Exp Oncol 2021
43, 2, 125–134

Experimental ONCOLOGY

CO-ADMINISTRATION OF APIGENIN WITH DOXORUBICIN ENHANCES ANTI-MIGRATION AND ANTIPROLIFERATIVE EFFECTS VIA PI3K/PTEN/AKT PATHWAY IN PROSTATE CANCER CELLS

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Prostate cancer is one of the leading cancers in men, and new approaches are needed for its treatment. *The aim* of this study was to investigate the effect of co-administration of naturally occurring flavone apigenin and doxorubicin to androgen-insensitive prostate cancer cells. *Methods*: The effect of the treatment on survival and migration of human PC3 cells was evaluated by MTT and scratch assay, respectively. Apoptosis and cell cycle distribution were detected by image-based cytometry. mRNA and protein expression were determined by real-time quantitative polymerase chain reaction and Western blot, respectively. *Results*: Apigenin and doxorubicin dose-dependently inhibited cell survival, and co-administration of both agents significantly induced cell death via upregulating the mRNA expression of caspases, Bax and cytochrome c, and downregulating Bcl-XL. Combination therapy caused cell cycle arrest by upregulating the expression of p21 and p27. The treatment modality inhibited cell migration via downregulating Snail, Twist and MMPs in which doxorubicin was ineffective. Apigenin dephosphorylated Akt strongly, significantly suppressed ERK phosphorylation, and increased PTEN expression 4.5-fold. The combination of apigenin and doxorubicin inhibited PI3K and AKT phosphorylation more strongly than a single administration. *Conclusions*: Our data indicate that a combination of the natural flavone apigenin with doxorubicin might have a potential in treatment of castration-resistant prostate cancer.
***Key Words*: prostate cancer, PC3, doxorubicin, apigenin, cell migration.**

DOI: 10.32471/exp-oncology.2312-8852.vol-43-no-2.16096

Prostate cancer (PCa) is the second most commonly occurring cancer in men worldwide and ranks sixth in cancer-related deaths [1]. In 2018, 1,276,106 men were diagnosed with PCa worldwide, which accounted for approximately 7.1% of all diagnosed cancers [2]. It is the fifth most common cause of cancer-related death worldwide, and 358,989 deaths in 2018. The signaling pathways associated with androgen receptor play a key role in the development and function of male reproductive organs. Basically, irregular hormonal stimulation is known to be the main route for proliferation of PCa cells. Thus, androgen deprivation therapy is the most common approach for the early stage of PCa treatment [3]. However, most cases eventually become castration-resistant, bypassing the need for androgen, which is often incurable, aggressive and deadly. Therefore, emerging effective therapeutic approaches are the hardest challenges in PCa research [4].

Although docetaxel therapy in combination with prednisone is used as a first line standard therapy in patients with androgen-independent PCa, the development of drug resistance and side effects such as neutropenia, nausea, vomiting, and anorexia are common [5, 6]. Doxorubicin, a natural anthracycline antibiotic, is a topoisomerase II inhibitor that is effectively used for cancer treatment [7]. Though, it has been used as a less conventional therapy for patients with PCa, its efficacy as a single agent was reported within the wide range of 5–84%, depending on the response criteria [8]. The effects of doxorubicin such as triggering a DNA damage response via damaging DNA and causing apoptosis by cell cycle arrest in the G2/M phase on solid tumors have been clinically approved, and doxorubicin is remarkably efficient in PCa treatment [9, 10]. However, doxorubicin therapy is limited due to its cardiotoxic adverse effects [11]. In order to optimize the treatment for the highest effect while minimizing the side effects, doxorubicin is often used in combination therapies with other drugs [7].

Flavonoids are secondary metabolites of the polyphenol structure that are naturally produced by plants. The plants use these compounds for protection against UV-B, microbial infection and herbivores [12]. Apigenin, (4′,5,7-trihydroxyflavone), is a naturally occurring flavon, which is widely found in plants, herbs, fruits, and vegetables such as parsley, onion, orange and tea [13]. Apart from the antitumor properties, apigenin exhibits antioxidant, anti-inflammatory, antibacterial and antimetastatic activities [12]. Apigenin has been proven as a chemopreventive agent against various malignancies including PCa [14] and hepatocellular cancer [15]. This flavone has also been shown to be effective against cancer stem cells, which are involved in therapy resistance development and recurrence [14, 16]. Apigenin provides its anticancer effects causing cell cycle arrest and inducting apoptosis in extrinsic caspase-dependent pathway, suppressing the migration and invasion via downregulation of matrix metallopeptidases-2,-9 and Snail expression [17, 18]. It has been shown that apigenin blocks signal transduction pathways such as nuclear factor-κB (NF-κB), phosphatidylinositol-3-kinase (PI3K)-AKT, insulin-like growth factor 1 axis and beta-catenin [19].

The therapeutic potential of apigenin in various cancers is being studied, but its combination with

Submitted: August 06, 2020.
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Abbreviations used: MMP – matrix metallopeptidases; NF-κB – nuclear factor-κB; PCa – prostate cancer; PI3K – phosphatidylinositol-3-kinase; RT – room temperature.

doxorubicin in androgen-insensitive PCa cells has not yet been elucidated. Therefore, in this study, the effects of apigenin and doxorubicin combination on cell viability and migration in castration-resistant PCa cells and their mechanism of action were analyzed.

MATERIALS AND METHODS

Cell culture. Androgen-insensitive human prostate cancer PC3 cells were purchased from ATCC (Manassas, USA). The cells were grown in complete DMEM/F-12K medium (Winsent, Canada) containing 2 mM L-glutamine, 1500 mg/L sodium bicarbonate, 10% fetal bovine serum (FBS; Life Technologies, USA) at 37 °C and 5% CO_2. Apigenin and doxorubicin (Sigma–Aldrich, USA) were dissolved in DMSO (Sigma-Aldrich, USA) and stored at –20 °C until use. The final concentration of DMSO in culture medium did not exceed 0.1%.

Cell survival test. PC3 cells were seeded into 96-well plates overnight, and then exposed to 1.6–100 μM apigenin or 0.16–10 μM doxorubicin for 48 h. In combination therapy, cells were exposed to 25 μM apigenin with 1.25 μM doxorubicin for 48 h. After incubations, MTT test was performed to determine the effect of pharmacological agents on cell survival. Briefly, 1 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to the wells. Following 3 h incubation at 37 °C, MTT solution was discarded, and then formazan crystals were dissolved by 200 μl of DMSO. The absorbance was measured at 570 nm using a plate reader (Multiscan GO, Thermo Scientific, Finland). Viability of the cells was calculated by the formula:

OD sample/OD blank control • 100.

