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Equol and daidzein decrease migration, invasion and matrix metalloproteinase (MMPs) gene expression in prostate cancer cell lines, DU-145 and PC-3

[Equol y daidzeina disminuyen la migración, invasión y la expresión génica de metaloproteinasa de la matriz (MMPs) en las líneas celulares prostáticas cancerosas DU-145 y PC-3]

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Abstract: This search is focused on the study of diet compounds that may have any potential chemopreventive effect against cancer. Some compounds that fulfill this requirement are phytoestrogens. Among them we find genistein (1), the most studied, daidzein (2) and equol (3) (figure 1). To compare the sensitivities of different prostate cancer cells to phytoestrogen treatment, sulphorhodamine B dye assay was performed to determine cell viability. DU-145 and PC-3 prostate cancer cell lines treated with various doses of phytoestrogen (0-12.5-25-50 and 100 µM) for different times (24, 48 and 72h). For cell invasion or migration assay cells were seeded in a Transwell chamber with or without coating Matrigel respectively. DU-145 and PC-3 cells were treated previously with phytoestrogen (50 µM) for 24h. The study showed that equol, daidzein and genistein inhibited migration and invasion in prostate cancer cell lines. Moreover, we analyzed the effects of phytoestrogens in MMP-2 and MMP-9 mRNA expression by RT-PCR. The results indicated that equol, daidzein and genistein diminished the expression of MMP-2 and MMP-9 in a cell-dependent manner. Our data suggested that equol, daidzein and genistein inhibited migration and invasion in prostate cancer cell lines. Moreover, the results also suggest that down-regulation of MMP-2 and MMP-9 might be involved in the inhibition of invasion of PC-3 and DU-145 cells after genistein, daidzein and equol treatment.

Keywords: equol, daidzein, migration, invasion, MMP, prostate cancer.

Resumen: Este trabajo se centra en el estudio de los compuestos de dieta que pueden tener potencial efecto quimiopreventivo contra el cáncer. Algunos de estos compuestos son los fitoestrógenos. Entre ellos encontramos la genisteína (1), el más estudiado, la daidzeína (2) y el equol (3) (figura 1). Para comparar el efecto de estos fitoestrogenos sobre las líneas celulares de cáncer de próstata, DU-145 y PC-3, se utilizó el ensayo de sulforodamina B para determinar la viabilidad celular tras los tratamientos con diferentes concentraciones de fitoestrógenos (0-12.5-25-50-100 µM) durante diferentes tiempos (24, 48, 72 h). Para analizar el efecto sobre la migración celular, las células DU-145 y PC-3 fueron tratadas previamente con una concentración de fitoestrógrno (50 µM) durante 24 horas y sembradas en una cámara Transwell sin recubrir. El estudio mostró que el equol, daidzeína y genisteína inhibió en MMP-2 y MMP-9 expresiones de genes en líneas celulares de cáncer de próstata, la PC-3 y DU-145. Los resultados indicaron que la daidzeína disminuyó la expresión de MMP- 2 y MMP-9 en DU-145 células. Nuestros datos sugieren que equol, daidzeína y genisteína inhiben la migración y la invasión de líneas celulares de cáncer de próstata.

Palabras clave: equol, daidzein, migración, invasión, MMP, cáncer de próstata.

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INTRODUCTION

Prostate cancer is one of the most commonly diagnosed cancers and the second leading cause of death in men in Western countries (Jemal *et al.*, 2008). Tumor metastasis is one of the major causes of morbidity in cancer patients (Partin *et al.*, 2001). Unfortunately, no therapy has been developed that successfully targets metastasis-associated processes of any human cancer type (Overall & Lopez-Otin, 2002). Cancer metastasis is a multistep process (Bajaj *et al.*, 2005) including detachment, invasion, angiogenesis, adhesion to endothelial cells, extravasation and regrowth in distant organs. Invasion consists of three major processes: adhesion to the extracellular matrix (ECM), degradation of ECM by different kinds of proteolytic enzymes and migration (Bellon *et al.*, 2004; Minard *et al.*, 2004). Many studies have demonstrated that inhibition of the migration and the degradation of the ECM results in the prevention of metastasis (Hwang & 2006; Slivova *et al.*, 2005; Valachovicova *et al.*, 2004). Elevated extracellular protease activity increases the invasive activity of many cancer cell types, including prostate cancer cells (Stetler-Stevenson & Yu, 2001).

