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Research Article
Effect of Sika Deer (*Cervus nippon*) Velvet Antler on Prostate-specific Antigen Levels and Migration of LNCaP Human Prostate Cancer Cells

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Abstract

Objective: This study investigated the anti-prostate cancer activity of sika deer (*Cervus nippon*) velvet antler (DVA) extract on the expression of Prostate-Specific Antigen (PSA) and migration-related genes in LNCaP human prostate cancer cells. **Methodology:** The DVA was divided into three sections: Upper DVA (U-DVA), middle DVA (M-DVA) and bottom DVA (B-DVA) and an aqueous extract was prepared. The U-DVA was non-toxic up to 1,000 μg mL^{-1} to RWPE-1 transformed human prostate cells. The U-DVA (125-1,000 μg mL^{-1}) which exhibited the most potent ORAC activity, attenuated the migration of LNCaP cells. **Results:** In addition, U-DVA inhibited the expression of PSA and migration-related genes such as matrix metalloproteinase-9 and vascular endothelial growth factor. In contrast, U-DVA stimulated the expression of tissue inhibitor of metalloproteinase TIMP-1 and TIMP-2. **Conclusion:** This is the first report of DVA having an effect on a human-specific cancer. These results indicate that U-DVA possesses anti-prostate cancer potential via modulation of several genes. Further investigations to explore individual bioactive compounds in U-DVA that are responsible for these *in vivo* effects as well as clinical trials in humans are needed.

Key words: Deer velvet antler, prostate cancer, matrix metalloproteinases, prostate-specific antigen, vascular endothelial growth factor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Prostate cancer is the cancer with the highest incidence in the United states. There were over 241,000 new diagnoses occurring in 2012 and it is the second most fatal cancer among men[1]. In Korea, prostate cancer deaths were very rare until 1980 with a mortality rate of only 0.13/100,000 in 1983 (National Statistics Office, Vital Statistics for Causes of Deaths Analysis, USA). However, this rate gradually increased to 2.95/100,000 by 2012 making prostate cancer the 7th most common cancer among Korean males (National Cancer Center, Republic of Korea).

Prostate cancer is usually asymptomatic in its early stage. Thus, diagnosis occurs in the later stages when treatments are less effective. There are several treatments for prostate cancer including surgery, radiation therapy, hormone therapy and chemotherapy[2]. Recently, targeted therapies are emerging[3]. However, most therapies have side effects such as bowel dysfunction and an increased risk of diabetes and myocardial infarction[4]. Therefore, prevention involving food science technology which has no side effects is a potentially effective strategy.

Prostate-Specific Antigen (PSA) is a glycoprotein produced by prostate cells. When the prostate is healthy and the cells are organized in a tight pattern, only a small amount of PSA leaks out into the bloodstream. In contrast, once prostate cells become disorganized and the layers between the prostate and blood vessels become disrupted, PSA can enter the blood stream. Moreover, prostate cancer cells also secrete PSA protein by themselves[5-7]. Therefore, the PSA is an important biomarker currently applied to help diagnose and follow the progression of prostate cancer[8,9].

Almost of cancer deaths are ascribable to metastases. Metastasis is the process by which a tumor cell leaves its primary location, travels to another via the circulatory system and establishes a secondary tumor. Degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is a trigger mechanism in tumor metastasis. Therefore, MMPs are common and essential target effectors for many oncogenes and tumor suppressor genes by facilitating tumor metastasis[10]. The MMPs are regulated by specific inhibitors, the tissue inhibitors of MMP (TIMPs) that are closely correlated with physiological and pathological processes by the degradation and accumulation of the ECM[11]. Angiogenesis is also required for the formation of tumor metastases. Tumors that have become neovascularized often express increased levels of pro-angiogenic proteins such as Vascular Endothelial Growth Factor (VEGF). Therefore, blocking the progression of a tumor is also a valuable approach to cancer therapy.

Deer Velvet Antler (DVA) has been used in Asia as an alternative medicinal substance for more than[12] 2,000 years. The DVA is shed and reproduced annually and it grows faster than any other mammalian tissue[13]. The DVA possesses a wide range of health benefits including anticancer[14], immunomodulatory[15] and anti-aging effects[16], the ability to strengthen bones and muscles[17] and tonify the kidney[12]. However, there has been little scientific investigation on the effect of DVA on hormone-sensitive diseases such as prostate cancer which is generally believed that the use of DVA might be aggravated because of the pro-stamina effect of DVA[18]. Despite of the general belief, until this study, there is no scientific evidence of the bioactivity and safety of velvet antler on prostate cancer.

