Original Article
Running Title: UDE Impairs Progression of PCa
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Nettle (*Urtica Dioica L.*) Modulates Angiogenesis by Targeting the *PI3K/AKT/eNOS* Pathway in Prostate Cancer

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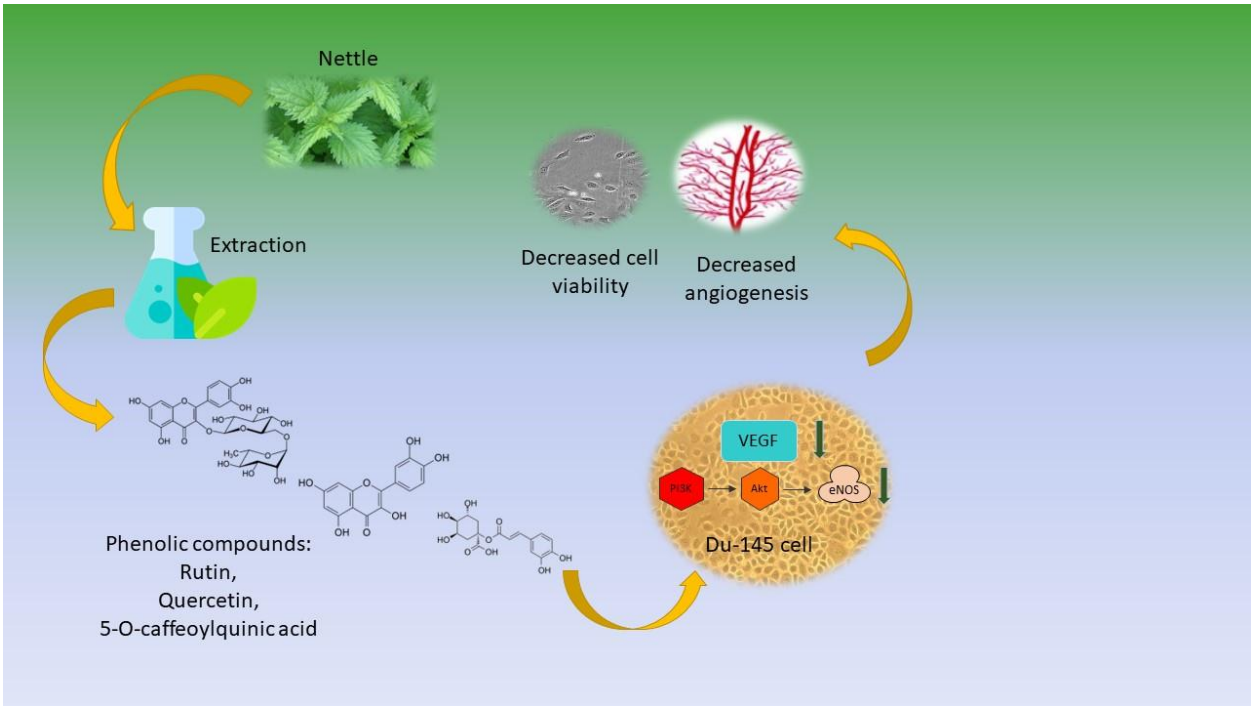
Abstract

Background: Medicinal plants, predominantly those rich in polyphenolic mixtures, have been recommended to have chemopreventive and chemotherapeutic effects. This study aimed to investigate the effects of Urtica dioica L. extract (UDE) on angiogenesis and prostate cancer (PCa) cell progression through the phosphoinositide 3-kinase/Protein kinase B/ endothelial nitric oxide synthase *(PI3K/AKT/eNOS)* signaling pathway.

Method: This study employed an in vitro experimental design using PCa cell lines. To gain mechanistic insights into the anti-proliferative properties of UDE, PCa cell proliferation was assessed using an MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay in DU-145 cells (incubated for 48h). Also, we explored expression patterns of *PI3K/AKT/eNOS* pathway genes with therapeutic potential (with 50 µg/ml of UDE) in DU-145 cells by quantitative polymerase chain reaction and western blotting assay. Furthermore, we applied the ELISA cell death assay kit to reveal the apoptotic effects of UDE on PCa cells. Statistical analysis was determined using the Mann-Whitney U test and $P \leq 0.05$ was considered statistically significant.