The activation of MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) enables the degradation of the extracellular matrix (ECM) by tumor cells, allowing tumor metastasis (Itoh & Nagase, 2002).

Both epidemiological and experimental evidence suggest that increased consumption of soy and soybased foods is associated with a decreased risk for prostate cancer (Hsing *et al.*, 2000; Kolonel *et al.*, 2000; Lee *et al.*, 2003; Akata *et al.*, 2004). Soy isoflavones are thought to be responsible, in part, for this observed protective effect. Soy contains two principal isoflavones, genistein and daidzein (Coward *et al.*, 1993), that can modulate a variety of biological processes associated with carcinogenesis. Genistein and daidzein have been extensively studied. Their anticancer effects mainly included inhibition of cell proliferation, induction of cell cycle arrest and induction of apoptosis (Chen & Donovan, 2004; Hedlund *et al.*, 2002; Hsu *et al.*, 2009; Li *et al.*, 2004; Li & Sarkar, 2002a; Li & Sarkar 2002b; Shen *et al.*, 2000; Shenouda *et al.*, 2004). Equol is an active metabolite formed by intestinal bacteria from daidzein. Several independent lines of evidence suggest that equol may be one of the most biologically active metabolites (Lampe, 2010). Equol have significantly longer half-live in the body than daidzein or genistein (Kelly *et al.*, 1995). Equol is more potent than daidzein and genistein in terms of its antioxidant properties (Arora *et al.*, 1998) and antiproliferative effects (Lund *et al.*, 2004) in various cell types. Interesting observations have been made suggesting that equol may also have a protective role in the prostate.

The expression of various metalloproteinases (MMPs) in human prostate cancer cells was inhibited by genistein treatment (Li *et al.*, 2004; Wang *et al.*, 2004), while there are a few studies on the migration and invasion effects of daidzein and equol previously in prostate cancer cells (Magee et al., 2006; Zheng et al., 2012]. A recent study provides the only evidence to date that equol has direct effects on the growth of human prostate cancer cells (Lampe, 2010; Lund *et al.*, 2004; Magee *et al.*, 2006; Mitchell *et al.*, 2000; Zheng *et al.*, 2012). In these studies there are controversie between used concentrations ranging from 10^{-5} to 10^{-4} M.

The current study was therefore undertaken to elucidate the effect of equol and daidzein phytoestrogen compounds in prostate cancer cell migration and invasion, for which there are few avalaible data. Furthermore, we evaluated the effects of these compounds on the expression of two matrix metalloproteinase genes (MMP-2 and MMP-9).

MATERIAL AND METHODS

Cell culture and phytoestrogens treatment

Human androgen-independent prostate cancer cells PC-3 and DU-145 were cultured in DMEM (Gibco, San Diego, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37° C in a humidified 5% CO_2 incubator.

For phytoestrogen treatment, appropriate volumes of stock solution (1 mM in ethanol) of phytoestrogen (Sigma, St Louis, MO, USA) were added to the medium to achieve the indicated concentrations (12.5, 25, 50, and 100 μM) and were then incubated with cells for the indicated periods of time (24, 48 and 72 hours). Stock solution of compounds was prepared in ethanol and the final concentration of this solvent was kept constant at 0.1%. Control cultures received 1% ethanol alone.

Cell viability by sulphorhodamine dye assay

To compare the sensitivities of different prostate cancer cells to phytoestrogen treatment, sulphorhodamine B (SRB) (Sigma, St Louis, MO, USA) dye assay was performed to determine cell viability. DU-145 and PC-3 prostate cancer cell lines

were seeded at the density of 3×10^3 cells/well in 96-well plates for 24 h and then treated with various doses of phytoestrogen (0-12.5-25-50 and 100 μM) for different times (24, 48, 72 h). After the exposure time, in vitro cytotoxicity was measured by the SRB dye assay. Cells were fixed by adding 25 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubated for 60 min at 4º C. Plates were washed with deionized water and dried; 50 μL of SRB solution (0.1% wt/vol in 1% acetic acid) were added to each microtiter well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried and bound stain was solubilized with 100 μL of Tris base (10 mM). Optical densities were read on an automated spectrophotometer plate reader at a single wavelength of 540 nm. Values are shown as the % viability vs. ctrl +/- SD, n = four independent experiments in triplicate.

