

Expression of prostate stem cell antigen is downregulated during flavonoid-induced cytotoxicity in prostate cancer cells

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Abstract. Prostate stem cell antigen (PSCA) is expressed in the majority of prostate cancer cases and may be a potential therapeutic target in the treatment of prostate cancer. The present study evaluated the cytotoxicity of three flavonoids (genistein, luteolin and quercetin) towards DU145 prostate cancer cells, and investigated the effect of these flavonoids on PSCA expression. The results demonstrated that genistein, luteolin and quercetin inhibited the growth of DU145 cells in a dose-dependent manner ($P < 0.05$) and induced morphological changes characteristic of apoptosis in DU145 cells. Flow cytometry analysis also indicated that the flavonoids induced S phase cycle arrest in DU145 cells. Notably, it was observed that expression of PSCA was inhibited at the mRNA ($P < 0.05$) and protein levels in DU145 cells following flavonoid treatment compared with the control. These results suggest that flavonoids may be potential therapeutic agents in the treatment and prevention of prostate cancer.

Introduction

Prostate cancer is the second most commonly diagnosed cancer and the sixth leading cause of mortality in men worldwide (1). The highest incidence rates of prostate cancer are observed in the United States, Canada and northwestern Europe, whereas this malignancy is less common across Asia and South America (1,2). Although the lowest incidence and mortality rates of prostate cancer worldwide are observed in Asian men, these rates have increased over the past

20-30 years (3). The advent of prostate-specific antigen (PSA) screening in the 1980s has improved early-stage diagnosis of prostate cancer and increased rates of diagnosis at potentially treatable stages (4). However, many prostate cancer patients that undergo definitive treatment that aims to permanently eradicate the malignancy, including radical prostatectomy and radiation therapy, suffer from biochemical recurrence and eventually progress to metastatic disease (4). However, therapeutic options currently available for advanced prostate cancer have short-lasting effects and a minimal effect on patient survival rates, thus the identification of novel treatment strategies is required (4).

Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell membrane protein that belongs to the Thy-1/Ly-6 family of cell surface antigens, and consists of 123 amino acids (5). Expression of PSCA in normal tissues is predominantly prostate-specific. However, low-level expression of PSCA has also been detected in other tissues, including the placenta, stomach and kidney (2). Elevated levels of PSCA have been documented in >80% of prostate cancer tissues and in all cases of bone metastatic prostate cancer in patients (6). In preclinical trials, administration of murine anti-PSCA monoclonal antibodies to mice bearing human prostate cancers led to the inhibition of tumor growth and metastasis, and prolonged survival of mice (7,8). This was observed for xenografts derived from both bone metastasis and lymph node malignancies, and for non-castration and castration-resistant tumors (7). Furthermore, silencing of PSCA using small interfering RNA has been demonstrated to inhibit the proliferation and reduce the migration and invasion of human prostate cancer PC-3M cells (9).

The present study investigated the effect of plant-derived flavonoid compounds, namely genistein, luteolin and quercetin, on the expression of PSCA and inhibition of prostate cancer cells *in vitro*.

Materials and methods

Materials. Genistein ($\geq 98\%$ purity), quercetin ($\geq 97\%$ purity) and luteolin ($\geq 98\%$ purity) were purchased from Aladdin Industrial Corp. (Shanghai, China). An Annexin V-FITC/PI apoptosis detection kit (40302ES20) and Hoechst 33342 nucleic

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acid stain were purchased from Shanghai Qcbio Science and Technologies Co., Ltd. (Shanghai, China). The following reagents were from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China): Propidium iodide (PI), ethidium bromide (EB), dimethyl sulfoxide (DMSO), RNase and materials for western blot analysis, including SDS gel, nitrocellulose membranes, blot filter paper, bovine serum albumin and Ponceau S dye. Human anti-PSCA (ab56338) and anti- β -actin (ab3280) antibodies were obtained from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse antibody (TA130003) was purchased from Origene Technologies, Inc. (Beijing, China).