Results: UDE significantly decreased the cell viability after 48 h of treatment in DU-145 cells. Also, reducing the *vascular endothelial growth factor (VEGF)* levels revealed anti-angiogenic

outcomes. Also, the *eNOS* level in the *PI3K/AKT/eNOS* pathway is dramatically alleviated upon treatment with UDE. Moreover, the apoptosis rate of DU-145 cells was enhanced compared with the control group.
Conclusion: The antitumoral activity of UDE was prominent in its persuasive anti-angiogenic potential, as UDE contributed to a striking diminish in *PI3K/AKT/eNOS* pathway in PCa and diminished the *VEGF* expression.



Graphical Abstract

Keywords: Prostatic neoplasms, Urtica dioica, Angiogenesis, *VEGF*, *PI3K/AKT/eNOS* pathway

Introduction

Based on Miller et al.'s report in 2022, the most predominant malignancy among the male population is prostate cancer (PCa), with 3,523,230, which is steadily increasing.[1] Some tumors grow slowly and pose little threat to the patient's life without treatment. However, if PCa grows aggressively, it will quickly metastasize and impose difficult consequences on the patient.[2,3] PCa management is a clinical dilemma, and there are many challenges to predicting patient survival in PCa. PCa is heterogeneous and incredibly diverse in its transcriptome.[4-6] Therefore, when cancer is confirmed in a person, an optimal method will be provided to keep patients safe from unnecessary treatment processes. Before starting treatment, the current clinical classification uses Gleason Score, tumor size estimation, and prostate-specific antigen level. To prepare GS, it is necessary to prepare biopsy samples, which increases complications after biopsy in patients and significantly burdens health care.[7,8]

Polyphenols are a great group of natural substances extensively present in plants. These substances are the product of the secondary metabolism of plants, and so far, 8000 types of this group have been identified.

Natural polyphenols are chemically various, ranging from simple molecules to polymerized compounds. Polyphenols are primarily associated with sugar residues (xylose, galactose, glucose, etc.), but their relationship with other molecules, such as lipids, organic acids, and amines, is also typical.[9,10]

Urtica dioica L. (UD), or stinging nettle, is a perennial herbaceous wild flowering plant. It is a widespread crop with several uses occasionally disregarded.[11] Acetylcholine, histamine, and 5-hydroxytryptamine (serotonin) are released when a nettle's caustic hair is touched. Dead UD has a variety of nutrients, including lignans, formic acid, potassium salts, flavonoids, sterols, enzymes, vitamin C, polysaccharides, significant amounts of chlorophyll, coumarins, phenylpropanoids, and terpenoids. The phenolic compounds of UD have antioxidant properties.[12] Phenolic substances stabilize lipid peroxidation. In a study, bioactive components of UD extract (UDE) were determined using high-performance liquid chromatography. Dar et al. explained that the leaves of UDE consist of neophyte diene (19.96 %), 2,6,10,15-tetramethylheptadecane (12.82 %), heptadecyl ester (9.45 %), hexyl octyl ester (6.31 %), butyl tetradecyl ester (4.73 %).[13] The extract comprises ethyl acetate, methanolic, and phenolic components, and flavonoids function as antioxidants with a 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity of about 62% and 98%, as reported in different studies.[14,15] Also, UD has polyphenolic substances that may reduce carcinogenesis and mutagenesis.[16] UD has been introduced as a medicinal plant for treating inflammation for decades. Antioxidant activity and its beneficial effects have been reported in various studies.[17] The positive effect of UDE in reducing inflammation has been shown, and this medicinal plant prevents tumor growth in prostate, colon, lung, and breast cancer cells.[18,19] Therefore, the present study aimed to investigate the effects of UD on angiogenesis and the progression of PCa tumorigenesis.

In the present study, we evaluated the effects of UDE on the DU-145 PCa cell line proliferation through the *phosphatidylinositol-3-kinase (PI3K)/AKT/endothelial nitric oxide synthase (eNOS)* signaling pathway. We also determined the angiogenesis and apoptosis of these cells when treated with UDE.

Materials and Methods

Study design

This study employed an in vitro experimental design using PCa cell lines.

Preparation of the UD extract

The leaves were shade-dried and ground into a powder with a mortar and pestle. The ready powder was utterly shielded from light and stored in airtight containers (due to oxidation by light). The process of extracting hydroalcoholic extract (HAE) involved macerating 100 g of dry plant powder for 48 hours at room temperature in 500 mL of 80% ethanol. Then, the macerated plant material was extracted with 80% ethanol solvent by percolator apparatus (2-liter volume) at 25 ˚C. The plant leaf extract was removed from the percolator, filtered using Whatman filter paper (NO. 4), and dried with a rotatory evaporator at 37°C under decreased pressure. The concentrated leaf extracts of the plant were treated in Phosphate Buffer Saline (PBS) and filtered to produce a 10 mg/ml stock solution. Working solutions with a 180 µg/ml concentration were created from stock and used in the cell culture treatments and subsequent quantitative polymerase chain reaction (qPCR).