The present study investigated the effects of sika deer (*Cervus nippon*) velvet antler on PSA levels and the expression of metastasis-related genes *in vitro* using the human prostate cancer cell line, LNCaP. The results indicate that DVA has beneficial effects.

MATERIALS AND METHODS

Materials: Seven sika DVAs were harvested and collected about 50 days after casting at the same farm (Fanrong farm, Shuang Yang, China). The DVAs were lyophilized, divided into three sections (upper, middle and bottom; U-DVA, M-DVA and B-DVA, respectively) and homogenized with a grinder before extraction. Ten grams of each section were put into 100 mL of distilled water and extracted using boiling water at 100°C for 1 h. The DVA extracts were filtered (0.25 μm) and lyophilized (yield by dry weight; top 6.7%, middle 6.3% and bottom 5.2%) for 5 days. The LNCaP, androgen-dependent human prostate cancer cells were obtained from the Korean Cell Line Bank (Seoul, Korea; KCLB numbers: 21740). The RWPE-1, normal transformed human prostate cells were supplied by American Type Culture Collection (Manassas, VA, USA ; ATCC numbers: CRL-11609). Dihydrotestosterone (DHT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The RPMI-1640 media and trizol reagent were from Invitrogen (Carlsbad, CA, USA). Primary antibodies against PSA (SC-7638), MMP-9 (SC-10737), TIMP-1 (SC-6823), TIMP-2 (SC-6835), VEGF (SC-152), β-actin (SC-1616), peroxidase-conjugated anti-goat (SC-2020) and anti-mouse (SC-2005) secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Oxygen Radical Absorbance Capacity (ORAC): Overexpression of free radicals is believed to be associated with tissue and cellular pathogenesis that can result to several

chronic diseases such as cancer and diabetes melitus as well as neurodegenerative and inflammatory diseases[19,20]. Therefore, chemicals that possess antioxidant activity may also have anticancer properties. To select the most effective anticancer section from the extracts of U-DVA, M-DVA and B-DVA this study estimated their antioxidant activity using the ORAC assay described by Ou *et al.*[21]. With some slight modifications. The working solution of fluorescein and the 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) radical was prepared daily. Sample, blank or standards were placed in a 96 microwell plate and AAPH was added. Fluorescence was measured every 5 min beginning immediately after the addition of AAPH. The ORAC was calculated as:

$$\text{ORAC (}\mu\text{M of trolox equivalents)} = \left(\frac{C_{\text{Trolox}} \times (AUC_{\text{sample}} - AUC_{\text{blank}}) \times \kappa}{AUC_{\text{sample}} - AUC_{\text{blank}}}\right)$$

$$AUC = \left(0.5 + \frac{f_1}{f_0} + \frac{f_2}{f_0} + ... + \frac{f_n}{f_0}\right) \times 5$$

where, C_{Trolox} is the concentration of trolox, AUC is the area under the curve and κ is the dilution factor. Excitation and emission wavelengths were 485 and 520 nm, respectively.

Cell culture: The LNCaP and RWPE-1 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum, 10 µg/100 mL penicillin/streptomycin in a 5% CO_2 atmosphere at 37°C. For the migration assay, the cells were seeded at a density of 5.0×10^5 cells per well in a 6 well culture plate. After 24 h, the cells were treated with 125-1,000 µg mL^{-1} of U-DVA extracts for 24 h and then harvested.

Cytotoxicity assay: Cell viability was determined by the 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay in 96-well plates as described previously[22]. The human transformed prostate cell line, RWPE-1 was incubated with various concentrations of U-DVA for 48 h followed by MTT for 4 h. One hundred microliters isopropanol (in 0.04 N HCl) was added to dissolve the formazan crystals. Absorbance was read at 570 nm using a spectrophotometer (Tecan, Männdorf, Switzerland). Cell viability was calculated as the absorbance relative to control absorbance.