Cell culture. The prostate cancer cell line DU145, which expresses PSCA (10), was obtained from the National Infrastructure of Cell Line Resource (Beijing, China). DU145 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 mg/ml streptomycin (all Beijing Solarbio Science and Technology Co., Ltd.) at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cell viability assay. DU145 cells (1x10⁴ cells/well) were plated into 96-well plates and incubated at 37°C for 24 h in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum to allow the cells to attach prior to treatment with flavonoids (genistein, luteolin and quercetin). Each flavonoid compound was dissolved in 0.1% DMSO and made up with RPMI-1640 medium to a final flavonoid concentration of 20, 40, 80 and 100 μ M. DU145 cells were individually treated with 20, 40, 80 and 100 μ M of each flavonoid compound at 37°C for 24 h in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Cells treated with 0.1% DMSO served as a negative control. Cell viability was measured by counting the cells with a Neubauer chamber (BRAND Trading Co., Ltd., Shanghai), according to the manufacturer's protocol. Cell viability was expressed relative to control cells. Half maximal inhibitory concentration (IC₅₀) values were subsequently determined as the concentration of compound that produced a 50% reduction in cell number, and were determined graphically from the concentration response curve.

Morphological observation. Morphological changes characteristic of cell death were observed using a normal inverted light microscope. Cells were individually treated with different concentrations of the three flavonoids for 24 h. Morphological changes of cells were observed at 24 h post-treatment.

Cell cycle analysis. DU145 cells (70% confluence in 6-well plates) were starved in 1% FBS-supplemented RPMI-1640 media for 24 h to arrest cells in the G0 phase of the cell cycle, after which they were individually treated with each flavonoid compound (80 μ M) for 24 h in 10% FBS-supplemented RPMI-1640 medium. The cells in the control group were treated with 0.1% DMSO under the same conditions. Following flavonoid treatment, cells were trypsinized, washed twice with cold phosphate-buffered saline (PBS) and centrifuged

at 110 x g for 5 min at room temperature. The pellet was resuspended in 75% ethanol for 24 h at -20°C. Cells were subsequently centrifuged at 110 x g for 5 min at room temperature and the supernatant containing dead and apoptotic cells was discarded, whereas the pellet was washed twice with cold PBS, suspended in 500 μ l PBS and incubated with 5 μ l RNase (final concentration, 20 μ g/ml) at 37°C for 30 min. Cells were incubated in the dark with PI (final concentration, 50 μ g/ml) for 1 h at room temperature and analyzed by flow cytometry using a BD FACSAria III Cell Sorter (BD Biosciences, San Jose, CA, USA) and WinMDI (version 2.8) software (Scripps Institute, La Jolla, CA, USA).

Observation of apoptosis by Hoechst 33342 staining. DU145 cells were seeded into 6-well plates (5x10⁵ cells/well) and individually incubated with each of the three flavonoid compounds (80 μ M) at 37°C for 24 h. Cells in the control group were treated with 0.1% DMSO under the same conditions. The cells were incubated with Hoechst 33342 (30 μ M in PBS) in the dark for 30 min. Cells were then washed twice with PBS and cell fluorescence was observed using a FluoView FV1000 confocal microscope (Olympus Corp., Tokyo, Japan).

Detection of apoptosis by flow cytometry. Levels of cell apoptosis were measured using an Annexin V-FITC apoptosis detection kit, according to the manufacturer's protocol. Briefly, cells were individually treated with each of the three flavonoids (80 μ M) or DMSO control (0.1%) for 24 h at 37°C. Cells were trypsinized and washed with PBS, then resuspended in Annexin V binding buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl₂. Cells were subsequently stained with FITC-conjugated Annexin V and PI at room temperature for 15 min in the dark prior to the addition of binding buffer. The number of apoptotic cells was measured by flow cytometry, as described above. Cells were sorted into intact (Annexin V-/PI-), early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+) and necrotic (Annexin V-/PI+) cell populations.