Prostate cell culture

The American Type Culture Collection (ATCC; USA) provided the human PCa cell line DU-145, purchased and grown at 37°C,

5% CO2, and 95% humidity in RPMI1640 media with 10% fetal bovine serum. The adequate number of cells for each experiment was calculated based on preliminary experiments and power analysis to ensure statistical significance.

Also, to ensure the identity and purity of the DU-145 cell line, we employed several verification methods. Short Tandem Repeat (STR) profiling was performed upon receipt of the cells and repeated every six months during the study period. The STR profile was compared with the ATCC database to confirm cell line authenticity. Additionally, cells were regularly tested for mycoplasma contamination using a PCR-based mycoplasma detection kit (MycoAlert™ Mycoplasma Detection Kit, Lonza) every three months. Strict aseptic techniques were employed to prevent cross-contamination, and separate media and reagents were used for each cell line. Morphological observations were conducted routinely using phase-contrast microscopy to detect unusual changes in cell appearance or growth patterns.

MTT assay

DU-145 cells were seeded in a 96-well plate with a density of 1×10^4 cells per well, and the cells were incubated in the presence of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/ml of UDE. Untreated DU-145 cells were considered as the control group. After 72 hours of incubation, 50 µl (25 mg/ml) (3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells' medium, and the cells were incubated for 4 h at 37°C. Then, 200 µl DMSO was added to each well to dissolve the formazan crystals and shake for 10 minutes. Then, the resulting color was read by a microplate reader (State Fax, Awareness Technology Inc, USA) at 490 nm. The experiment was performed in triplicate.

Selection of target genes

Vascular endothelial growth factor *(VEGF)*, *PI3K*, *AKT*, and *eNOS* were selected as target genes due to their crucial roles in angiogenesis and the *PI3K/AKT/eNOS* signaling pathway, which is known to be involved in PCa progression. *VEGF* is a key regulator of angiogenesis, while *PI3K*, *AKT*, and *eNOS* are important components of a signaling cascade that promotes cell survival and proliferation in cancer cells.

RNA isolation and qPCR measurement

To extract RNA from cell cultures, cells were seeded in 6-well plates with a density of 2×10^5 cells/ well and treated with defined concentrations of UDE. Total RNA was extracted by TRIzol (Ambion, USA) and quantified by nanodrop (Thermo Scientific, USA).

After synthesizing cDNA using the EURx kit according to the manufacturer's protocol using a cDNA synthesis kit, the expression of *VEGF*, *PI3K*, *AKT*, and *eNOS* genes was assessed in the DU-145 cell line using a qPCR device (Bio-Rad, USA). Relative gene expression was analyzed using the $2^{-\Delta\Delta C_t}$ method (Livak method). *GAPDH* was used as the internal control gene. ΔCt was calculated by subtracting *GAPDH's* Ct value from the target gene Ct value. ΔΔCt was then calculated by subtracting the ΔCt of the control sample from the ΔCt of the treated sample. Sequences of primers used to amplify were listed in table 1.

Protein extraction and western blotting

Cells were seeded in a 6-well plate with a density of 2×10^5 cells /well and treated with defined concentrations of UDE. Proteins were extracted after 72 hours of incubation in 100 µl of NENT buffer (TRIS-HCl 20 mM, NaCl 50 mM, NP40 2%, SDS 6.2%, pH 7.5). Total protein concentration was determined with a BCA assay kit. The extracted proteins were separated on a polyacrylamide gel, and proteins were transferred to a nitrocellulose membrane. Membranes were blocked using a blocking buffer and incubated with rabbit

anti-*VEGF* antibody (BioSS and 1:2000). Horseradish peroxidative conjugated goat anti-rabbit (ab6721, Abcam; 1:2500) was used as the secondary antibody; finally, immunoblots were visualized using an ECL solution.

Cell death assay

The enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics gmbH, Germany) evaluated apoptosis.