Cell viability tests were repeated at least three times and performed in eight wells for each agent.

Morphological assessment of cells. Hoechst 33342 dye (Sigma–Aldrich, USA) was used to label nuclear DNA of the cells. The cells were cultured in 12-well plates at a density of 2.5 • 10^5 per well in 1 ml of medium containing 10% FBS overnight. Then, cells were treated with 25 μM apigenin, 1.25 μM doxorubicin or both for 48 h. Next, the cells were washed twice with PBS and stained with Hoechst dye (5 μg/ml) at 37 °C for 5 min. The morphological alterations of the cells with fragmented nuclei were observed immediately under an inverted fluorescence microscope (ZEISS, Axio Vert A.1, Germany) at 340–510 nm emission at × 40 magnification and images were captured. The assessment was made twice and performed in three replicates.

Determination of apoptosis. The percentage of apoptosis generated by apigenin, doxorubicin and combined treatment in cells was quantitatively determined by the cell-based cytometric method. Following the treatment of PC3 cells with 25 μM apigenin, 1.25 μM doxorubicin or both for 48 h at a density of 2.5 • 10^5 for 48 h, the cells were washed twice with ice-cold PBS, suspended in annexin V binding buffer and incubated with Annexin V Alexa Fluor 488 (Invitrogen/Life Technologies, USA) at room temperature (RT) in the dark for 15 min. Next, cells were centrifuged at 300 × *g* for 5 min, then resuspended in annexin V binding buffer and incubated with PI in the dark for 5 min at RT. The images were evaluated by Tali image-based cytometer (Invitrogen/Life Technologies, USA) within 30 min. Apoptosis evaluation was performed twice and performed in three replicates.

Cell cycle analysis. The effect of combined therapy on the cell cycle progression was examined by cytometric method. For this, PC3 cells were cultured at a density of 2.5 × 10^5 per 12-well plate and treated with 25 μM apigenin, 1.25 μM doxorubicin or both for 48 h. Then, the cells were detached by trypsinization, fixed in 70% ethanol and kept at –20 °C, overnight. The cells were washed in PBS, and treated with propidium iodide kit (Tali Cell Cycle Kit, Life Technologies, USA) at RT for 30 min. The cell population found in cell cycle phases was determined by image-based cytometer. The results were stated as percentage of G_1, S and G_2 cells. Analyzes were repeated three times in three replicates.

mRNA expression. Cells were seeded at a density of 1 × 10^6 in 25 cm^2 flask, after overnight incubation, they were then treated with 25 μM apigenin, 1.25 μM doxorubicin or their combination for 48 h. The total RNA samples were isolated according to the manufacturer instructions (Thermo Fisher Scientific, USA). Then, mRNA was converted into complementary DNA using cDNA synthesis kit (Thermo Fisher Scientific, Applied Biosystems, USA). The mRNA expression levels found in samples were assessed by Step One Plus Real-Time PCR Systems (Applied Biosystems, USA) using power SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA). A representative reaction condition consisted of an initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s and 72 °C for 15 s. The fold enrichments of the targets were calculated using the relative quantification according to the $2^{-\Delta\Delta Ct}$. The primer sequences used in specific amplifications (PRZ Biotech, Turkey) are given in the Table. In the analysis, triple wells were used and GAPDH was used as internal reference. PCR analysis were performed using two different RNAs and triple repeats for each gene.

Assessment of cell migration. The effect of treatment on cell migration was assessed by scratch assay. Prostate cancer cells were cultured in 6-well tissue culture plates with serum-free medium to reach 80–90% confluence. After the cell monolayers were scratched with 200 μl micropipette tips along the center of each well, they were allowed to migrate for 48 h by applying control, 25 μM apigenin, 1.25 μM doxorubicin and a combination thereof. The gap was photographed under an inverted microscope at 12 h, 24 h and 48 h, and gap closure was measured. Cell migration assay was repeated twice in triplicates.

Table. Sequence of forward (F) and reverse (R) primers used in mRNA expression analysis of selected genes

Target gene	Accession number	Primer sequence
Caspase 3	NM_001354779.1	F:5´-GGCATTGAGACAGACAGTGG-3´ R: 5´-CATGGAATCTGTTTCTTTGC-3´
Bax	XM_017027077.1	F:5´-TTGCTTCAGGGTTTCATCCA-3´ R:5´-CAGCCTTGAGCACCAGTTTG-3´
Caspase 8	NM_001080125.1	F:5´-CTGCTGGGGATGGCCACTGTG-3´ R:5´-TCGCCTCGAGGACATCGCTCTC-3´
Cytochrome c	NM_018947.5	F:5'-AGTGGCTAGAGTGGTCATTCATTTACA-3´ R:5´-TCATGATCTGAATTCTGGTGTATGAGA-3´
Bcl-XL	NM_138578	F:5´-GTAAACTGGGGTCGCATTGT-3´ R:5´-TGGATCCAAGGCTCTAGGTG-3´
p21	NM_001291549.1	F:5´-GGCGTTTGGAGTGGTAGAAA-3´ R:5´-GACTCTCAGGGTCGAAAACG-3´
p27	NM_004064.4	F:5´-CCGGCTAACTCTGAGGACAC-3´ R:5´-TTGCAGGTCGCTTCCTTATT-3´
Snail	NM_005985.3	F:5´-CAACCCACTCAGATGTCAA-3´ R:5´-CATAGTTAGTCACACCTCGT-3´
Twist	NM_000474.3	F:5´-GGGAGTCCGCAGTCTTAC-3´ R:5´-CCTGTCTCGCTTTCTCTTT-3´
MMP1	NM_001145938.1	F:5´-ACAGCCCAGTACTTATTCCCTTTG-3´ R:5´-GGGCTTGAAGCTGCTTACGA-3´
MMP7	NM_ 002423.4	F:5´-TGAGCTACAGTGGGAACAGG-3´ R:5´-TCATCGAAGTGAGCATCTCC-3´
MMP9	NM_004994.2	F:5´-CCTTGTGCTCTTCCCTGGAG-3´ R:5´-GGCCCCAGAGATTTCGACTC-3´
GAPDH	NM_001289745.2	F:5´-TTGGTATCGTGGAAGGACTCA-3´ R:5´-TGTCATCATATTTGGCAGGTTT-3´