Isolation of RNA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from DU145 cells individually treated with each of the three flavonoids (80 μ M) or DMSO control (0.1%) for 24 h using the TRIzol reagent-phenol chloroform procedure (Gibco; Thermo Fisher Scientific, Inc.). DNase I (2 U, D7076; Beyotime Institute of Biotechnology, Haimen, China) was added to the RNA and incubated in a 37°C water bath for 20 min. DNase Inactivation reagent (2 μ l, AM1906; Ambion; Thermo Fisher Scientific, Inc.) was added to the RNA and incubated for 2 min at room temperature. The tube was flicked once more during the incubation to redisperse the DNase Inactivation reagent and centrifuged at 10,000 x g for 1 min at 4°C. A total of 2 μ g total RNA from each sample, quantified with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc.) was subjected to RT using a SYBR PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. For each sample, qPCR was carried out in triplicate in a total reaction mixture of 20 μ l [2 μ l cDNA, 10 μ l SYBR Premix Ex Taq, 0.4 μ l ROX Reference

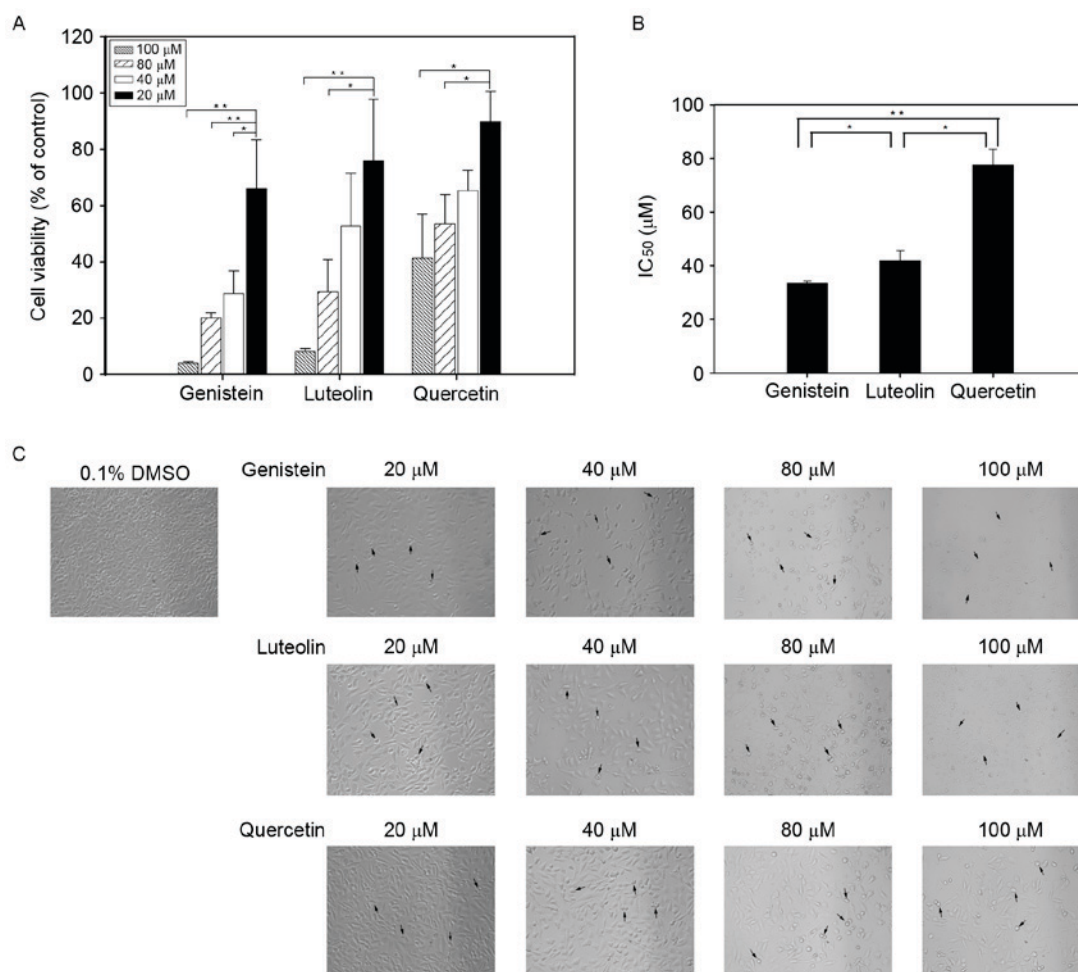


Figure 1. Cytotoxic effects of flavonoids (genistein, luteolin and quercetin) in DU145 cells. (A) Inhibitory effect of flavonoids on the viability of DU145 cells. (B) Comparative effects of flavonoids on the growth of DU145 cells. IC₅₀ was defined as the concentration of compound that produced a 50% reduction in cell number and was determined graphically from the concentration response curve. Values are presented as the mean \pm standard error of the mean of three independent experiments each conducted in triplicate. (C) Representative light microscopy images of DU145 cells treated with flavonoids or DMSO control. Black arrows indicate cells with altered morphology compared with control cells. Magnification, x200. *P<0.05 and **P<0.01 as indicated. IC₅₀, half maximal inhibitory concentration.