This test measures the number of monoligonucleosomes in the cytoplasmic fragments of apoptotic cells. DU-145 cells were treated with UDE concentrations according to the concentrations obtained from the MTT assay. After 72 hours of incubation, the cells were collected, and an ELISA assay was performed according to the manufacturer's guidelines (Roche Diagnostics, Mannheim, Germany; References: 11774425001). Briefly, cells were lysed and centrifuged at 200 g for 10 minutes. Then, 20 µl of supernatant and 80 µl of anti-DNA peroxidase and anti-histone-biotin were added to each of the wells covered with streptavidin, and the plate was incubated at room temperature for 2 hours. After washing with incubation buffer, 100 µl of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) solution (Roche Diagnostics GmbH, Mannheim, Germany) was added to each well, and the absorbance was measured using a plate reader at 405 nm.

Data analysis

Statistical analysis was achieved using Prism version 6.01 software. The significance of the data was determined using the Mann-Whitney U test. All data were reported as mean ± SD, and P-value ≤ 0.05 was considered statistically significant.

Ethical issues

No animals or human tissue were used in this study. All the human cell lines used were by the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

Results

MTT assay

The cell viability of DU-145 cultured in various UDE concentrations decreased with increasing drug concentration. IC50 was measured for DU-145 cells, 3.8 mg/ml (Figure 1).

UDE decreases the expression and protein synthesis of VEGF in PCa cells

After treating DU-145 cells in the presence of UDE, the amount of *VEGF* mRNA and protein in these cells was measured before and after treatment with UDE. The results showed that the expression of *VEGF* in DU-145 cells before the treatment with UDE was about 10 times that of the control group (Figure 2A), while after treatment with UDE, about 25% of the expressed values decreased in the control group (P-value ≤ 0.05; Figure 2B). The data obtained from western blotting also confirmed the data obtained from measuring the amount of cellular mRNA (Figure 2A).

UDE decreases the expression of PI3K/AKT/eNOS pathway genes

Western blot analysis showed that the *PI3K/AKT/eNOS* pathway was inhibited at the protein level in the presence of defined concentrations of UDE. Moreover, the synthesis of *PI3K/AKT/eNOS* proteins was significantly decreased in DU-145 cells compared with the control group (Figure 3).

PCa cells show high mortality in the presence of UDE

ELISA cell death assay was used to investigate the apoptosis rate in DU-145 cells after incubation with defined concentrations of UDE. The results showed 60% death in DU-145 cells in the groups receiving UDE, which showed a significant difference from the control group. (P-value ≤ 0.05; Figure 4).

Discussion

Our study findings demonstrate that UDE exhibits potent anti-cancer effects on PCa cells. We observed that UDE significantly reduced cell viability, decreased *VEGF* expression, inhibited the *PI3K/AKT/eNOS* pathway, and increased apoptosis in DU-145 cells. These findings suggest that UDE may have therapeutic potential in PCa treatment through its anti-proliferative and anti-angiogenic properties.

Due to the enhanced prevalence and mortality rate, PCa is becoming the most common male urinary system malignancy. PCa shows a high invasion rate, metastasis, and proliferation, the main reason for PCa-related mortality. In this regard, the *PI3K/AKT* signaling pathway is upregulated in different malignancies, including PCa, and highly activated in castration-resistant prostate cancer (CRPC). Thus, inhibiting the *phosphate, and tensin homolog (PTEN)/PI3K/AKT* pathway can be a therapeutic target in hormone-sensitive PCa and CRPC.[20,21] Further, the tumor suppressor, *PTEN* is a negative regulator of the *PI3K/AKT* signaling pathway, which converts PIP3 to PIP2. Activated *AKT* protein phosphorylates a wide range of downstream proteins, including *FOXOs*, *NFkB*, and *TSC2*.[22] This pathway eventually leads to the activation of *mTORC1*, which leads to autophagy prevention and promotes cell growth and proliferation. In this regard, new investigations have recommended the capability of nettle to limit or postpone the dispersal of cells, particularly tumor cells, into surrounding tissues as one of its features. Multiple reflections have demonstrated that UDE disrupts various pathways involved in the genesis of malignant cells.[13]

Indeed, medicinal plants provided many innovative chemotherapeutic factors that revolutionized anti-tumor therapies, improving patient treatment outcomes. Many studies demonstrated the anti-inflammatory and anti-tumor properties of UD. To date, it has been understood that UDE has beneficial effects in treating many malignancies. In this regard, D'Abrosca et al. findings spotlighted that UDE differentially impeded cell proliferation in A549, H1299, H460, and H322 cells. At the same time, it did not show considerable cytotoxicity in normal lung fibroblast and bronchial epithelial cells.[23] Also, the study by Ghasemi et al. on HT29 and MKN45 tumor cells revealed that UDE actuated cytotoxic effects on these cells.[24] With this approach, in the present study, UDE could decrease the potential of cell proliferation stress as well as cause the death of DU-145 cells.

