate cancer cells with different metastatic sites and their differential response to the treatment which in turn may lead us to reach suitable therapeutic outcomes to combat cancer *(Fig. 3, Ref. 36)*. Text in PDF *www.elis.sk*
KEY WORDS: prostate cancer, chemokine receptor, integrin, quercetin, vitamin C.

Introduction

Prostate cancer is the most prevalent and leading cause of cancer related mortality in men. Its onset and progression is dormant and diagnosed late in elderly men (1). More frequently it metastasizes to the bone, brain and lymph nodes which is usually associated with poor prognosis (2). Two commonly investigated prostate carcinoma cell lines are DU145 and PC3 which are established from brain and bone metastasis of prostate cancer. In spite of common features like androgen independent, they have some important biological differences. PC3 cells do not express α-catenin (3), PTEN (4), E-cadherin, p53 antigen and have more metastatic potential compared to DU145 cell line (5). These features may be related to the tendency of them to metastasize to distinct organs and differences in the sensitivity and response to the treatments. Study of Jayakumar and colleagues demonstrated the different response of DU145 and PC3 cells to ionizing radiation. The basal and reduced glutathione content of DU145 cells was higher than PC3 cell line, against both basal and inducible levels of reactive oxygen species that were higher in PC3 than DU145 cells (6). An important issue in invasion and metastasis of prostate cancer is interaction of tumor cells with their microenvironments such as chemokines receptors along with integrins which are involved in promotion of tumor cell proliferation, differentiation and migration (7). Chemokine receptor 4 (CXCR4) considered as primary receptor and CXCR7 as alternative receptor for Chemokine CXC ligand 12 (CXCL12), contribute to the development and function of organs through regulation of cell hemostasis and trafficking (8, 9). Integrin family consist of 18 α and 8 β subunits that assemble into 24 distinct heterodimers. According to their subunits composition, they attach to their ligands and promote signal transduction inside or outside the cell (10). Fibronectin-binding integrins such as α4β1

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and α5β1 are major regulators of cell adhesion and trafficking that are involved in prostate cancer cells metastasis (11). As mentioned, after becoming metastatic, prostate cancer gets resistant to conventional therapies. Foods and nutrients, may have role in the etiology of cancers. The effect of natural sources driven compounds such as flavonoids and polyphenols in the prevention and treatment of cancers is investigated in some studies. There is a negative correlation between cancer prevalence and flavonoids rich diet (12, 13). Quercetin is the most abundant dietary flavonoid found in fruits and vegetables. It has an anti-cancer effect in cervix, breast, colon, lung and prostate cancers (14, 15). It has been demonstrated that quercetin reduced cell viability by reducing cyclin D and C, CDK2, CDC25c and increasing expression of p21, p53, p18 and p27 in prostate cancer cells (16). Selenium and quercetin have synergistic effects in endometrial adenocarcinoma cells by modulating oxidative stress (17). Vitamin C is also another supplement that may be effective against cancers. Epidemiological studies suggested that there is a reverse relationship between plasma levels of vitamin C and cancer incidence (18). It is supposed that it may prevent cancer in a variety of ways like enhancing the immune system and reducing chronic inflammation and ameliorating oxidative stress (19). It must be mentioned that due to the lower efficiency of dietary supplements, they are often used in combination to synergize and enhance each other's effects. In previous studies in our lab, it was shown that sequential treatment of vitamin C and quercetin had anticancer effect by modulating oxidative stress, increasing apoptosis and cell cycle arrest and ultimately it increased the effects of doxorubicin and paclitaxel in breast cancer cell lines (20, 21).We hypothesize that although these supplements are often considered to be antioxidants, their anticancer effect is not just due to this property. They may inhibit other factors involved in metastasis. As mentioned above, chemokines receptors and integrins have pivotal roles in metastasis. Therefore, in this study we intended to investigate the effect of quercetin and vitamin C on these factors that are involved in proliferation and metastasis of two androgen independent prostate cancer cell lines.