Western blot analysis. PC3 cells were treated with 25 μM apigenin, 1.25 μM doxorubicin or 25 μM apigenin + 1.5 μM doxorubicin for 48 h to reveal the mechanisms underlying the effect of combination therapy of apigenin and doxorubicin on PCa cells. Following, protein lysates were extracted using ice cold RIPA buffer containing a cocktail of protease inhibitors. After denaturation, 50 μg samples were separated by 8–12% polyacrylamide gel and transferred onto PVDF membrane (Life Technologies, USA). After nonspecific binding sites were blocked, they were incubated overnight at 4 °C with anti-p-PI3K (LifeSpan, Bioscience, USA), anti-PTEN, anti-p-Akt and anti-p-ERK (Santa Cruz Biotechnology Inc., USA) and anti-β-actin antibodies (Novus Biologicals, USA). Bound secondary antibodies were then detected using appropriate anti-rabbit or anti-mouse immunoglobulin G with a chemiluminescence substrate kit (Thermo Fisher Scientific, USA). The density of the bands obtained was determined using the gel imaging System (Bio-Rad ChemiDoc MP System, USA). β-actin was used as the loading control. Protein expression was assessed using two different homogenates and triple replicates for each sample.

Statistical analysis. The differences between the groups were evaluated by one-way analysis of variance. Subsequently, Duncan's multiple comparisons test was used for multiple comparisons (SPSS, 19.0; SPSS, Chicago, IL for Windows). $P < 0.05$ was considered statistically significant. Each data point represents the mean ± SD.

RESULTS

Increasing concentrations of apigenin, doxorubicin and their combinations were administrated to PC3 cells in order to examine the cytotoxic effects of the agents. As shown in Fig. 1, both agents dose-dependently inhibit cell survival. We then determined the efficacy of co-administration by combining approximately median concentrations of 25 μM apigenin and 1.25 μM doxorubicin. According to MTT (Fig. 1) and cytometric (Fig. 2) analysis, combination therapy reduced cell survival by 62%, while apigenin and doxorubicin as single agents decreased cell survival by 41% and 43%, respectively.

To study the apoptotic effects of these two agents, cells were treated with 1.25 μM doxorubicin, 25 μM apigenin, or combinations thereof for 48 h. First, we checked by Hoechst staining at the cellular level whether treatment strategies caused apoptosis. The result showed that both agents caused apoptosis (Fig. 2, *a*). It was noteworthy that co-administration led to higher apoptosis rate detected by Hoechst staining. Then, the cells treated with the same agents were subjected to annexin V/PI cytometric analysis. As in case of microscopic evaluation, the combined apigenin-doxorubicin treatment increased the apoptosis rate compared to single use of these agents. While apigenin and doxorubicin caused apoptosis at about 30% and 25%, respectively, the rate was 36% in combined application (Fig. 2, *b*). Similarly, while apigenin and doxorubicin induced non-apoptotic cell death by about 11% relative to the untreated cells, dual administration enhanced this effect to 25% (Fig. 2, *b*).To identify the changes in expression of the apoptosis-involved genes induced by apigenin and doxorubicin combination, RT-qPCR analysis was performed with RNA samples obtained from the cells treated similarly to the above. Both doxorubicin and apigenin significantly induced the mRNA expression of caspase-3, -8 and cytochrome c (Fig. 3), but combination treatment did not increase their effects as single agents.

Treatment modality significantly arrested cell cycle progression at G_1 phase. Both treatment applications increased the G_1 phase cell population above 80% as compared to 58% in untreated cells (Fig. 4, *a*). mRNA expression of cell cycle inhibitors p21 and

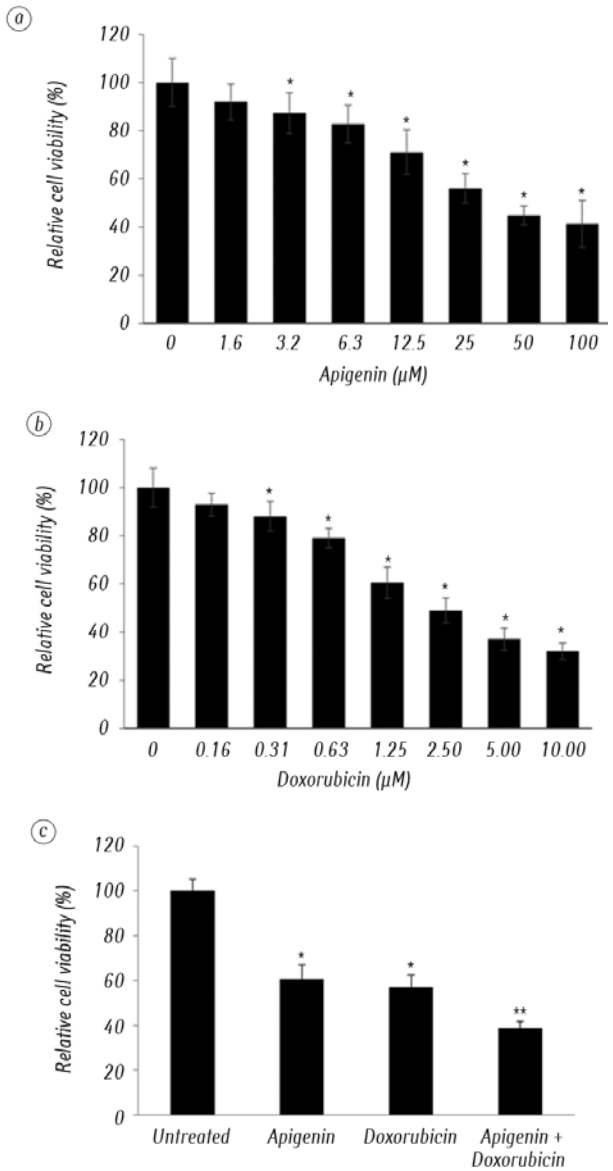

Fig. 1. Co-administration of apigenin enhances the effect of doxorubicin on reducing cancer cell survival. PC3 cells were exposed to different concentrations of apigenin (*a*) or doxorubicin (*b*) for 48 h and then subjected to the MTT cell survival test. Cells were treated for 48 h with 25 μM apigenin, 1.25 μM doxorubicin or a combination of both (*c*), and then a cell survival test was performed. *$p < 0.05$ compared with untreated control. **$p < 0.01$ compared with apigenin and doxorubicin