Moreover, the induction of apoptosis in cancer cells is an essential method of controlling cancer cell progression without inducing damage to normal dividing cells. In a wide variety of cancers, apoptosis is inhibited, and malignant cells are found as a way to escape death signals, so the induction of apoptosis is considered a therapeutic approach.[25] Fattahi et al. showed that UDE exerted dose-dependent antioxidant and apoptotic results on the MCF-7 cell line after 72 h of exposure. Likewise, the same extract exerted cytotoxic and apoptotic impacts on the LNCap cell line after 24 h of treatment.[26] In accordance with previous studies, our results revealed that UDE dramatically improved the apoptosis rate in the treated group compared with the control group.

Also, herein, we applied a mechanistic framework that, at a molecular level, accentuated the mechanisms fundamental to the particular anti-angiogenic impact of the UDE in DU-145 cells. In this regard, attention should be paid to the subject in which *VEGF* increases the permeability of blood vessels and generates new capillary networks, which are highly needed in tumor progression and are known as tumor angiogenesis.[27] Therefore, the repression of atypical angiogenesis may afford therapeutic

approaches in treating angiogenesis-dependent disorders. Thus, understanding the underlying molecular mechanisms of natural compounds with anti-angiogenesis effects may improve natural-based agent treatment. This study showed that UDE could inhibit angiogenesis by down-regulation of *VEGF* in the DU-145 cells.

Briefly, our data showed a robust anti-cancer effect of UDE on PCa cell lines which were exerted through the down-regulation of *PTEN/PI3K/AKT/eNOS* genes and inhibition of *VEGF* and thus angiogenesis. Furthermore, UDE limits the tumor through the induction of apoptosis.

This study has several limitations that should be considered. First, as an in vitro study, it may not fully represent the complex interactions within a living organism. Second, while we focused on the DU-145 cell line, results may vary with other PCa cell lines. Finally, the mechanisms of UDE's effects may be more complex than those explored in this study. Additionally, the exact bioactive compounds in UDE responsible for the observed effects need to be identified and characterized. Future in vivo studies and clinical trials must confirm these findings and explore their translational potential.

Conclusion

Natural components such as UDE can be used as a potential anti-cancer drug. In this study, we demonstrated that UDE could reduce the proliferation and angiogenesis of PCa cells and increase the apoptosis rate in these cells. Therefore, this component can be a promising therapeutic factor for patients with PCa.

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Authors' Contribution

A.A.A: Conception, design, and drafting of the work; M.S.M., N.A.Y.A.K., and S.A.A: Data gathering and drafting; M.S.M: interpretation of data for the work and critical reviewing; M.A.A and Y.F.M: drafting and critical reviewing of the manuscript. All authors have read and approved the final manuscript and agreed to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

None declared.

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Table 1. Primers used for qPCR analysis

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')	Product length (bp)	Annealing temperature (°C)
VEGF	AGGAGGAGGGCAGAATCATCA	CTCGATTGGATGGCAGTAGCT	137	60
PI3K	CCTGATCTTCCTCGTGCTGCTC	ATGCCAATGGACAGTGTTCCTCTT	150	58
AKT	TCTATGGCGCTGAGATTGTG	CTTAATGTGCCCGTCCTTGT	113	59
eNOS	GTGGCTGTCTGCATGGACCT	CCACGATGGTGACTTTGGCT	159	61
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226	60

qPCR: Quantitative polymerase chain reaction; *VEGF: Vascular endothelial growth factor*; *GAPDH: Glyceraldehyde 3-phosphate dehydrogenase*; *PI3K: Phosphoinositide 3-kinase*; *AKT: Protein kinase B*; *eNOS: Endothelial nitric oxide synthase*; bp: Base pair