Dye II, 0.5 μ l of each forward and reverse primer (both 10 μ M) and 6.6 μ l H₂O] on an ABI PRISM 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primers (Sangon Biotech Co., Ltd., Shanghai, China) used were as follows: PSCA forward, 5'-ATC AGG AGG GCC CAG TAA AG-3' and reverse, 5'-TCC CAG GAA CTC ACG TCA AC-3'; and β -actin forward, 5'-AAC ACC CCA GCC ATG TAC G-3' and reverse, 5'-ATG TCA CGC ACG ATT TCC C-3'.

qPCR thermal cycling was initiated at 95°C for 10 sec, followed by 40 thermal cycles, each at 95°C for 5 sec and 60°C for 34 sec. Levels of PSCA mRNA expression were measured using the 2^{- Δ ACq} method (11) and were normalized to the expression of β -actin in each sample. Melting curves for each qPCR analysis were generated to verify the purity of the amplification product.

Western blot analysis. DU145 cells (70% confluent) were individually treated with each of the three flavonoid compounds (80 μ M) or DMSO control (0.1%) at 37°C for 24 h. Following treatment, the media was aspirated and cells were washed with cold PBS and subsequently incubated with ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA,

1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride; pH 7.4) over ice for 30 min. Cells were then scraped and the lysate was collected in a microfuge tube. The lysate was cleared by centrifugation at 14,000 \times g for 15 min at 4°C and the supernatant (total cell lysate) was immediately used or stored at -80°C. Protein concentration of the supernatant was determined using a Bradford Protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Proteins (50 μ g) from each sample were separated by 10-12% SDS-PAGE and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% non-fat dry milk and 0.05% Tween-20 in 20 mM TBS; pH 7.6) for 1 h at room temperature. Blots were then incubated with anti-PSCA (1:1,000) overnight at 4°C and anti- β -actin antibodies (1:1,000) for 2 h at room temperature, followed by incubation with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:10,000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection system (Beyotime Institute of Biotechnology). β -actin was used as an internal control.