Materials and methods

Reagents

RPMI-1640 cell culture medium was purchased from BIOIDEA, Iran. Fetal bovine serum (FBS), Penicillin-Streptomycin (pen/strep) were purchased from Gibco (Thermo Fisher Scientific USA), Quercetin, vitamin C, di-methyl sulfoxide (DMSO) and Trypsin-EDTA(1x) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). RNA isolation kit was Tripure RNA extraction reagent (Roche) and cDNA synthesis kit was from Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA. Specific primers for genes was ordered to © metabion international AG (Germany).

Cell culture

The human prostate cancer cells, PC3 and DU145 were obtained from the National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, Iran). Cells cultured in RPMI-1640 medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5 % CO_2 incubator.

Quantitative reverse transcription PCR (RT-qPCR)

Cancer cells were cultured in T25 culture flask and incubated for 12 h until to get 50% confluence, treatments were as follows, cells were treated with vitamin C (100 µM) and quercetin (75 µM) for 30 h. While in combination group, cells were treated with vitamin C (100 µM) for 24 h and then after washing the wells, quercetin (75 µM) was added to the wells for 6 h in sequential manner. Total treatment duration was 30 h for all groups. Total RNA was isolated using Tripure RNA extraction reagent (Roche) and the amount of RNA content was measured using a nanodrop 1000 spectrophotometer (nanodrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). 2 µg of RNA was used to prepare cDNA using reverse transcriptase kit (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), according to the manufacturer's protocol. Specific primers for each gene were used to detect their mRNA expression. The primers sequences used were as follows: Marker of proliferation Ki-67 (MKI67): Forward Primer 5′-TTCTCACAGCGTCATCCAT-3′ and Reverse Primer 5′-GAGCCACTCTTCCTTGAACAC-3′, Integrin subunit beta 1 (ITGB1): Forward Primer 5′-CCTTACATTAGCACAACACCAG-3′ and Reverse Primer 5′-ACATTCCTCCAGCCAATCAG-3′, Integrin subunit alpha 5 (ITGA5): Forward Primer 5′-TGCCGAGTTCACCAAGAC-3′ and Reverse Primer 5′-ACAGCCACAGAGTATCCT-3′, Integrin subunit alpha 4 (ITGA4): Forward Primer 5′-GTTCGGCTACTCGGTCGT-3′ and Reverse Primer 5′-TTCCACAAGGTTCTCCATTAGG-3′, C-X-C chemokine receptor 4 (CXCR4): Forward Primer 5′-CAGTGAGGCAGATGACAGA-3′ and Reverse Primer 5′-ATGACAATACCAGGCAGGAT-3′, chemokine receptor 7 (CXCR7): Forward Primer 5′-CAGCAGGAAGAAGATGGTA-3′ and Reverse Primer 5′-GCAGTAGGTCTCATTGTTG-3′, Glucuronidase beta (GUSB): Forward Primer 5′-TCGCTCACACCAAATCCTT-3′ and Reverse Primer 5′-GGCTTCTGATACTTCTTATACCA-3′. GUSB mRNA were analyzed as reference gene. RT-qPCR was performed by steponeplus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) Using Amplicon RealQ Plus Master Mix Green. Amplification was carried out for 40 cycles using the following protocol: 95 °C for 10 min then 95 °C for 10 secs, Annealing Temperature (Ta), specific for each primer pair for 15 secs and 72 °C for 30 secs for each amplification cycle. The Pfaffl method was used to calculate the relative mRNA expression, as described previously (22).

Statistical analysis

Results data are expressed as the mean ± SEM from three independent experiments. Statistical analysis was done using GraphPad Prism software version 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS software version 16; SPSS, Chicago, IL, USA) One-way ANOVA and Tukey's test were applied to compare the untreated control against the treated groups. $p < 0.05$ was considered to indicate a statistically significant difference.