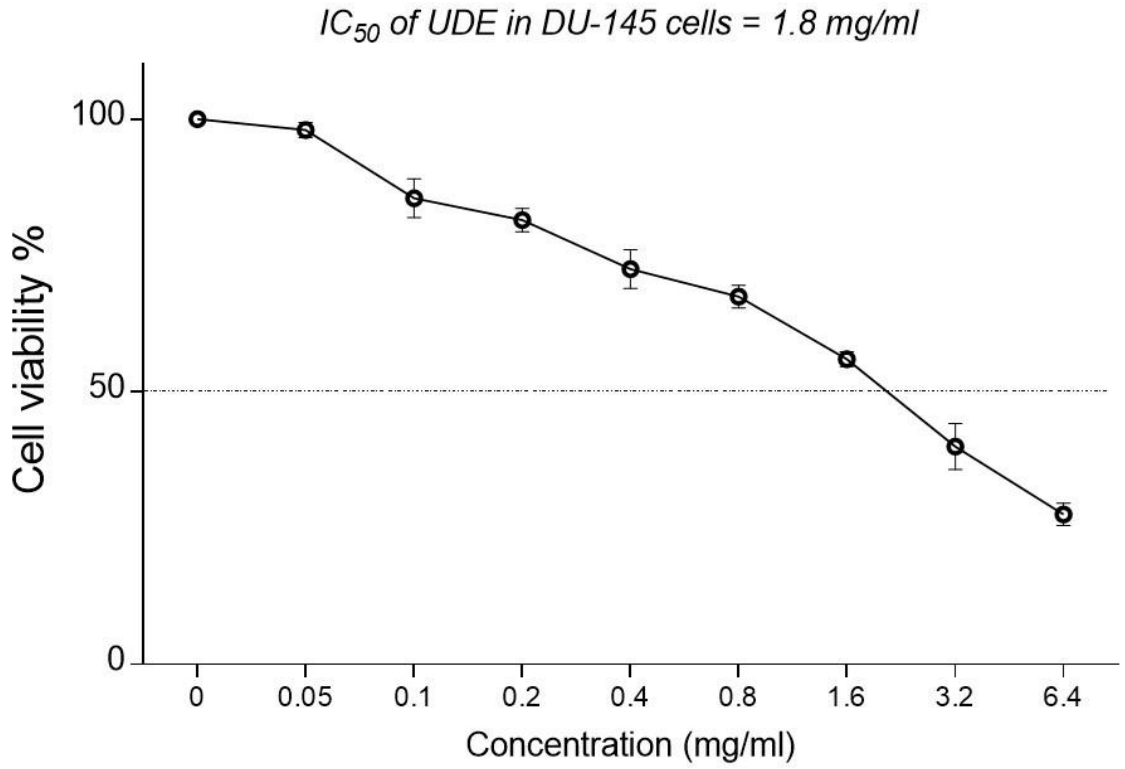

Figure 1. Cell viability was determined by MTT assay. UDE inhibits in vitro cell viability of the DU-145 cells (after 72 h). Results are depicted as mean ± SD (n = 3).
MTT: 2,5-diphenyl-2H-tetrazolium bromide; UDE: Urtica dioica L. extract; SD: Standard deviation