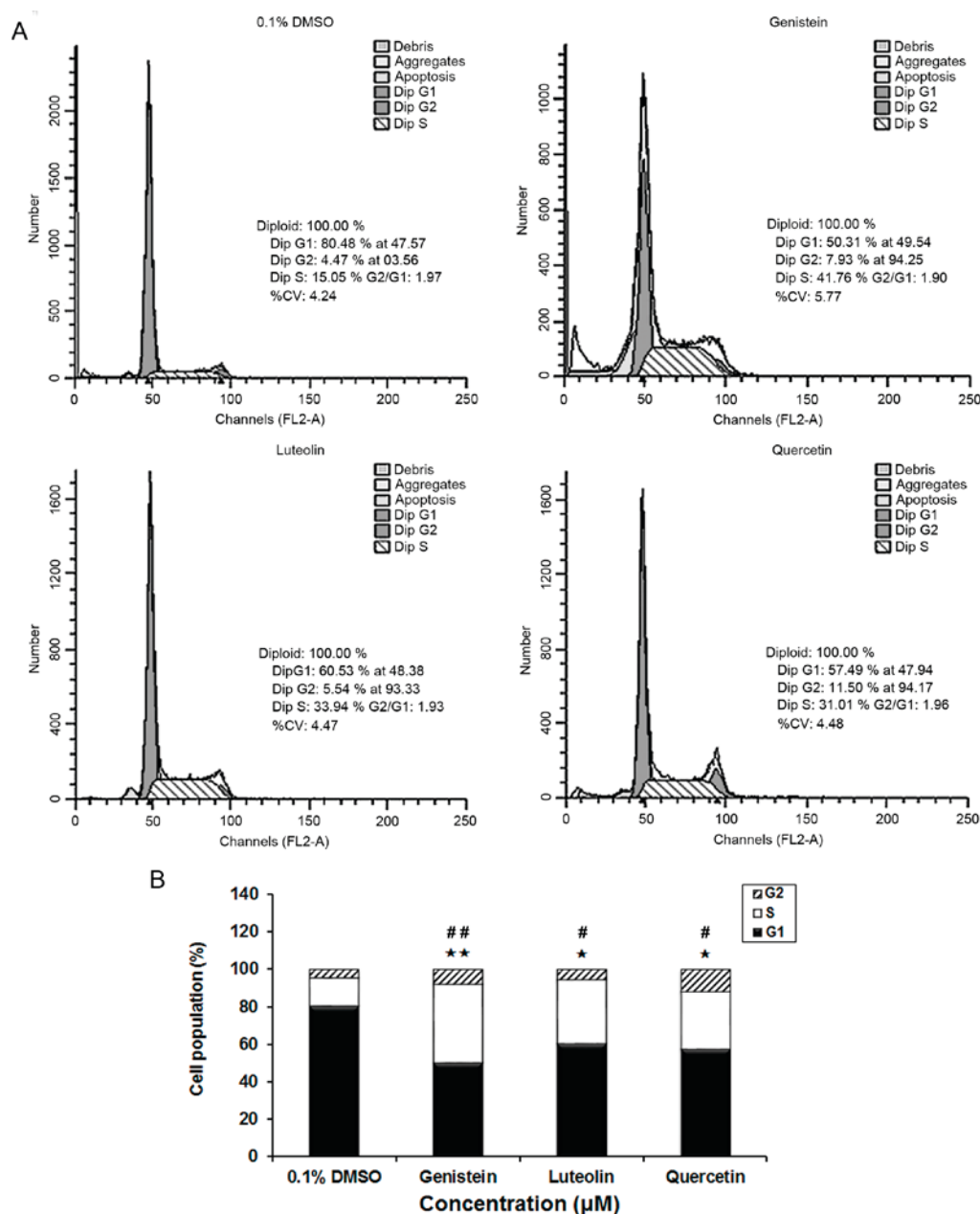


Figure 2. Effect of flavonoids (genistein, luteolin and quercetin) on cell cycle progression in DU145 cells. (A) Analysis of cell cycle progression in DU145 cells treated with flavonoids by flow cytometry. (B) Comparative effects of flavonoids on cell cycle progression in DU145 cells. DMSO served as the control. Cell cycle progression of DU145 cells in response to 80 μ M flavonoids was analyzed by flow cytometry 24 h after treatment. $^{\#}P<0.05$, $^{##}P<0.01$ in S phase; $^{*}P<0.05$, $^{**}P<0.01$ in G1 phase.

Statistical analyses. All data are expressed as the mean \pm standard error of the mean of at least three independent experiments. Differences between the mean values of multiple groups were analyzed using one-way analysis of variance. Statistical analysis was performed with SPSS 19.0 software (IBM SPSS, Armonk, NY, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cytotoxic effects of flavonoids in DU145 cells. To determine the effect of flavonoids on the inhibition of cell viability, the cytotoxicity of various concentrations (20-100 μ M) of genistein, luteolin and quercetin were evaluated by counting

cells in a Neubauer chamber at 24 h post-treatment. As depicted in Fig. 1A, flavonoids inhibited cell viability in a dose-dependent manner ($P<0.05$). Genistein exhibited the greatest inhibitory effect on cell cycle inhibition with an estimated IC_{50} value of 33 μ M, which was significantly lower compared with that of luteolin (42 μ M; $P<0.05$) and quercetin (78 μ M; $P<0.01$; Fig. 1B). Observations by light microscope also indicated that, following flavonoid treatment, numbers of DU145 cells decreased in a dose-dependent manner, and cell morphology was altered relative to that of control cells. Notably, fewer DU145 cells were observed in cell cultures treated with increasing concentrations of each flavonoid, while swelling and damage of the plasma membrane was observed in the remaining cells (Fig. 1C).