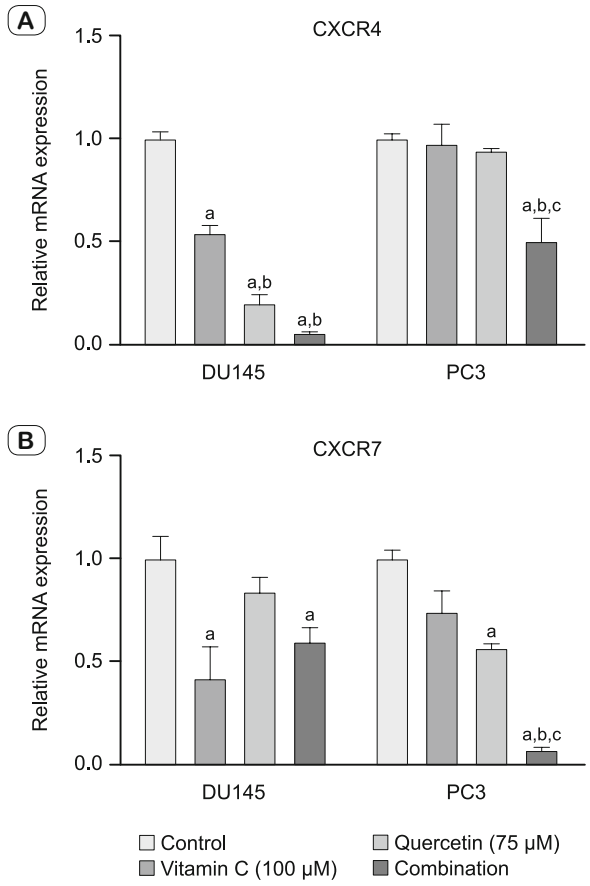

Fig. 1. Effects of vitamin C and quercetin on chemokine receptors gene expression in the prostate cancer cell lines. RT-qPCR data represent results of (A): CXCR4, (B): CXCR7. Data are presented as the mean ± SEM in 3 independent experiments and p < 0.05 considered as significant difference between the groups. Alphabet letters indicate significant differences. a; compared to untreated control, b; compared to vitamin C and c; compared to quercetin group. CXCR4; C-X-C chemokine receptor type 4, CXCR7; C-X-C chemokine receptor type 7.