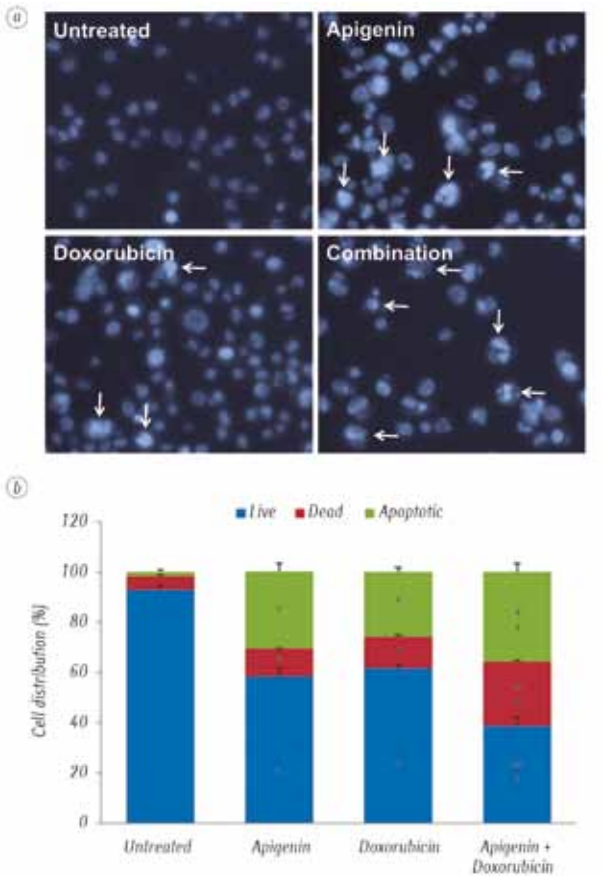

Fig. 2. The combination of apigenin with doxorubicin increases the rate of apoptosis in prostate cancer cells. PC3 cells were treated with 25 μM apigenin, 1.25 μM doxorubicin or a combination of both agents for 48 h. Subsequently, cells were stained with Hoechst 33342 dye and examined under fluorescence microscope (*a*) or after annexin V/PI staining apoptotic and necrotic cell counts were determined by flow cytometry (*b*). Arrows point to apoptotic cells. *$p < 0.05$ compared with untreated control. **$p < 0.01$ compared with apigenin and doxorubicin. $^{+}p < 0.05$ compared with doxorubicin

p27 were significantly induced by apigenin and doxorubicin, by 2.5-fold and 6-fold, respectively, while co-administration increased p21 mRNA expression by 5.8-fold compared to untreated cells (Fig. 4, *b*). The efficacy of the applied agents on p27 expression was slightly different from p21. While each agent increased p27 expression by 2-fold compared to untreated cells, the combined administration enhanced p27 expression by 3-fold (Fig. 4, *c*).

Since cell migration is one of the crucial steps of metastasis, the effectiveness of the applications to cell migration was evaluated by scratch assay. According to the observations made, 1.25 μM doxorubicin treatment did not significantly prevent cell migration compared to untreated cells (Fig. 5 *a, b*).

On the other hand, apigenin significantly inhibited the migration (Fig. 5, *a*). The degree of inhibition of cell migration by apigenin was similar when combined with doxorubicin. The remaining wound area after apigenin applications were significant compared to the untreated cells (Fig. 5, *b*). To evaluate the mechanisms underlying the anti-migration effects of apigenin combination, mRNA expression of several cell migration-related transcription factors and enzymes was analyzed. The results showed that doxorubicin exerted no effect on *Snail* (Fig. 6, *a*), *Twist* (Fig. 6, *b*) and *MMP1* (Fig. 6, *c*) expression, similar to the scratch assay. On the other hand, apigenin significantly reduced the expression of the specified genes, both alone and in combination (Fig. 6, *a–c*). Both agents suppressed MMP7 expression, but combination treatment further enhanced the effect (Fig. 6, *d*). While doxorubicin strongly suppresses MMP7 expression, apigenin was less effective. However, combination administration was still effective relative to the untreated cells (Fig. 6, *e*).The expression and phosphorylation states of some intracellular signal molecules were analyzed to determine the inhibitory