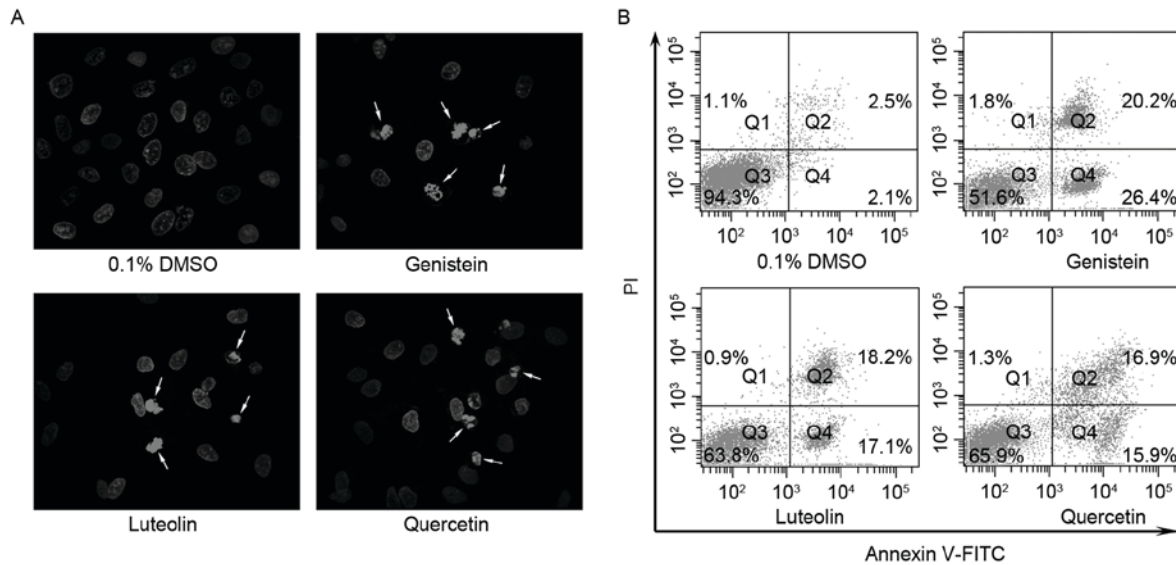


Figure 3. Effect of flavonoids (genistein, luteolin and quercetin) on the induction of apoptosis in DU145 cells. (A) Morphological changes associated with apoptosis were observed in DU145 cells treated with 80 μ M flavonoids for 24 h. Apoptotic body formation (indicated by white arrows) as an indicator of apoptosis was analyzed using a confocal laser scanning microscope after Hoechst 33342 staining. Magnification, x600. (B) Levels of cell apoptosis induced by flavonoids were measured by flow cytometry. PI, propidium iodide; FITC, fluorescein isothiocyanate; Q, quartile.