Gap closure migration assay: Migration was assessed using the Radius™ 24 well cell migration assay from Cell Biolabs (San Diego, CA, USA). For the analysis, 500 µL of Radius™ gel pretreatment solution was added slowly to each well by carefully pipetting down the wall of the well. The plate was then covered and incubated at room temperature for 20 min. The Radius™ gel pretreatment solution was aspirated carefully from the wells and 500 µL of Radius™ wash solution added to each well. Cells were harvested and resuspended in culture medium at 0.2×10^6 cell mL^{-1}. The Radius™ wash solution was aspirated carefully from the wells and 500 µL of the cell suspension added to each well by carefully pipetting down the wall of the well. The plate was transferred to a cell culture incubator for 24 h to allow firm attachment. After 24 h, the media was aspirated from each well and the attached cells were washed 3 times with 0.5 mL of fresh media. Sufficient 1× Radius™ gel removal solution for all wells was prepared by diluting the stock 1:100 in culture media. The media was aspirated from the wells and 0.5 mL of 1× Radius™ gel removal solution was added. Cells were washed 3 times with 0.5 mL of fresh media. After the final washing was complete, 1 mL of complete medium containing U-DVA extracts (125-1,000 µg mL^{-1}) was added to each well. Photos of the cells were taken at 0, 8, 12 and 24 h. To compare the differences in migratory gap, images were captured at the same size. Gap closure was determined at each time using CellProfiler™ software (Broad Institute, Cambridge, MA, USA).

RNA isolation and mRNA expression analysis: For RT-PCR, total cellular RNA was isolated using trizol according to the manufacturer's protocol. The first-strand complementary DNA (cDNA) was synthesized using superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The following primers were used: PSA (s 5'-AGC CCC AAG CTT ACC ACC-3'; as 5'-GCT GAC CTG AAA TAC CTG-3'), MMP-9 (s 5'-CGA CGT CTT CCA GTA CCG AG-3'; as 5'-GTT GGT CCC AGT GGG GAT TT-3'), TIMP-1 (s 5'-CAA GAT GAC CAA GAT GTA TAA AGG-3'; as 5'-AAC AGT GTA GGT CTT GGT GAA G-3'), TIMP-2 (s 5'-CAG CTT TGC TTT ATC CGG GC-3'; as 5'-ATG CTT AGC TGG CGT CAC AT-3'), VEGF (s 5'-GGG GCA GAA TCA TCA CGA AG-3'; as 5'-TTT CTC CGC TCTGAG CAA GG-3') and GAPDH (s 5'-CCA TGG GGA AGG TGA AGG TC-3'; as 5'-AAA TGA GCC CCA GCC TTC TC-3') was used as the internal control. The conditions for RT-PCR were described[23].

Western blot analysis: Cell extracts were prepared by the detergent lysis procedure as described elsewhere[24]. Samples of protein (40 µg) were electrophoresed using Novex 4-12% bis-tris gel (Life Technologies, Carlsbad, CA, USA) and then transferred to nitrocellulose membranes for 7 min in the iBlot dry blotting system (Life technologies). The transferred proteins were blocked overnight at 4°C with clear milk (Thermo Scientific, Rockford, IL, USA). Blots were subsequently

incubated with primary antibodies diluted 1:2000 in 1×TBST for 1 h. Goat anti-rabbit or goat anti-mouse horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) were used at 1:2000 dilution in 1×TBST. Blots were treated with Western lightning Western blot Chemiluminescence reagent (Advansta, Menlo Park, CA, USA) and the proteins were detected by autoradiography. Equal protein loading was ascertained by Ponceau S staining of blotted membranes as well as Western blotting for β-actin. Immunodetection was done using an enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ, USA).

Statistical analyses: The results shown are summaries of the data sourced from atleast three experiments. All data are presented as Means±SE. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA). Treatment effects were analyzed using one-way analysis of variance followed by Dunnett's multiple range tests. The results were considered significant at $p<0.05$.

RESULTS

Selection of one from three sections; U-DVA, M-DVA and B-DVA: Generally, DVA is divided into three sections, each of which is used to treat different diseases such as anemia, arthritis, hypercholesterolemia and cancer[25]. The U-DVA including the tip which is the most expensive and sought-after part of DVA called a wax piece is used as a growth reagent for children. The M-DVA is also called a blood part, used to treat adults with arthritis and related disorders. The B-DVA is also called a bone part, used for calcium deficiency and geriatric therapies[26]. However, there is little information regarding which section is best for prostate cancer prevention or therapy.

In the current study, to determine which DVA section was the most effective inhibitor of prostate cancer, antioxidant activity was assessed by the ORAC assay on U-DVA, M-DVA and B-DVA (Fig. 1). The U-DVA, M-DVA and B-DVA exhibited ORAC activities of 112, 88 and 74%, respectively, at 1,000 μg mL^{-1}. Finally, significant differences ($p<0.05$) were found between U-DVA, M-DVA and B-DVA at the same concentration. Therefore, U-DVA was selected for the anti-prostate cancer study.