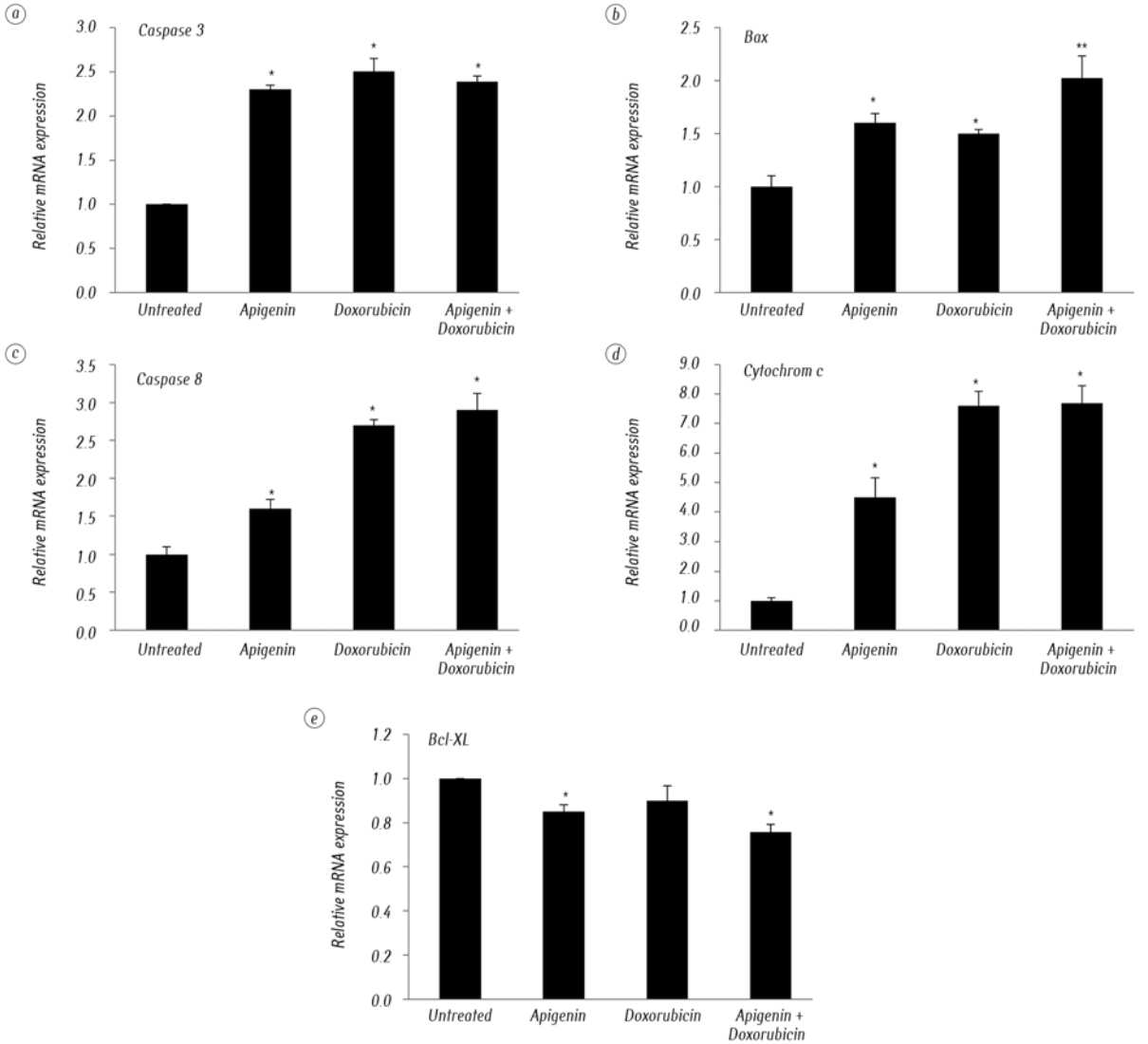

Fig. 3. Treatment modality regulates the expression of apoptosis-related genes. RNA samples were isolated after cells were exposed to 25 μM apigenin, 1.25 μM doxorubicin or their combination for 48 h. *$p < 0.01$ compared with untreated control. ** $p < 0.05$ compared with apigenin and doxorubicin as single agents

effectiveness of the applied treatment regimen for cell survival and migration. Neither single apigenin nor doxorubicin altered PI3K phosphorylation, but co-administration of both agents significantly inhibited PI3K phosphorylation (Fig. 7, *a*). However, these pharmacological agents used alone significantly increased anti-tumor PTEN protein expression. The effect did not increase further upon the combined treatment (Fig. 7, *b*). Unexpectedly, doxorubicin administration increased AKT phosphorylation, while apigenin and combined treatment significantly reduced AKT phosphorylation (Fig. 7, *c*). Both treatments significantly reduced the expression levels of phospho-ERK proteins, while co-administration did not change this effect (Fig. 7, *d*).

DISCUSSION

Although taxol derivatives are used as a first line therapy in the treatment of metastatic castration-resistant PCa, side effects and drug resistance development are common in patients [20]. According to previous reports, naturally occurring bioactive apigenin makes several cancer cells sensitive to cisplatin [21], 5-fluorouracil [22], docetaxel [14], etoposide and doxorubicin [23]. Doxorubicin has been effectively used in cancer therapy; nevertheless, its use is limited due to cardiotoxic side effects [11]. On the other hand, cardiotoxic side effects of doxorubicin can be attenuated by apigenin administration [24, 25]. It has been shown that apigenin makes hepatocellular carcinoma cells susceptible to doxorubicin [15] through inhibiting PI3K/Akt/Nrf2 pathway. In cancer treatments, a combination therapeutic strategy is preferred for effective treatment, since tumor cells have multiple mutations and are intended to attenuate the side effects. However, the concomitant use of apigenin with doxorubicin has not been studied in PCa. Thus, we hypothesized that co-administration of apigenin may make PCa cells susceptible to doxorubicin.

Previous studies [26, 27] and current data show that apigenin dose- and time-dependently inhibit the proliferation of PCa cells as well as cancer stem

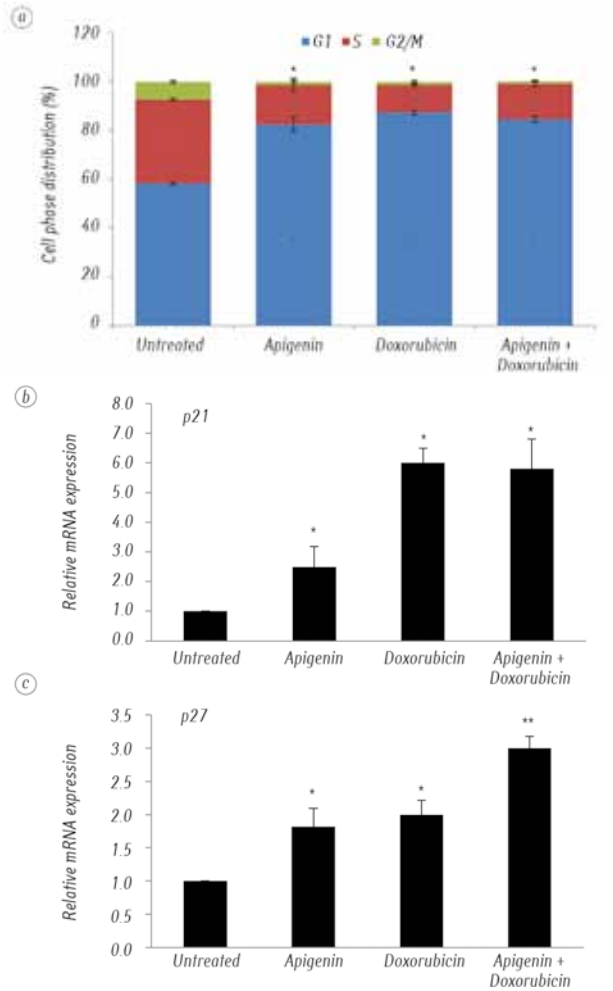

Fig. 4. Applications of pharmacological agents regulate the cell cycle. After PC3 cells were exposed to 25 μM apigenin, 1.25 μM doxorubicin or their combination for 48 h, analysis of cell cycle phases was performed by cytometric method (*a*). After similar application, mRNA expression analyzes of cell cycle-related genes were evaluated by RT-qPCR (*b, c*). *$p <$ 0.01 compared to untreated control. **$p < 0.01$ compared to apigenin and doxorubicin