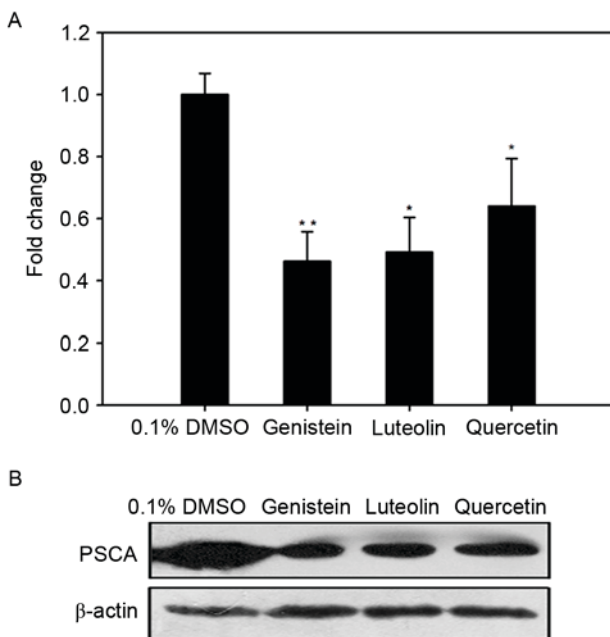


Figure 4. Effect of flavonoids (genistein, luteolin and quercetin) on PSCA expression in DU145 cells. (A) Inhibitory effect of flavonoids on PSCA mRNA expression. Levels of PSCA mRNA were measured relative to that of β -actin and are expressed as a fold change relative to the DMSO control. Values are presented as the mean \pm standard error of the mean of three independent experiments each conducted in triplicate. (B) Inhibitory effect of flavonoids on PSCA protein expression. Representative western blot images from three independent experiments are shown. β -actin was used as an internal control. * P <0.05 and ** P <0.01 vs. 0.1% DMSO control. PSCA, prostate stem cell antigen.

Flavonoids induce S phase cell cycle arrest in DU145 cells. To determine whether the inhibitory effects of flavonoids on cell growth were mediated by alterations in cell cycle progression, synchronized cells were incubated with each flavonoid (80 μ M) for 24 h, and the effect of the flavonoids on

cell cycle phase distribution was determined. Representative histograms are depicted in Fig. 2. Relative to control cells, cell groups treated with genistein, luteolin and quercetin exhibited a significantly increased population of S phase cells, and a corresponding significant decrease in G1 phase cells (both P <0.01 for genistein; both P <0.05 for luteolin and quercetin). This was consistent with the aforementioned growth inhibitory effects of genistein, luteolin and quercetin.

Flavonoids induce apoptosis in DU145 cells. Flavonoids may have inhibited the viability of DU145 cells through the induction of apoptosis, and thus cell morphologies were assessed to determine the extent of apoptosis. Hoechst 33342 staining demonstrated that treatment with genistein, luteolin and quercetin induced chromatin condensation and fragmentation of the nuclei into oligonucleosomes (Fig. 3A). Apoptotic cells were subsequently quantified using a flow cytometry assay. Individual treatment with each of the three flavonoid compounds (80 μ M) for 24 h decreased the proportion of intact cells (Fig. 3B) and increased the proportion of apoptotic cells. Notably, treatment with genistein, luteolin and quercetin decreased the proportions of intact cells from 94.3%, as observed for control cells, to 51.6, 63.8 and 65.9%, respectively. By contrast, genistein, luteolin and quercetin increased the proportions of early apoptotic cells from 2.1% (control) to 26.4, 17.1 and 15.9%, respectively. These data indicate that flavonoids may induce apoptosis in DU145 cells.

Flavonoids inhibit the expression of PSCA in DU145 cells. To determine whether the inhibitory effects of genistein, luteolin and quercetin were associated with a downregulation in PSCA, RT-qPCR was used to measure the levels of PSCA mRNA expression in DU145 cells following treatment with each of the three flavonoids (80 μ M) for 24 h. As depicted in Fig. 4A, levels of PSCA mRNA in DU145 cells treated with genistein, luteolin and quercetin were decreased by 0.46-fold (P <0.01),

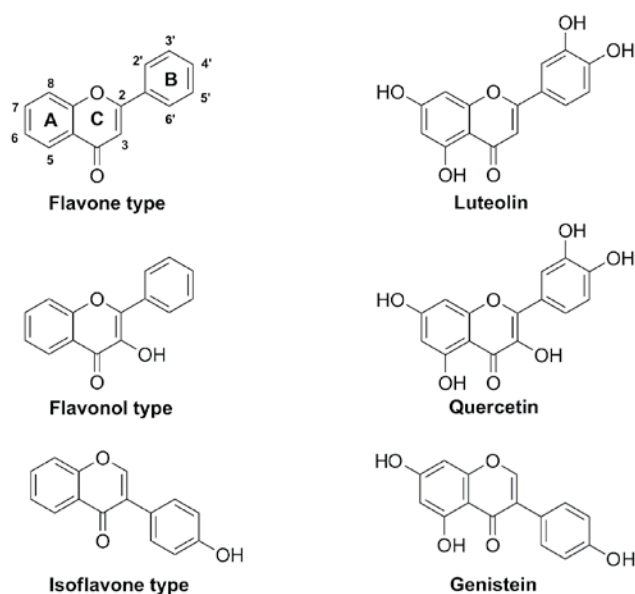


Figure 5. Chemical structures of flavonoids.