Effect of U-DVA on the expression of PSA in human prostate cancer cells: Before the anticancer study, the cytotoxicity of U-DVA was determined on normal transformed RWPE-1 human prostate cells. The U-DVA had no cytotoxic effect on these cells (Fig. 2). The expression of PSA mRNA was investigated by RT-PCR after treatment of LNCaP cells with or without DHT (0.1 nM) and U-DVA (125-1,000 μg mL^{-1}) for 24 h. The U-DVA significantly decreased PSA mRNA expression (Fig. 3a, b). In addition, the expression of PSA protein was significantly suppressed by U-DVA (Fig. 3c, d).

Mobility inhibitory effect of U-DVA on human prostate cancer cells: Cell migration involves the movement of cells from one location to another in response to events, biological signals or environmental changes. Tumor cell migration is crucial for invasion and dissemination from primary solid tumors and for the establishment of lethal secondary metastases at distant organs[27,28]. To compare differences in the migratory gap, images were captured at the same size. Gap closure was determined at 0, 8 and 24 h, compared to control and DHT groups. After 24 h, the gap was closed approximately 33.8% in DHT alone-treated cells. In contrast,

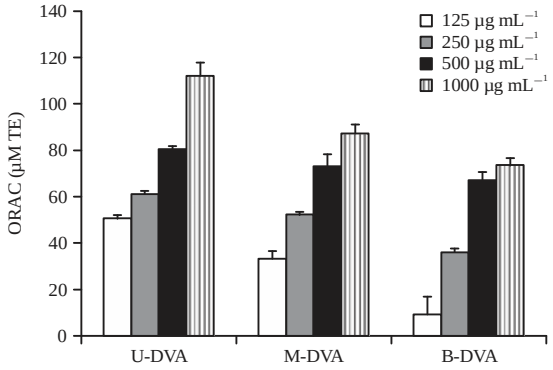

Fig. 1: Effect of DVA on ORAC and values not sharing a common letter are significantly different at $p<0.05$ by Dunnett's multiple range tests[47]

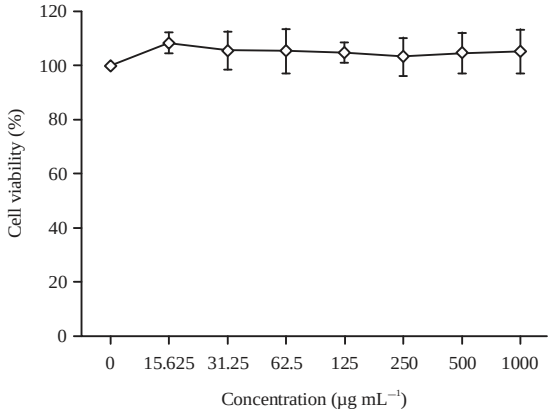

Fig. 2: Effect of U-DVA on the viability of RWPE-1 cells, cells were treated with various concentrations of U-DVA for 48 h followed by the MTT assay and cell viability was calculated as the relative absorbance compared to control absorbance[47]