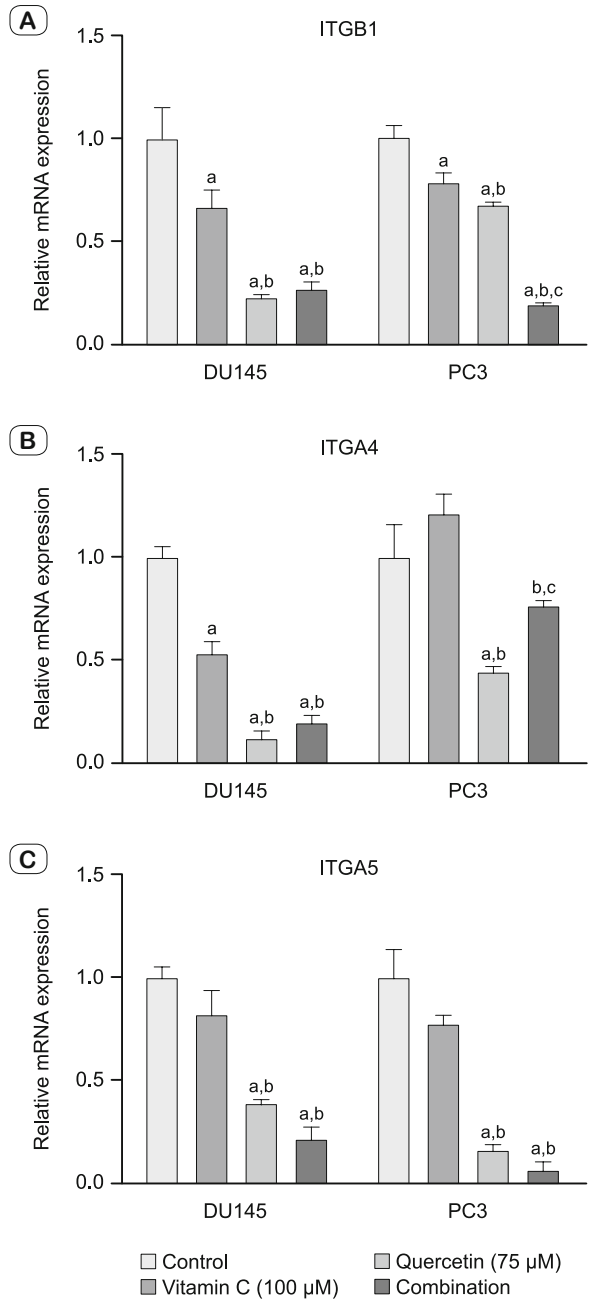

Fig. 2. Effects of vitamin C and quercetin on integrin subunits gene expression in the prostate cancer cell lines. RT-qPCR data represent results of (A): ITGB1, (B): ITGA4, (C): ITGA5. Data are presented as the mean ± SEM in 3 independent experiments and p < 0.05 is considered as significant difference between the groups. Alphabet letters indicate significance differences. a; compared to untreated control, b; compared to vitamin C and c; compared to quercetin group. ITGB1; Integrin Subunit beta 1, ITGA4; Integrin Subunit Alpha 4, ITGA5; Integrin Subunit Alpha.5

Results

The effects of quercetin and vitamin C on chemokine receptors gene expression on the prostate cancer cell lines

Quantitative reverse transcription PCR (RT-qPCR) was performed to detect the expression levels of the CXCR4 and CXCR7 mRNA. As shown in Figure 1A, in DU145 cell lines, CXCR4 mRNA expression decreased down 46 % in vitamin C ($p < 0.001$), 81 % in quercetin ($p < 0.001$) and 95 % ($p < 0.001$) in combination groups compared to the untreated control group. In PC3 cell lines, relative changes in CXCR4 mRNA expression in quercetin and vitamin C groups were not significant, yet in the combination group it decreased by 50 % ($p = 0.03$). The effect observed in the combination group was the same as that of DU145. These data indicated that despite the different effect of vitamin C and quercetin in these cell lines, combination of them was found to be more effective in both cell lines. Figure 1B illustrates that in DU145 cell, mRNA expression of chemokine receptor, CXCR7 decreased by 58 % in vitamin C ($p = 0.02$) and 40 % in the combination group ($p = 0.04$) while it was not significant in quercetin group ($p = 0.73$). In

PC3 cell lines, the expression of CXCR7 mRNA decreased down 44 % in quercetin ($p = 0.003$), 26 % in vitamin C ($p = 0.051$) and 93 % in the combination group ($p < 0.001$). These obtained results also suggest that combination of the treatments is more effective than a single agent treatment.

The effects of quercetin and vitamin C on integrins gene expression in the prostate cancer cell lines

As demonstrated in Figure 2A, in DU145 cell line, mRNA expression of ITGB1 was reduced by 33 %, 78 % and 74 % respectively in vitamin C ($p = 0.047$), quercetin ($p = 0.011$), and the combination ($p = 0.014$) groups compared to the untreated control group. In PC3 cell line, ITGB1 mRNA expression decreased by 22 % ($p = 0.055$), 33 % ($p = 0.014$) and 81 % ($p < 0.001$) in vitamin C, quercetin and combination groups, respectively. According to these results, the expression of ITGB1 decreased in all the treatment groups, and the reduction was greater in the combination groups. Integrin α4 subunit is one of the common heterodimers assemble with β1 subunit of integrin. As illustrated in Figure 2B, in DU145 cell line, ITGA4 expression decreased by 47% in vitamin C ($p = 0.001$), 88 % in quercetin ($p < 0.001$) and 80 % in the combination ($p < 0.001$) groups compared to the untreated control group. In PC3 cell lines, ITGA4 expression was reduced by 56 % in quercetin ($p = 0.043$) while this reduction was not significant in vitamin C group. Accordingly, combination of the treatments is believed to be more effective than each treatment alone and it seems that the effects of combination treatments were greater in DU145 cells compared to those in PC3 cell line. Another common heterodimer of β1 is α5 subunit. As shown in Figure 2C, the expression of ITGA5 decreased by 61 % in quercetin ($p = 0.02$) and 79 % in the combination group ($p = 0.005$) in DU145 cell line. In PC3 cells, it decreased by 74 % in quercetin ($p = 0.002$) and 99 % in the combination group ($p < 0.001$). In vitamin C group, the effect was not significant. In summary, our results revealed that quercetin and vitamin C were of different effects on DU145 and PC3 cells integrin expression, yet the combination of the treatments was found to be more efficient in both cells.