Wound healing assay

To determine the effect of phytoestrogens on migration of DU-145 and PC-3 cells in vitro, cells were seeded at a density of 2×10^5 cells in 24 well plates and grew to about 90 - 95% confluence after 48 h. Medium was removed and cell monolayers were wounded by manually scraping cell layer with a 1 ml plastic pipette tip. Debris was removed from the culture by washing with PBS twice, and cells were then cultured with fresh medium containing phytoestrogen (50 μM) for 24 h. Images were captured immediately after wounding and 24 h later. Wound closure was monitored with a Olympus® IX 81 model inverted microscope. Wound sizes were measured with a scale to ensure that all wounds had initially the same width.

The wound area was measured with ImageJ software at time = 0 and time = 24 h After that the following formula was applied:

% Wound Area t24 = (Wound Area (t0) – Wound Area (t24)/Wound Area (t0))x 100

Cell migration and invasion assays

For cell migration assay cells were seeded in a Transwell chamber without coating Matrigel. DU-145 and PC-3 cells were treated previously with phytoestrogen (50 μM) for 24h. Treated cells were then seeded into the upper chamber with 2×10^5 cells/well in 300 μL of serum-free medium, and 0.5 ml medium containing 20% FBS was added to the lower chamber as a chemoattractant. After incubation for 24 h at 37° C in 5% CO_2, the upper surface of the porous membrane was wiped off with a cotton swab. Cells that migrated to the lower surface of the porous membrane were fixed with 50% methanol and stained with 0.1% Crystal Violet.

Cell invasion assay was conducted similarly in a Transwell chamber with coating Matrigel for 48h. Matrigel (Sigma, Santiago, Chile) was diluted with serum-free medium to a final concentration of 2 mg/mL, and 8 μm diameter pore polycarbonate membrane filters were coated with 50 μL of Matrigel. In both migration and invasion assays cell numbers were counted in twenty random fields (×100) per filter, in a total of three filter (n = 3).

% Migration = (number of treated cells/number of control cells) x 100
% Invasion = (number treated cells/number of control cells) x 100

Quantitative Reverse transcription-polymerase chain reaction (qRT-PCR)

After phytoestrogen treatment, total RNA was isolated from DU-145 and PC-3 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantitated by absorbance at 260 nm. RNA (2 μg) was reverse transcribed using MMLV reverse transcriptase according to the manufacturer's instructions. MMP-2,-9 and GADPH mRNA expressions were determined by quantitative real-time PCR which was conducted in a Light Cycler machine (Roche,) with capillary tubes. Briefly, each amplification mixture (20 μL) contains:

2 ul of cDNA,
10 ul of FAST EVAGreen qPCR Master Mix
0,5 ul forward primer (F) 10 uM
0,5 ul reverse primer (R) 10 uM
2 ul BSA 0,1 mg/ml
5 ul ultra pure water

Cycling conditions were as follows: 96° C, 2 min, 30 cycles of 96° C, 5 s; 58° C, 30 s; 72° C, 25 s. Reactions were finished with 72° C, 25s extension.

Primers used for MMP-2, MMP-9 and GAPDH:

MMP-2
(133 bp): 5'-CCTCCCTGCCCCTCCCTTCA-3' (forward)
and 5'-GCTTCTGGCTGGGTCTGTGGC-3' (reverse);

MMP-9
(174 bp): 5'-GCCTTTGGACACGCACGACG-3' (forward)
and 5'-GCCAAAGCAGGACGGGAGCC-3' (reverse);

GAPDH
(226 bp): 5'- GAAGGTGAAGGTCGGAGTC-3' (forward)
and 5'-GAAGATGGTGATGGGATTTC-3' (reverse).

After cycling conditions, a fusion curve was programmed so as to determine the Tm of the PCR product. This ensures the specificity of the amplified product.

Statistical analysis

All assays were repeated in triplicate in three independent experiments, and all data were expressed as means ± SEM. Analysis of variance (ANOVA) for multiple comparisons was used as noted. In all cases, $P < 0.05$ was considered significant. All statistical tests were performed with statistical analysis software.