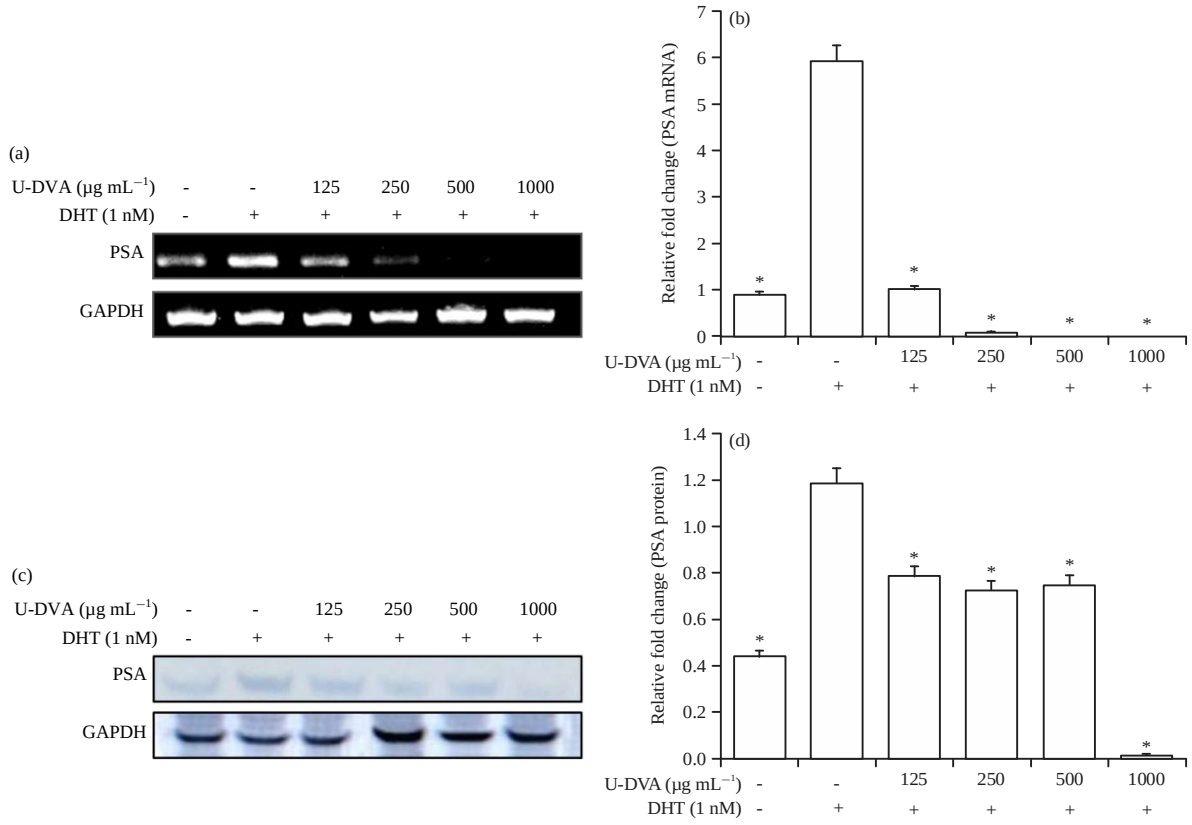

Fig. 3(a-d): (a, b) Expression of PSA using RT-PCR and Western blots PSA mRNA and (c, d) Protein expression were quantified by Image Gauge (Fujifilm, Japan), cells were treated with or without DHT and various concentrations of U-DVA for 24 h, GAPDH and β-actin were used as loading controls for RT-PCR and Western blots, respectively, the results were similar in three independent experiments and *Significantly different from the DHT alone group[47] ($p<0.05$)

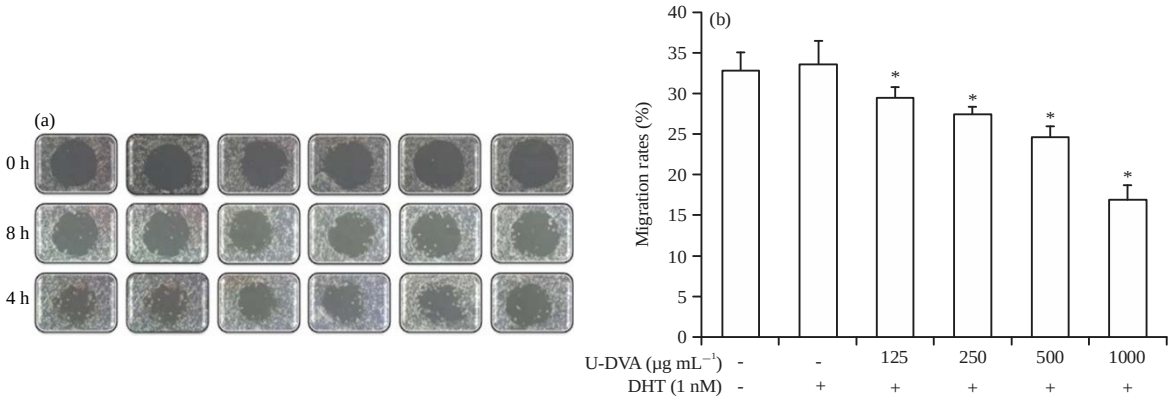

Fig. 4(a-b): Migration rates of U-DVA-treated LNCaP cells, (a) Cell migration time course and (b) Images were captured at the same size, gap closure was determined after 0, 8, 12 and 24 h using CellProfiler™ software and values not sharing a common letter are significantly different at $p<0.05$ by Dunnett's multiple range tests[47]

U-DVA significantly reduced cell motility up to 17.2% in a dose-dependent manner, compared with DHT alone-treated cells (Fig. 4).