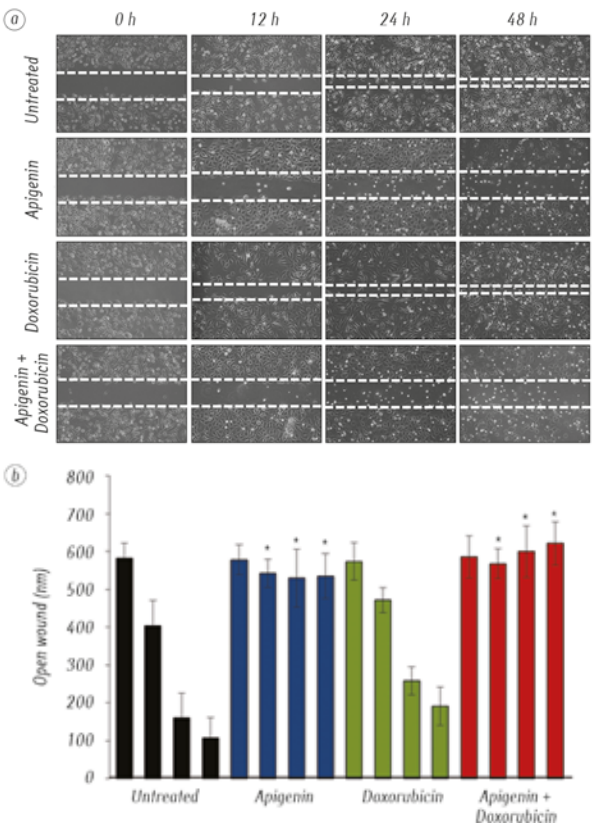

Fig. 5. Apigenin but not doxorubicin inhibits cell migration. Cells were incubated for 48 h with 25 μM apigenin, 1.25 μM doxorubicin and their combination, and subjected to scratch assay (*a*). During the wound healing period, width of the gaps was measured under a microscope at 12 h, 24 h and 48 h (*b*). *$p <$ 0.01 compared to untreated control and doxorubicin

cells [14, 21]. The anti-proliferative potential of this agent on breast [28], colon [29] and gastric [30] cancer cells has also been demonstrated. These results indicate that apigenin may be a candidate pharmacological molecule that can prevent tumor growth. In this study, we showed that co-administration of doxorubicin with apigenin reduced cells viability by approximately 32% more than a single administration of each agent. This result supports previous reports that apigenin may make various cancer cells susceptible to doxorubicin [23, 31]. Today, strategies to eliminate cancer cells include triggering apoptosis or preventing cancer cell proliferation by arresting the cell cycle. Apigenin shows an anti-tumor effect by inducing apoptosis [14, 32], arresting the cell cycle [21, 33] and preventing metastasis [34] in various cancer cells. Likewise, doxorubicin binds to DNA, induces apoptosis by inhibiting DNA and RNA synthesis [35]. In this study, the treatment modality was shown to cause cell death by causing apoptosis and cell cycle arrest. At the molecular level, the co-administration of the two agents upregulated Bax expression, while the Bcl-XL expression is downregulated compared to doxorubicin. These results strongly support that combination of apigenin with doxorubicin can significantly decrease cell proliferation via inducing apoptosis. The antitumor activity of apigenin is mediated by modulating major intracellular signaling pathways such as PI3K/AKT/mTOR [14, 36], MAPK/ERK [33], JAK/STAT [37], NF-κB [34, 38], Wnt/β-catenin [39] and increasing the intracellular concentration of free radical level [30]. Similar to previous reports, apigenin induced anti-tumor PTEN expression, while significantly inhibiting AKT and ERK phosphorylation in PCa cells. Undoubtedly, co-administration of apigenin with doxorubicin significantly reduced the phosphorylation of PI3K and AKT compared to both single applications. This result indicates that the suppression of cell proliferation in combined therapy may occur via PI3K/AKT signaling pathway inhibition.

Since inhibition of the cell cycle progression will negatively affect mitotic entry, strategies to arrest the cell cycle play an important role in cancer treatment [40, 41]. It has been well documented that apigenin and doxorubicin induces cell cycle arrest at G_1 or G_2/M phases by modulating the expression of different cyclin-dependent kinases and other cell cycle-associated genes such as p21 and p27 [21,