RESULTS AND DISCUSSION

We exposed two human prostate cancer cell lines (PC-3, DU-145) to increasing concentrations of equol, daidzein or genistein and at different periods of time. Their effect on growth inhibition was compared using sulphorhodamine B dye assay (Table 1). These cells displayed marked homogeneity in their responsiveness. As shown Table 1, daidzein and equol significantly inhibited cell viability in a dose- and time-dependent manner. Consistent with previous data, genistein significantly inhibited cell proliferation of PC-3 and DU-145 cells in a dose- and time-dependent manner. In dose-dependent experiments (72 h), the higher dosis of equol, daidzein and genistein (50-100 μM) was enough as to significantly decrease cell viability of PC-3 and DU-145 cells. Lower concentrations of equol, daidzein and genistein (12.5-25 μM) not showed a significant growth inhibition of PC-3 and DU-145. Similarly, in time-dependent experiments, at the concentration analyzed (50 μM) of genistein, daidzein and equol the treatment was not enough to significantly decrease cell viability in PC-3 and DU-145 cells respectively at 24 h. At a concentration of 50 μM of genistein showed significant growth inhibition on PC-3 and DU-145 cells after 48h of treatment ($p < 0.05$). Hence, treatments at a concentration of 50 μM for 24h were applied in the following experiments. These data indicated that equol, daidzein and genistein significantly inhibited growth of PC-3 and DU-145 in a dose- and time-dependent manner.

Inhibitory effect on migration of PC-3 and DU-145 cells by genistein, daidzein and equol

The effect of phytoestrogens on migration was determined using a wound healing assay. As shown Figure 1 genistein, daidzein and equol inhibited migration in vitro for PC-3 and DU-145 cells. As shown Figure 1A the wounds in control healed better than the wounds treated with genistein, daidzein and equol (50 μM) in DU-145 cells. This inhibitory effect was similar for PC-3 cells (Figure 1B). In a quantitative analysis (Figure 1C), after 24 h treatment with either genistein, daidzein or equol, reduced the wound area by 68.1%, 57.1% and 62.0%, respectively in PC-3 cells. Similarly, the inhibition of migration after 24 h of treatment with genistein, daidzein or equol was 68.7%, 58.1% and 65.3% respectively in DU-145 cells. This inhibitory effect is significantly different ($p < 0.001$) in all treatments with respect to control cells in both PC-3 and DU-145 cells. Our results indicate that used doses of phytoestrogens could lead to significant inhibition of wound healing (migration) of PC-3 and DU-145 cells.

The effect of phytoestrogens on migration was further analyzed using a Transwell chamber, as shown Figure 2A equol, daidzein and genistein could significantly reduce the migration of PC-3 (similar results are observed in DU-145 cells). A quantitative analysis, in the case of PC-3 cells (Figure 2B white columns) migrated cells after 50 μM equol, daidzein

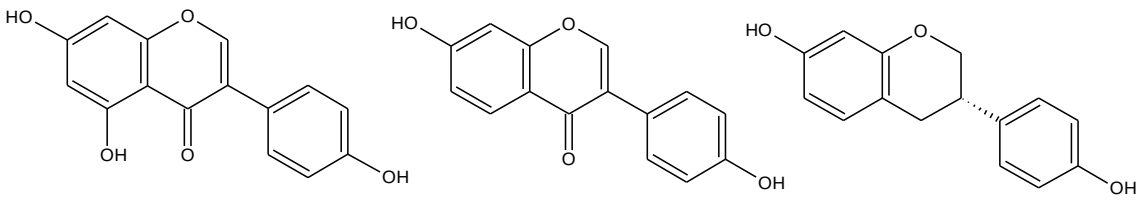
or genistein treatment represent 54.8%, 65.1% or 45.1% vs. control respectively. After genistein, daidzein and equol treatment (50 μM, 24 h), 49.4%, 68.7% and 59.7% of migration (100%) were observed respectively in DU-145 (Figure 2B, black columns) versus control cells

Table 1
Effects of genistein, daidzein and equol on cell viability of prostate cancer cell lines, PC-3 and DU-145