Effect of U-DVA on the expression of migration-related genes in human prostate cancer cells: Figure 5 shows that U-DVA significantly suppressed MMP-9 mRNA (Fig. 5a, b) and

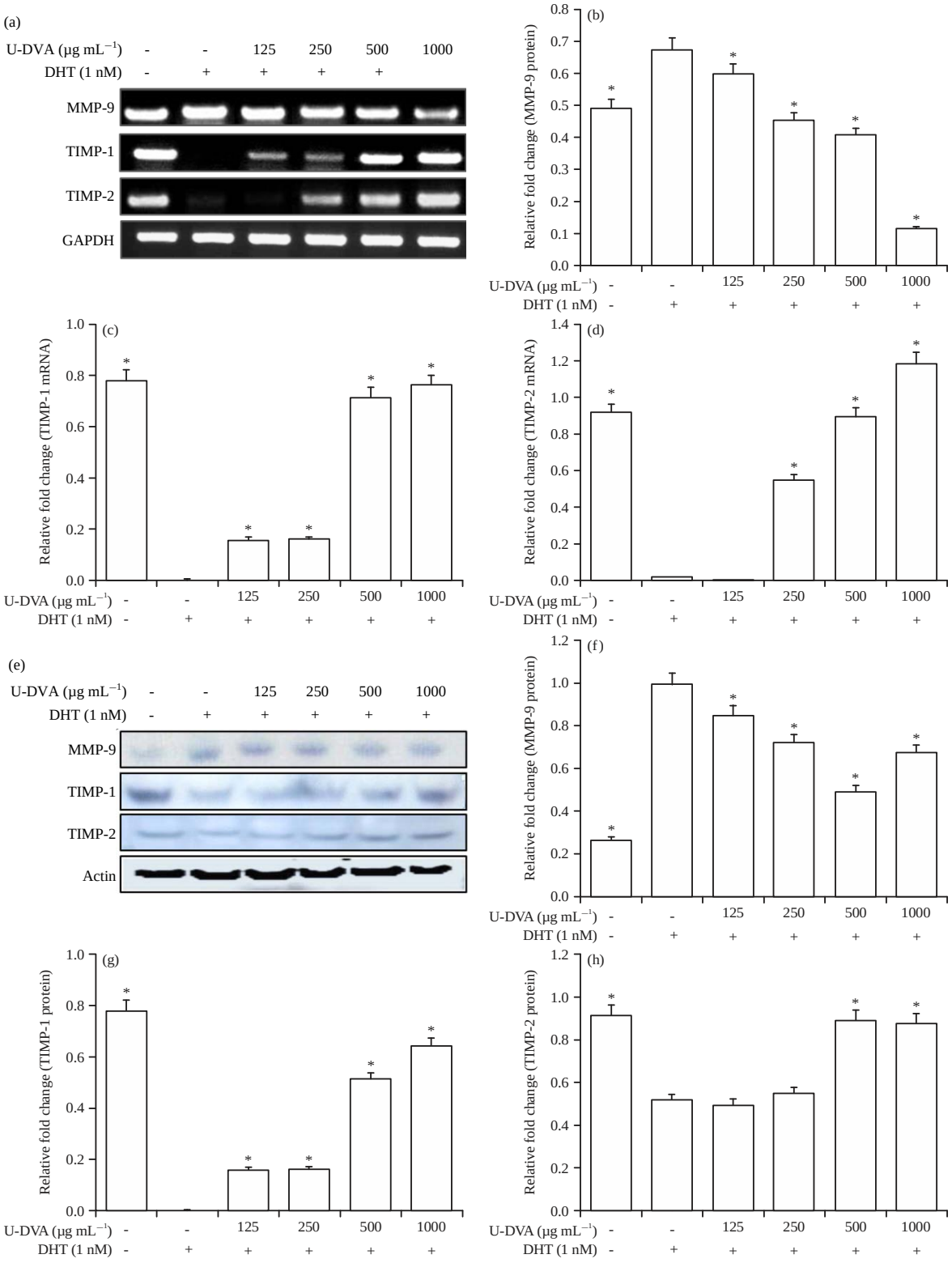

Fig. 5(a-h): (a-d) Expression of migration-related genes using RT-PCR and Western blots mRNA and (e-h) Protein expression of migration-related genes that were quantified by Image Gauge (Fujifilm, Japan), cells were treated with or without DHT and various concentrations of U-DVA for 24 h, GAPDH and β-actin were used as loading controls for RT-PCR and Western blots, respectively, the results were similar in three independent experiments and *Significantly different from the DHT alone group[47] ($p<0.05$)