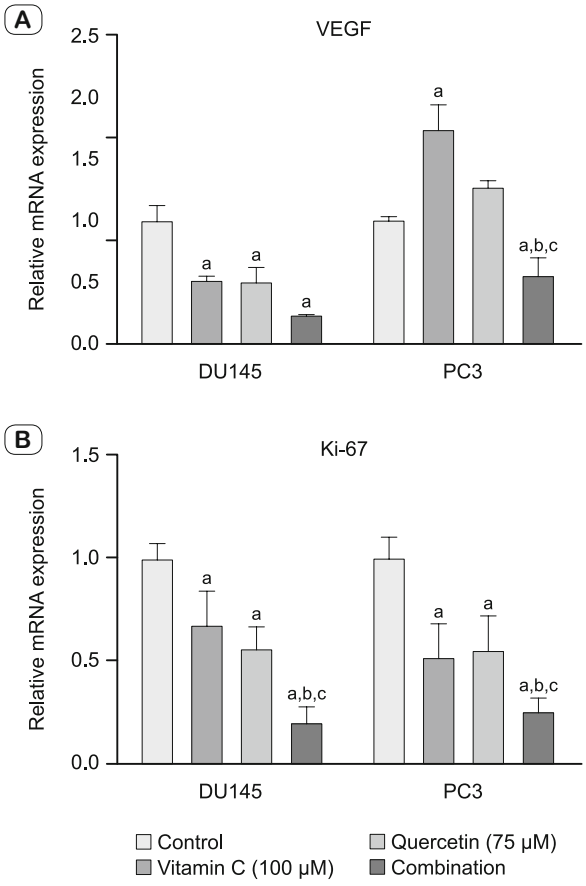

Fig. 3. Effects of vitamin C and quercetin on VEGF and MKI67 gene expression in the prostate cancer cell lines. RT-qPCR data represent results of (A): VEGF, (B): Ki-67. Data are presented as the mean ± SEM in 3 independent experiments and $p < 0.05$ considered as significant difference between the groups. Alphabet letters indicate significant differences. a; compared to untreated control, b; compared to vitamin C and c; compared to quercetin group. Ki-67; marker of proliferation Ki-67, VEGF; vascular endothelial growth factor.

The effects of quercetin and vitamin C on angiogenesis and proliferation markers genes expression in the prostate cancer cell lines

As shown in Figure 3A, RT-qPCR results indicated that in DU145 cell line, the expression level of VEGF decreased by 48% in vitamin C ($p = 0.03$), 50 % in quercetin ($p = 0.025$) and 77 % in the combination group ($p = 0.005$). In PC3 cells, the expression of VEGF increased up to 74 % in vitamin C ($p = 0.047$) whereas it decreased by 45 % in the combination group ($p < 0.043$). The effect of quercetin was not significant. Based on Figure 3B, in DU145 cells, Ki-67 expression decreased by 33 % in vitamin C ($p = 0.045$), 44 % in quercetin ($p = 0.038$) and 80 % in the combination group ($p = 0.02$). The results also demonstrated the different effects of vitamin C and quercetin on DU145 and PC3 cells and that the combination treatment had greater effects on the markers of angiogenesis and proliferation expression.