Cell viability (% of control)				
Cell line	**Treatment (μM)**	**24 h**	**48 h**	**72 h**
PC-3	**Equol 12.5**	**99.1 ± 7.5**	**97.4 ± 6.8**	**87.3 ± 7.9**
	Equol 25	**92.3 ± 5.7**	**91.2 ± 7.1**	**79.0 ± 7.4**
	Equol 50	**82.1 ± 9.1**	**78.1 ± 10.2**	**65.4 ± 6.3***
	Equol 100	**66.4 ± 5.2**	**61.6 ± 3.4**	**53.9 ± 4.4**
	Daidzein 12.5	**100.3 ± 9.3**	**97.3 ± 7.8**	**84.2 ± 7.9**
	Daidzein 25	**96.3 ± 5.1**	**94.7 ± 8.2**	**78.5 ± 6.9**
	Daidzein 50	**80.9 ± 9.7**	**79.8 ± 8.3**	**72.1 ± 7.9***
	Daidzein 100	**70.8 ± 9.1**	**61.3 ± 6.6**	**58.5 ± 3.1**
	Genistein 12.5	**98.1 ± 8.8**	**98.0 ± 7.4**	**83.1 ± 8.5**
	Genistein 25	**94.1 ± 7.8**	**90.6 ± 7.9**	**76.7 ± 8.1**
	Genistein 50	**81.2 ± 7.8**	**74.0 ± 5.8***	**62.4 ± 7.4***
	Genistein 100	**60.8 ± 7.0**	**58.1 ± 6.2**	**51.7 ± 5.0**
	Equol 12.5	**98.4 ± 8.6**	**98.1 ± 8.9**	**91.1 ± 10.3**
	Equol 25	**96.3 ± 8.4**	**93.2 ± 6.8**	**81.4 ± 7.8**
DU-145	**Equol 50**	**78.8 ± 6.6**	**76.5 ± 7.9**	**74.7 ± 6.1***
	Equol 100	**70.1 ± 8.1**	**68.2 ± 3.9**	**66.9 ± 4.8**
	Daidzein 12.5	**99.3 ± 5.4**	**98.2 ± 8.6**	**93.5 ± 7.4**
	Daidzein 25	**96.6 ± 9.8**	**90.1 ± 7.5**	**82.2 ± 8.9**
	Daidzein 50	**84.7 ± 8.7**	**82.6 ± 7.8**	**78.3 ± 6.9***
	Daidzein 100	**78.2 ± 5.3**	**71.2 ± 5.9**	**68.9 ± 7.3**
	Genistein 12.5	**100.3 ± 9.7**	**101.2 ± 9.2**	**93.4 ± 7.7**
	Genistein 25	**87.4 ± 7.6**	**85.8 ± 9.3**	**82.1 ± 6.9**
	Genistein 50	**78.3 ± 8.2**	**72.8 ± 6.7***	**71.4 ± 7.8***
	Genistein 100	**75.1 ± 6.6**	**70.4 ± 8.1**	**67.6 ± 7.8**

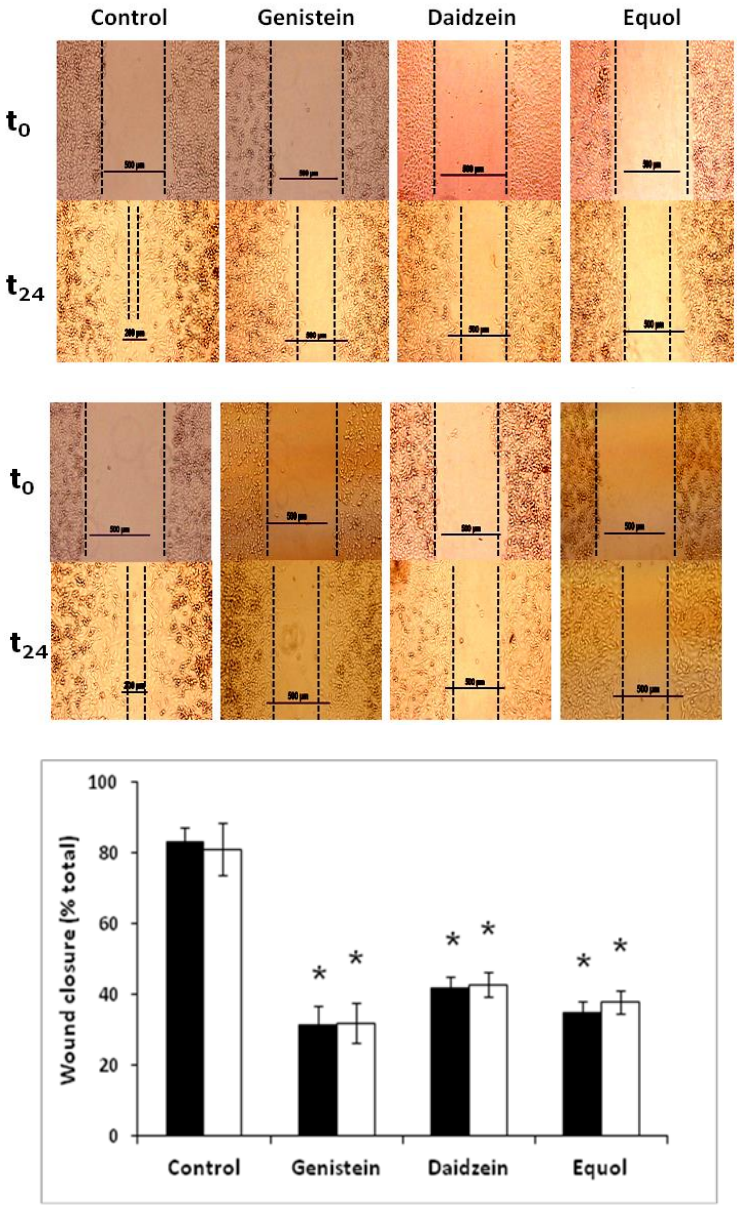
After cells were treated with the indicated doses of phytoestrogen (0, 12.5, 25, 50 100 μM) at different times (24, 48, and 72h), cell viabilities were determined by sulphorhodamine B dye assay. Data shown are means±SD of four independent experiments. * p<0.05 vs. control