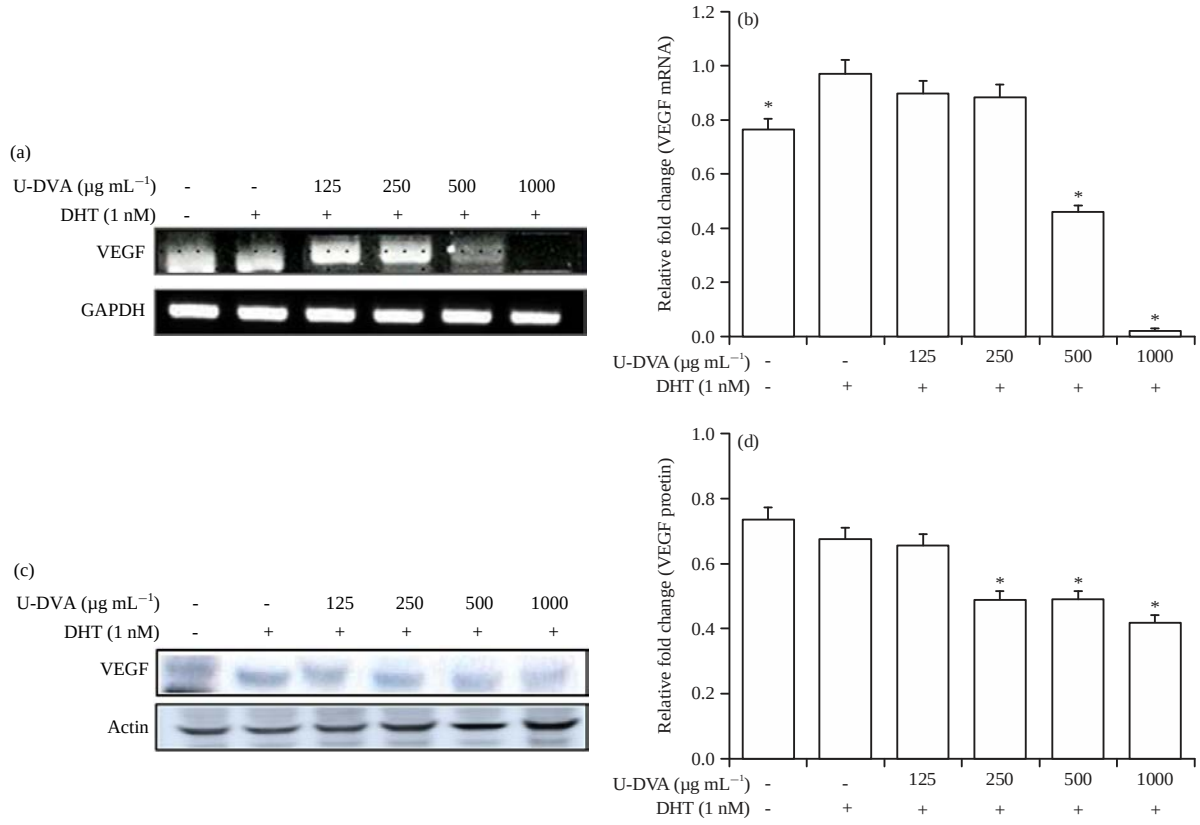

Fig. 6(a-d): (a, b) Expression of VEGF using RT-PCR and Western blot mRNA and (c, d) Protein expression of VEGF were quantified by Image Gauge (Fujifilm, Japan), cells were treated with or without DHT and various concentrations of U-DVA for 24 h, GAPDH and β-actin were used a loading controls for RT-PCR and Western blots, respectively, The results were similar in three independent experiments and *Significantly different from the DHT alone group[47] ($p<0.05$)

protein (Fig. 5e, f) in a dose-dependent fashion. Meanwhile, the glycoprotein TIMPs are natural inhibitors of the MMPs. The TIMP-1 and TIMP-2 mRNA (Fig. 5a, c, d) and protein (Fig. 5e, g, h) were significantly and dose-dependently increased in U-DVA-treated LNCaP cells. These data suggest that U-DVA suppresses migration by up-regulating the levels of TIMP-1 and TIMP-2 in human prostate cancer cells. The VEGF-mediated signaling occurs in tumor cells and this signaling contributes to crucial aspects of tumorigenesis including the function of cancer stem cells and tumor initiation[29]. The U-DVA significantly reduced mRNA (Fig. 6a, b) and protein (Fig. 6c, d) expression of VEGF in a dose-dependent manner.