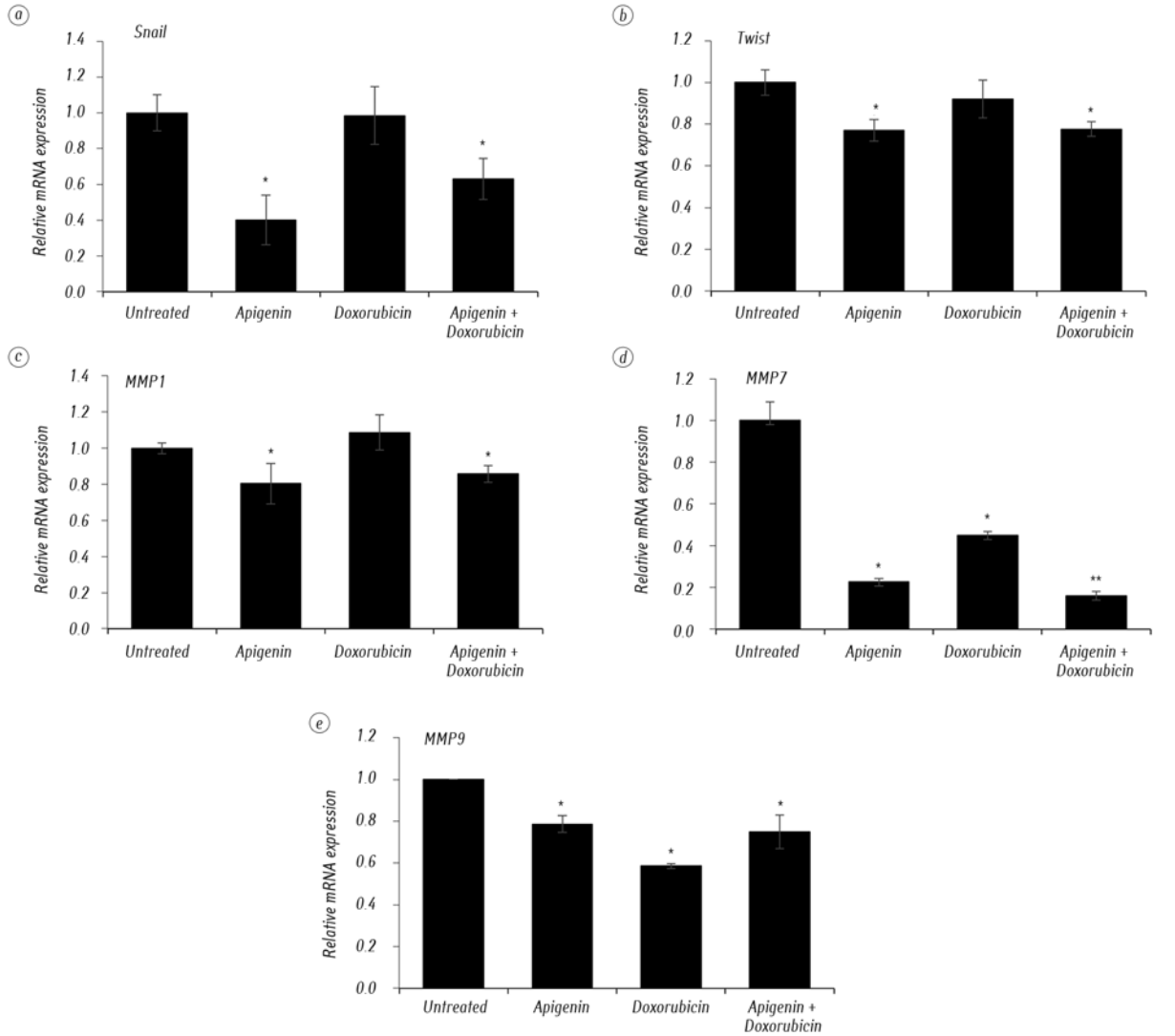

Fig. 6. The combined application regulates the expression of genes involved in cell migration. RNA samples were isolated after cells were exposed to 25 μM apigenin, 1.25 μM doxorubicin or combination of both agents for 48 h. mRNA expression of selected genes were determined by RT-qPCR using specific primers (*a–d*). *$p < 0.05$ compared with untreated control. **$p < 0.01$ compared with apigenin and doxorubicin

38, 42]. We showed that apigenin and doxorubicin arrested cell cycle at G_1 phase, which was supported by an upregulation in the expression of cyclin-dependent kinases inhibitors p21 and p27. Although the combination of both pharmacological agents did not alter the distribution of cell cycle phase population, it caused an increase in non-apoptotic cell death compared to untreated cells. The results obtained from this study are in agreement with previous reports [23, 43, 44] that the combination of flavonoids such as apigenin with doxorubicin increases the cell cycle arrest related to non-apoptotic cell death in different cancer cells.

Metastatic recurrence or migration of cancer cells to distant tissues is considered a distinctive feature of malignant tumor progression and is one of the most common causes of cancer-related death. Therefore, prevention of metastasis is a crucial strategy in cancer treatment. We and others have previously shown that apigenin significantly inhibits migration and invasion of not only prostate cancer cells [27, 45] but also cancer stem cells [14, 21]. It has been reported that doxorubicin does not significantly inhibit the migration of castration-resistant DU-145 PCa cells [46] and breast cancer cells [47]. Similar to previous reports, we found that doxorubicin was ineffective in preventing PC3 cell migration. Therefore, we examined whether PCa cell migration can be prevented with apigenin combined with doxorubicin. Certainly, the combined administration of apigenin with doxorubicin almost completely inhibits the migration of the cells. It has been revealed that apigenin decreases cancer cell migration though downregulation of PI3K/AKT/mTOR [14, 36], Wnt/beta-catenin [39] and NF-κB activity [48]. In this study, combined use of apigenin and doxorubicin significantly reduced PI3K and AKT expressions. In addition, both pharmacological agents stimulate PTEN protein expression, while suppressing ERK phosphorylation. Decrease or loss of PTEN expression leads to hyperactivity in AKT in human prostate cancer [49]. Thus, it can be concluded that inhibition