Figure 1
Isoflavones compounds.



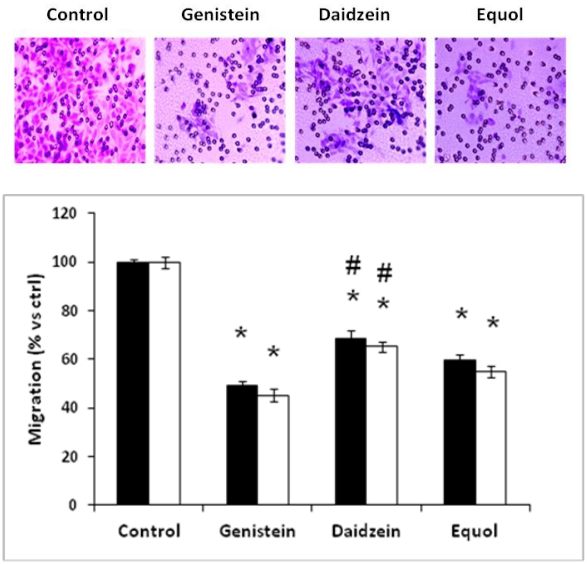
(1) **(2)** **(3)**

Figure 2
Inhibitory effects of phytoestrogens on the wound healing of PC-3 and DU-145 cells



Cells were treated with different phytoestrogens (genistein, daidzein, equol) (50 µM) for 24 h. (A) Representative photos of the wound healing inhibition of PC-3 after 50 µM genistein, daidzein and equol treatment (magnification×100). (B) Representative photos of the wound healing inhibition of PC-3 after 50 µM genistein, daidzein and equol treatment (magnification×100). (C) Quantification of the wound healing assay of DU-145 (black column) and PC-3 (open column). Data represent means±SEM of three independent experiments. * p < 0.001 vs. control.

Figure 3
Inhibitory effects of phytoestrogens on the migration assay of PC-3 and DU-145 cells.



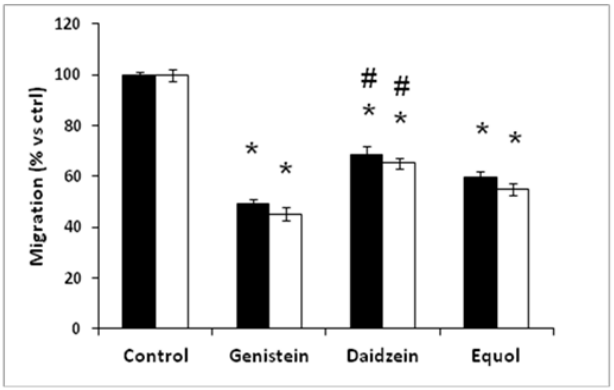
Cells were treated with different phytoestrogens (genistein, daidzein, equol) (50 µM) for 24 h. (A) Representative photos of the wound healing inhibition of PC-3 after 50 µM genistein, daidzein and equol treatment (magnification×100). (B) Representative photos of the wound healing inhibition of PC-3 after 50 µM genistein, daidzein and equol treatment (magnification×100). (C) Quantification of the wound healing assay of DU-145 (black column) and PC-3 (open column). Data represent means±SEM of three independent experiments. *p < 0.001 vs. control.

Additionally, the effect of phytoestrogens on invasion was determined using a Transwell chamber coated with Matrigel. As is shown in Figure 3, genistein, daidzein and equol significantly reduced the invasion of DU-145 and PC-3 cells. In the case of PC-3 cells, equol, daidzein and genistein (50 µM) reduced significantly the invasion by 59.8%, 51.7% and 64.3% vs. control cells ($p < 0.001$). Treatment with equol, daidzein and genistein (50 µM, 48 h) diminished significantly ($p < 0.001$) the invasion of DU-145 by 60.5%, 51.2% and 63.4% respectively versus control cells. In both cell lines significant differences were observed between daidzein and equol and genistein treatments ($p < 0.05$).