DISCUSSION

Damage to cells caused by free radicals, especially damage to DNA may play a role in the development of cancer and other health conditions[30,31]. Previously conducted studies of bioactive compounds and biological activities of velvet antler[32]. Figure 1 shows that the antioxidant activity of the antler differed depending on the segment tested. The ORAC approach has been used previously for evaluation of the antioxidant activity of a wide variety of samples[33]. The ORAC assay evaluates the decrease in fluorescence of a probe (β-phycoerythrin) due to the action of peroxyl radicals generated by thermal decomposition of AAPH[34].

Disruption of the normal prostatic architecture as caused by prostate cancer, allows greater amounts of PSA to reach the blood circulation[35]. In addition, prostate cancer cells secrete PSA protein by themselves[5-7]. Therefore, an elevated PSA level has become an important marker of many prostate diseases including prostate cancer to detect recurrence following local therapies and to follow response to therapies[36,37]. The results found significantly decrease PSA level which was higher inhibitory activity than that found from the genistein[38].

In the East for over 2000 year, DVA has been believed that it can increase sexual ability[18]. In addition, Clark *et al.*[39] reported deer velvet extract promotes angiogenesis to improve wound healing. Angiogenesis is a crucial process

which required for further growth and metastasis of cancers[40]. Due to these sexual and angiogenic effects of DVA, it is considered that DVA should be avoided for hormone-sensitive cancers such as prostate, uterine and breast. However, there is no scientific evidence of the influence of DVA for those hormone-sensitive cancers. Meanwhile, there are some studies which investigate the cancer inhibitory effects of DVA. Fraser *et al.*[41] reported that oral administration of DVA effectively decreased the severity of colon cancer. The DVA also prolonged the living time and reduced tumor weight of mice injected abdominally with sarcoma 180 cells[42,43]. In addition, DVA suppressed hormone-sensitive women disease via regulating of MMP-2 and MMP-9[14]. Yegorova[44] reported DVA inhibited 5α-reductase which gives possibility of reducing excessive DHT which is the primary contributing factor in prostate cancer. The results of this study show that DVA inhibited prostate cancer migration.

Tumor neovascularization can be initiated by neoplastic cell hypoxia which induces the activity of the gene encoding VEGF and the influence of VEGF on neovascularization has also been attributed to some MMPs such as MMP-9[45]. Therefore, this study investigated the expression of MMP-9 (Fig. 5) and VEGF (Fig. 6) in prostate cancer cells. The results are similar to the findings of Ganapathy *et al.*[46] and Tang *et al.*[47] showing that anti-prostate cancer on MMP-9 from resveratrol and on TIMPs from taurine. Elevated expressions of matrix metalloproteinases (MMPs) are associated with increased metastatic potential in many tumor cells. As key regulators of MMPs and TIMPs play a pivotal role in determining the influence of the extracellular matrix, of cell adhesion molecules, there is evidence that TIMPs have biological activities independent of metalloproteinase inhibition including effects on cell growth, differentiation, cell migration, apoptosis and angiogenesis[48]. The VEGF-mediated signaling occurs in tumor cells and this signaling contributes to crucial aspects of tumorigenesis. New blood vessel formation (i.e., angiogenesis) is an essential event in the process of tumor growth and metastatic dissemination[49]. Angiogenesis is part of the mechanism that increases the oxygen supply to tissues when blood circulation is insufficient. The VEGF was identified and isolated as an endothelial cell-specific mitogen that has the capacity to induce physiological and pathological angiogenesis[50,51] and VEGF also appears to play a significant role in androgen regulation of vascular growth in the prostate as a reduction of VEGF expression and an induction of endothelial cell apoptosis were observed in prostate tumor[52]. Similar results were obtained in taurne by Tang *et al.*[47].

CONCLUSION

This study demonstrated that U-DVA attenuated one of the key markers of prostate cancer, PSA. In addition, U-DVA inhibited the migration rate of prostate cancer cells and the expression of migration-related genes. Moreover, U-DVA also suppressed the expression of an angiogenesis-related gene. These results indicated that U-DVA possesses anti-metastatic potential for prostate cancer and may be a candidate for future *in vivo* and clinical study on prostate cancer treatments.

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