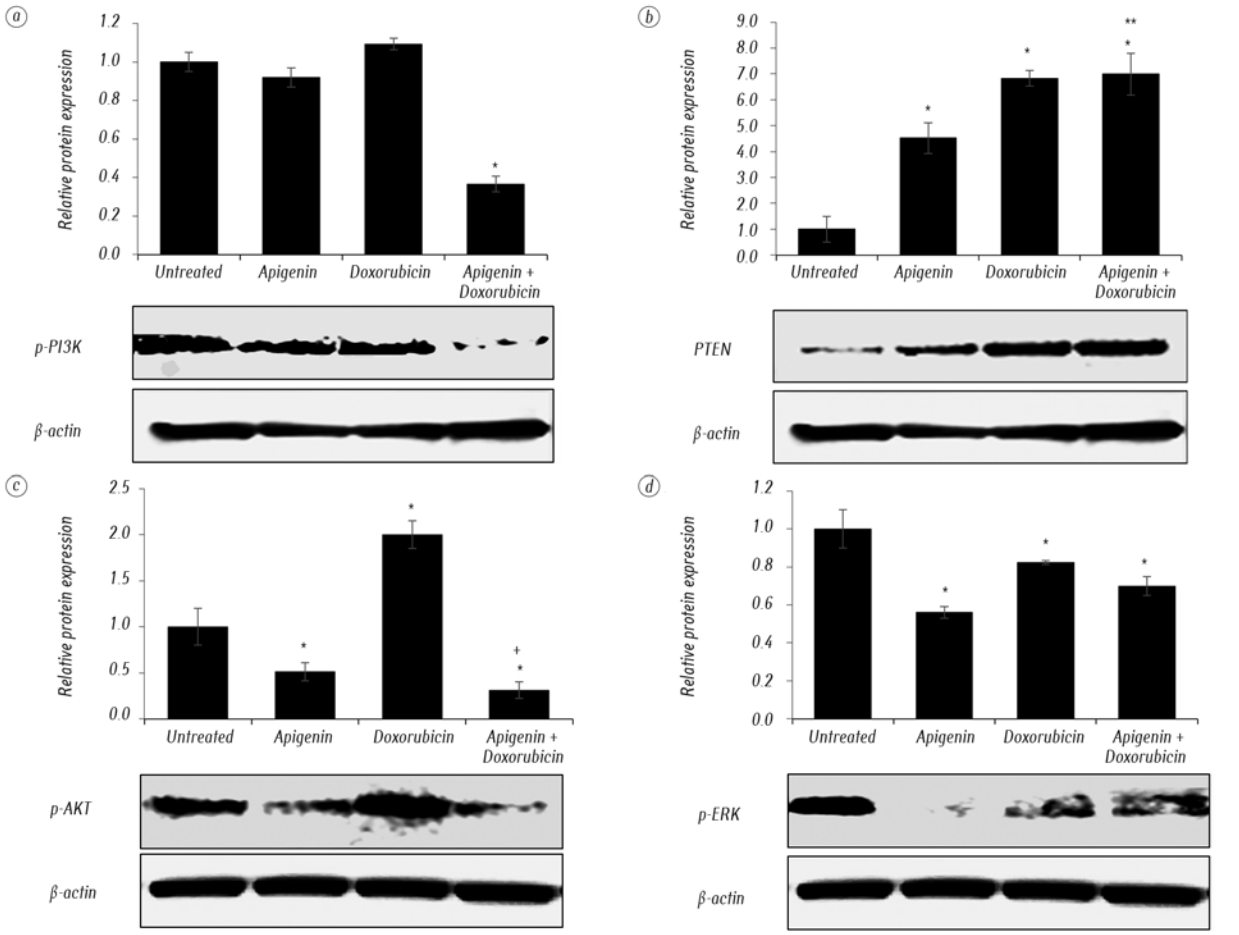

Fig. 7. Apigenin combination regulates the expression of proteins. Protein samples were extracted 48 h after exposure to 25 μM apigenin, 1.25 μM doxorubicin or their combination. The internal loading control and normalization was performed by housekeeping protein β-actin. Anti-p-PI3K (*a*) anti-PTEN (*b*), anti-p-Akt (*c*) and anti-p-ERK (*d*). *$p < 0.05$ compared with untreated control. **$p < 0.01$ compared with apigenin. $^{+}p < 0.01$ compared with apigenin and doxorubicin

of cell migration in which doxorubicin is ineffective can be achieved by its combination with apigenin.

Epithelial-mesenchymal transition is regulated by transcription factors such as Snail and Twist and plays an important role in PCa metastasis [14, 50, 51]. Though doxorubicin administration did not alter *Snail* and *Twist* mRNA expression, apigenin and its combination with doxorubicin significantly downregulated these genes. This result is in parallel with the modulation of the main signaling pathway PTEN/PI3K/AKT, which is associated with cell migration. *Snail* gene not only functions as the main regulator of epithelial-mesenchymal transition, but also mediates cell proliferation and survival through the downregulation of PTEN [52]. We have previously shown that apigenin inhibits PCa stem cell migration by downregulating *Snail, Slug* and matrix metallopeptidases *(MMP)-2, -9*, [14, 21]. Other groups have also shown that apigenin inhibits PCa cell migration by suppressing Wnt/beta-catenin [39] and Snail [27] expression. MMPs are the main modulators of the tumor microenvironment and participate in tumor growth, angiogenesis and metastasis. Accordingly, MMP inhibition seems to be an ideal solution to control cancer. Although doxorubicin inhibits the mRNA expression of *MMP7* and *MMP9*, the combination of this agent with apigenin also reduces *MMP1*, while further reducing *MMP7* expression. Therefore, it can be suggested that the ineffectiveness of doxorubicin in preventing PCa cell migration can be prevented by downregulating the expression of crucial genes in migration such as *MMPs, Snail* and *Twist* with the combination of apigenin.

In conclusion, our findings showed that the combination of apigenin and doxorubicin may be an effective therapeutic strategy to inhibit cancer cell proliferation and cell migration associated with tumor growth and metastasis. However, the results obtained from the study should be supported by *in vivo* experiments.

ACKNOWLEDGEMENTS

This study was funded by the Trakya University Research Project Foundation (TUBAP: 2018 — 17).

AUTHORS' CONTRIBUTIONS

SE designed the study, write up, drafted and critically revised the manuscript. AA, HK and KT performed experiments and made data analysis. All authors read and approved the final manuscript.

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СУМІСНЕ ВВЕДЕННЯ АПІГЕНІНУ З ДОКСОРУБІЦІНОМ ПІДВИЩУЄ АНТИМІГРАЦІЙНИЙ ТА АНТИПРОЛІФЕРАТИВНИЙ ЕФЕКТИ ЧЕРЕЗ PI3K/PTEN/AKT ШЛЯХИ У КЛІТИНАХ РАКУ ПЕРЕДМІХУРОВОЇ ЗАЛОЗИ

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Стан питання*:** Рак передміхурової залози є одним з основних видів злоякісних пухлин у чоловіків, і для його лікування потрібні нові підходи. ***Мета: Метою цього дослідження було вивчити вплив спільного введення природного флавону апігеніну і доксорубіцину *in vitro* на клітини раку передміхурової залози, нечутливі до андрогенів. ***Матеріали і методи:*** Вплив апігеніну і доксорубіцину на виживання і міграцію людських клітин PC3 оцінювали за допомогою MTT-тесту і аналізу міграції клітин в зону подряпини відповідно. Апоптоз і розподіл за фазами клітинного циклу визначали за допомогою проточної цитометрії. Експресію мРНК і білка визначали за допомогою ЗТ-кПЛР і вестерн-блоту відповідно. ***Результати*:** Апігенін і доксорубіцин дозозалежно зменшували кількість життєздатних клітин, і спільне застосування обох агентів значно підсилювало загибель клітин за рахунок посилення експресії мРНК каспаз, Bax і цитохрому с і пригнічення Bcl-XL. Комбінована дія апігеніну і доксорубіцину викликала затримку клітинного циклу за рахунок посилення експресії p21 і p27. Пригнічення міграції клітин відбувалося за рахунок зниження експресії Snail, Twist і MMP, для чого доксорубіцин був неефективним. Апігенин значно дефосфорилював Akt, суттєво пригнічував фосфорилювання ERK і збільшував експресію *PTEN* в 4,5 раза. Комбінація апігеніну і доксорубіцину сприяла сильнішому інгібуванню фосфорилювання PI3K і AKT, ніж їх застосування окремо. ***Висновки:*** Наші дані показують, що поєднання природного флавону апігеніну з доксорубіцином потенційно може покращити лікування кастраційно-резистентного раку передміхурової залози.

***Ключові слова*:** рак передміхурової залози, PC3, доксорубіцин, апігенін, клітинна міграція.