This inhibitory effect is significantly different ($p < 0.001$) in all treatments with respect to control cells in both DU-145 and PC-3 cells. Moreover, differences between treatments are shown specifically between daidzein treatment and genistein and equol treatments ($p < 0.05$). These results corroborate that equol, daidzein and genistein reduced significantly the migration of PC-3 and DU-145 cells.

To elucidate the possible underlying mechanisms of anti-invasive activities of genistein, daidzein and equol on PC-3 and DU-145 cells, we analyzed changes in MMP-2 and MMP-9 mRNA expression by RT-PCR. As shown in Table 2, 50 µM genistein treatment decreased MMP-2 and MMP-9 mRNA by 61% and 57% respectively in DU-145 cells. However, in PC-3 cells genistein had not effect on MMP-2 and reduced MMP-9 mRNA expression by 32%. Daidzein treatment also decreased MMP-2 and MMP-9 mRNA by 53% and 37% respectively in DU-145 cells but had not effect on MMP-2 and -9 mRNA in PC-3 cells. Finally, equol (50 µM) had not effect on MMP-2 and -9 mRNA in DU-145 cells in contrast to PC-3 cells where equol decreased 28% and 39% on MMP-2 and -9 mRNA respectively (Table 2).

Figure 4
Inhibitory effects of phytoestrogens on the invasion assay of PC-3 and DU-145 cells.



Cells were treated with different phytoestrogens (genistein, daidzein, equol) (50 µM) for 48 h and then harvested and seeded into the upper chamber coated with Matrigel. The number of cells that invaded into the lower chamber represented the invasion capability. Quantification of the invasion assay of DU-145 (black column) and PC-3 (open column). Data represent means±SD of three independent experiments. * p<0.001 vs. control; # p<0.05 vs. genistein and equol treatments.

Table 2
Effects of genistein, daidzein and equol (50 µM) on MMP-2 and MMP-9 gene expression in prostate cancer cell lines DU-145 and PC-3

		MMP-2	MMP-9
DU-145			
	Genistein	**0.39 ± 0.12****	**0.43 ± 0.14****
	Daidzein	**0.47 ± 0.13****	**0.63 ± 0.16***
	Equol		**1.02 ± 0.15**
PC-3			
	Genistein	**0.99 ± 0.16**	**0.68*± 0.10**
	Daidzein	**1.09 ± 0.21**	**1.13 ± 0.18**
	Equol	**0.72 ± 0.09***	**0.61 ± 0.11***

Numbers show up-regulation (>1) or down-regulation (<1) of each gene in each of the treatments. Values are already normalized against GAPDH expression, which was used as reference gene.Data represent means±SD of three independent experiments.

Together, these findings suggest that down-regulation of MMP-2 and MMP-9 might be involved in the inhibition of invasion of PC-3 and DU-145 cells after genistein, daidzein and equol treatment. Prostate cancer is one of the most commonly diagnosed cancers in Western countries (Jemal *et al.*, 2008). Epidemiological evidence suggests that increased consumption of soy and soy-based foods is associated with a decreased risk for prostate cancer (Hsing *et al.*, 2000; Kolonel *et al.*, 2000; Lee *et al.*, 2003). Interest in the effects of soy isoflavones focused mainly on genistein. Previous studies have reported that genistein inhibited the growth in LNCaP, DU-145 and PC-3 prostatic cancer cells (Li & Sarkar, 2002; Ouchi *et al.*, 2005; Shen *et al.*, 2000; Shenouda *et al.*, 2004). Daidzein also inhibited

proliferation in LNCaP, PC-3 and DU-145 (Cao *et al.,* 2006; Li & Sarkar 2002).

The current study extends these findings to include equol, a secondary metabolite of daidzein because very few studies have analyzed the direct actions of equol in the prostate. A recent study by Mitchell *et al.*. provides evidence to date that equol has direct effects on the growth of human prostate cancer cells (Mitchell *et al.,* 2000; Lund *et al.,* 2004; Lampe 2010). Another study indicates that equol reduced in a dose and time-dependent manner the viability of benign and malignant prostatic cells, PC-3 and DU-145 (Hedlund *et al.,* 2002; Magee *et al.,* 2006). Our data corroborate these findings showing similar effects for equol and genistein and a minor effect for daidzein on cell viability decrease.