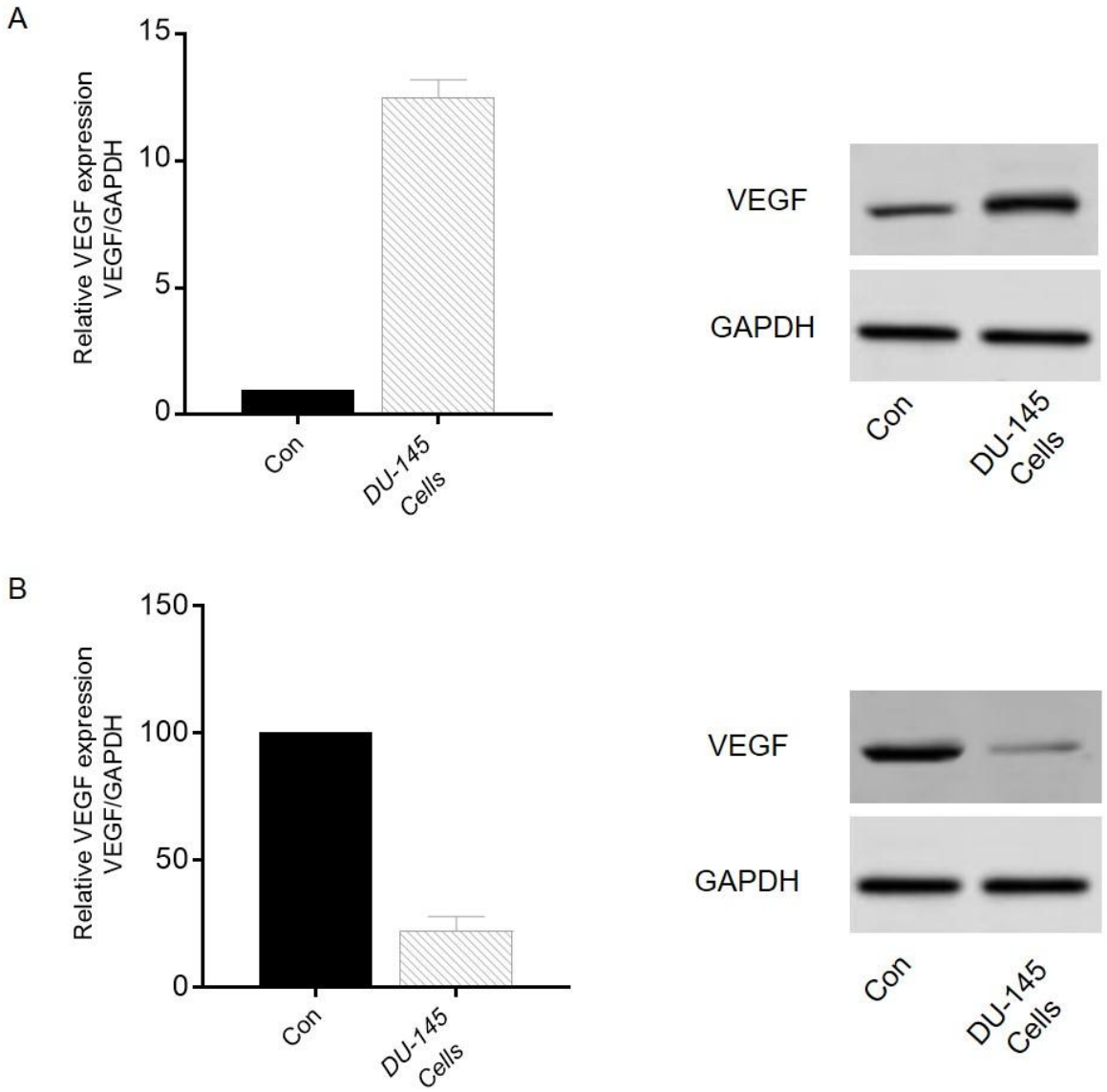

Figure 2. Relative expression levels of *VEGF* in (A) DU-145 cells compared with the (B) control group (untreated DU-145 cells) after treatment with UDE. Results are depicted as mean ± SD (n = 3).

Con: Control; UDE: Urtica dioica L. extract; *VEGF: Vascular endothelial growth factor*; *GAPDH: Glyceraldehyde 3-phosphate dehydrogenase*; SD: Standard deviation