0.49-fold ($P<0.05$) and 0.64-fold ($P<0.05$), respectively, when compared with controls. Thus, genistein, luteolin, and quercetin may inhibit the expression of PSCA at the mRNA level.

In addition, western blot analysis was conducted to verify whether alterations in the expression of PSCA mRNA led to alterations at the protein level. As depicted in Fig. 4B, genistein, luteolin and quercetin markedly decreased the expression of PSCA at the protein level after 24-h treatment.

Discussion

Flavonoids are polyphenolic compounds that are considered to have potent preventative and chemotherapeutic properties in the treatment of cancer (12). Flavonoids are principally categorized into flavones, flavonols, flavanols, flavanones, flavanonols and isoflavones (13,14). Genistein (isoflavone), luteolin (flavone) and quercetin (flavonol) are typically present in numerous fruits and vegetables, and have been shown to inhibit cell proliferation with variable efficacy in human prostate cancer (15-17). Consistent with these findings, the present study observed that genistein, luteolin and quercetin inhibited the proliferation of prostate cancer cells in a dose-dependent manner and in the following order of potency: Genistein>luteolin>quercetin. Based on the chemical characteristics of genistein, luteolin and quercetin (Fig. 5), the inhibitory effect of these flavonoids on cell proliferation may have been dependent on the number of hydroxyl groups in the molecules.

Cell cycle progression mediates the growth and proliferation of mammalian cells, and cell cycle inhibition is a potential therapeutic strategy in the management of cancer (18). The present study observed that genistein, luteolin, and quercetin exerted inhibitory effects on the growth of DU145 prostate cancer cells by arresting cells in S phase of the cell cycle. Similarly, previous studies have demonstrated that flavonoids inhibit cell growth by inducing S phase cell cycle arrest in prostate cancer cells (19-21). Flavonoids have also been documented to arrest cell-cycle progression at G0/G1 and G2/M in prostate

cancer cells (22-24). These results indicate that flavonoids may block cell growth at multiple stages of the cell cycle (25).

Previous studies have focused on cell cycle-mediated apoptosis as a potential strategy for cancer cell elimination (26,27). Analogous to previous studies in human prostate cancer cells (24,28-30), the present study demonstrated that genistein, luteolin and quercetin induced apoptosis in DU145 cells. Thus, genistein, luteolin and quercetin may inhibit the growth of prostate cancer cells through the induction of cell cycle arrest and apoptosis.

Biomarkers are considered to be critical in the development of anticancer therapies (31,32). Due to improved insight into the molecular basis of cancer, anticancer strategies now focus on targeting specific molecular alterations that occur in cancer cells (33,34). In prostate cancer, numerous biomarkers have been identified, including PSA, prostate-specific membrane antigen, PSCA, early prostate cancer antigen, B7-H3, chromogranin A, α -methylacyl coenzyme A racemase, glutathione S-transferase P1 (GSTP1), sarcosine, caveolin-1, TMPRSS2-ERG, Ki-67, prostate cancer antigen 3 (PCA3) and disabled homolog 2-interacting protein (35). Previous studies have demonstrated that a number of these biomarkers, including PSA, GSTP1, Ki-67 and PCA3, were the targets by which flavonoids exerted anticancer effects in prostate cancer (36-39). Using human xenografts grown in mice with severe combined immunodeficiency, it has also been demonstrated that anti-PSCA monoclonal antibodies inhibited the growth and metastasis of tumors (40), thus indicating that PSCA may be an immunotherapeutic target in the treatment of prostate cancer (40,41).

In conclusion, the current study was the first to demonstrate that the anticancer effects of genistein, luteolin and quercetin in DU145 prostate cancer cells were associated with a downregulation in PSCA at the mRNA and protein levels. The present findings indicate that flavonoids may be potential therapeutic agents in the treatment and prevention of prostate cancer.

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