Moreover, we compared the effects of the three phytoestrogens in prostate cancer cells (DU-145 and PC-3) migration, invasion and MMP-2 and -9 expressions.

One important characteristic of metastasis is the migratory and invasive ability of tumor cells. We used wound healing and Boyden chamber assay to quantify the effect of phytoestrogens, equol, daidzein and genistein on the migratory potential of PC-3 and DU-145 cells. The results demonstrated that equol, daidzein and genistein significantly diminished migration in both cell lines, PC-3 and DU-145, in wound healing and Boyden chamber assay. Daidzein in both cases has a minor effect compared to genistein and equol according to previous data (Ju *et al.,* 2006; Kang *et al.,* 2007). These results were consistent with previous studies. Other data indicates that these phytoestrogens reduced migration in breast cancer cell lines, MCF-7 and MDA-MB-231 (Magee *et al.,* 2006) and in ovarian cancer cell line, BG-1 Hsu *et al.,* 2009). Little data are available about the effect of equol on cellular migration (Zheng *et al.,* 2012).

Furthermore, we used Boyden chamber assay to quantify the effect of phytoestrogens on invasion of PC-3 and DU-145 cells. The compounds also showed similar effects in inhibiting the invasion of prostatic cells through Matrigel at the concentration used. Daidzein has a significant minor effect compared to genistein and equol. Little data are avalaible about the effect of equol and daidzein on cellular invasion in prostate cancer cells (Magee *et al.,* 2006; Zheng *et al.,* 2012).

Many reports have addressed the importance of interactions between cells and ECM, which could enhance cell migration, invasion, proliferation, and ECM degradation. Metastasis has been found to be accompanied by various physiological alterations involved in the degradation of ECM, such as the overexpression of proteolytic enzyme activity, such as MMPs, as well as the migration and invasion of tumor cells (Bellon *et al.,* 2004; Minard *et al.,* 2004). The expression of both MMP-2 and MMP-9 has been shown to play a critical role in degrading the basement membrane in cancer invasion and migration (Attiga et al., 2000). Moreover, the overexpression of MMP-9 is related to tumor invasion and metastasis in gastric carcinoma (Sun *et al.,* 2005). Li *et al.,* reported that the expression of MMP-2 and -9 has significant prognostic value in node-negative patients for predicting relapse free survival (Li *et al.,* 2004). Increased MMP-2 expression has been associated with a higher Gleason score, a more advanced disease stage and decreased disease-free survival (Trudel *et al.,* 2003).

Our study indicates that equol, daidzein and genistein inhibited on MMP-2 and -9 gene expressions in prostate cancer cell lines, PC-3 and DU 145. As shown in Table 1, our results indicated that daidzein diminished the expression of MMP-2 and MMP-9 in DU-145 cells. However daidzein treatment has no effect on these two genes expression in PC-3 cells. By the other hand, equol decreased expression of both, MMP-2 and MMP -9 mRNA, in PC-3 cells having no effects in DU-145 cells. Finally, genistein reduced significantly the expression of MMP-9 in both cell lines but only diminished expression of MMP-2 in DU-145 cell line. These data are not in agreement with recent data indicating that equol and genistein inhibited MMP-2 and -9 in DU-145 cells (Zheng *et al.,* 2012) and that genistein reduced MMP-2 levels in PC-3 cells (Kumi-Diaka *et al.,* 2006). Previously, it has been found that genistein down-regulated the expression of MMP-9, MMP-2, uPAR and other genes related to angiogenesis and metastasis by gene expression profiling of genistein treated PC-3 prostate cancer cells and PC-3 bone tumor (Li & Sarkar, 2002) and these data was further reported by studies showing inhibition of MMP-9 expression in PC-3 cells in vitro (Li *et al.,* 2004) and the inhibition of MMP-2 activation and reduction of prostate cancer cell invasion by genistein (Huang *et al.,* 2005; Xu & Bergan, 2006). Taken together our data suggested that equol, daidzein and genistein inhibited migration and invasion in prostate cancer cell lines. Moreover, these results suggested that the anti-invasive effect of equol, daidzein and genistein was related to the

inhibition of enzymatically degradative processes of tumor metastasis associated to MMP-2 and -9 in a cell-type manner. These data indicates that it is possible to target motility-associated processes with equol, daidzein and genistein.

Nevertheless, the underlying molecular mechanism of action of MMP-2 and -9 remain unknown and further research is required.

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