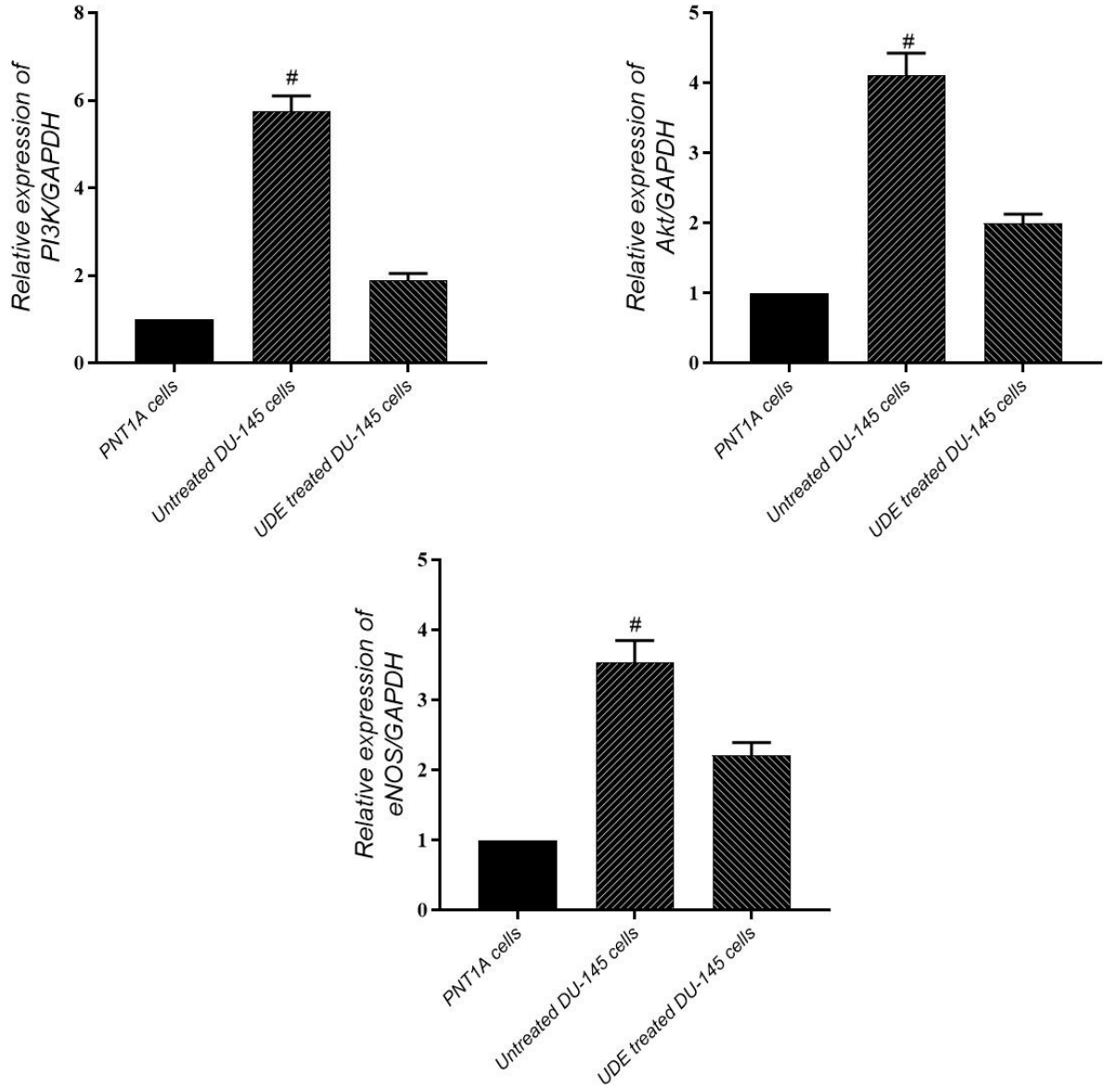

Figure 3. UDE significantly decreased the expression of *PI3K/AKT/eNOS* pathway genes in mRNA levels. The mRNA and protein expression levels before and after 72 hours of exposure to UDE (3.8 mg/ml) in DU-145 cells. Results are depicted as mean ± SD (n=3). PNT1A cells are human prostatic normal cells.

[#]: *P*-value ≤ 0.05 (significant change); UDE: Urtica dioica L. extract; *PI3K: Phosphoinositide 3-kinase*; *AKT: Protein kinase B*; *eNOS: Endothelial nitric oxide synthase*; *GAPDH: Glyceraldehyde 3-phosphate dehydrogenase*; SD: Standard deviation

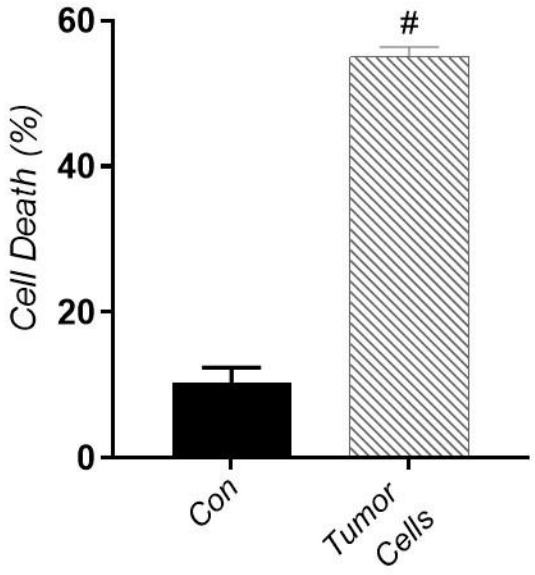

Figure 4. Apoptotic rate analysis of PCa cells using ELISA cell death assay kit upon 72 hours exposure to UDE with a concentration of 3.8 mg/ml. The control group was untreated DU-145 cells. Results are depicted as mean ± SD (n = 3).

[#]: *P*-value ≤ 0.05 (significant change); Con: Control; PCa: Prostate cancer; UDE: Urtica dioica L. extract; ELISA: The enzyme-linked immunosorbent assay; SD: Standard deviation