Discussion

Prostate cancer becomes resistant against conventional chemotherapies and metastasizes in most cases to bone and brain in late stages. PC3 and DU145 are the two prostate cancer cell lines established from bone and brain, which are rather different in the aggressiveness and metastatic pattern. We investigated the effects of quercetin (75μM) and vitamin C (100μM) treatments on the factors contributing to proliferation, metastasis and angiogenesis of prostate cancer. According to our results, the treatments of prostate cancer cells with either quercetin or vitamin C decreased the expression of CXCR4 and CXCR7 chemokine receptors and their combination treatment had greater effects. In agreement with our findings, Wang and colleagues demonstrated that quercetin decreased the expression of CXCR4 in breast cancer stem cells (23). To the best of our knowledge, the effects of quercetin

or vitamin C on CXCR7 have not been investigated yet, but similar flavonoid, OroxylinA, which was driven from Scutellaria baicalensis, enhanced the effects of Imatinib through an increase in apoptosis and a decrease in the expression of CXCR7 in chronic myeloid leukemia (24). CXCR4 and CXCR7 are both receptors for CXCL12 chemokine. It was observed that the expression of CXCR4 and CXCL12 increased in clinically localized prostate cancer samples (25). Cancer cells migrate to the organs with higher concentrations of CXCL12, it is hypothesized that chemokines act as chemoattractant and promote the migration of cancer cells to the tissues with a high concentration of them (26, 27). The role of CXCR7 in metastasis process is illusive, it might act as an alternative receptor for CXCL12 by modulating its activity via scavenging or sequestering of it (28). Furthermore, our study depicted that quercetin and vitamin C decreased the expression of α4, α5 and β1 subunits of integrin in DU145 and PC3 cells and the effects of the combination treatment was greater. In accordance with our results, He and colleagues reported that quercetin reduced the expression of CXCR4, β1 and α5 integrin subunits, inhibited proliferation, and migration of pulmonary artery smooth muscle cells (29). Moreover, Doersch and colleagues demonstrated that quercetin reduced β1 integrin in fibroblast cells and caused less fibrosis in the wound site (30). The roles of α4, α5 and β1 subunits of integrin in prostate cancer cell adhesion and metastasis was investigated in the previous studies in our lab (9, 11, 31). Integrin β1 is the most abundant subunit that assemble with most of the integrin α subunits. Integrin α4β1 binds to fibronectin and VCAM-1 and α5β1 integrin is the major receptor for fibronectin. Their overexpression in cancer cells promotes migration, metastasis and resistance against therapies (32, 33).

In the present study, we found that quercetin and vitamin C decreased the expression of VEGF as an angiogenesis marker, and Ki-67 as a proliferation marker, in prostate cancer cells. In agreement with our results, it was demonstrated that co-treatment of quercetin and green tea extract had chemopreventive effect in the prostate cancer (34) and increased the therapeutic efficacy of docetaxel by reducing VEGF and Ki-67 expression and enhanced the inhibition of PC3 xenograft tumor growth in SCID mice (35). Daker and colleagues exhibited that quercetin could synergistically increase the effects of cisplatin by reducing the expression of Ki-67 in nasopharyngeal carcinoma cell lines. The authors suggested that the co-administration of quercetin with cisplatin might reduce the dosage required for the treatment and reduced chemotherapy associated toxicity (36). In conclusion, our results implied the beneficial effects of two abundant dietary supplements against PC3 and DU145 prostate cancer cell lines. We found that even though the treatment of these cells with vitamin C or/and quercetin had different effects on PC3 and DU145 cell lines, the sequential treatment of vitamin C and quercetin had greater effects. They were observed to be capable of reducing the expression of the important family of genes involved in cell proliferation and metastasis of cancer cells which in turn could be used in combination with other conventional therapies in prostate cancer. However to prove their other possible mechanisms of effect, more in vitro and in vivo studies are needed.

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