Zanthoxyli Fructus induces growth arrest and apoptosis of LNCaP human prostate cancer cells *in vitro* and *in vivo* in association with blockade of the AKT and AR signal pathways

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Abstract. *Zanthoxyli* Fructus belongs to the family of oranges and is used as a seasoning in Asian countries including Japan. This study found that a water extract of *Zanthoxyli* Fructus possessed anti-tumor activity against a wide variety of cancer cells including those from prostate (LNCaP, DU145, PC-3), breast (MCF-7, T47D, MDA-MB231), lung (NCI-H460, -H520), as well as leukemia (HL-60, NB4, Jurkat) *in vitro*, as measured by the trypan blue exclusion test. Importantly, *Zanthoxyli* Fructus slowed the proliferation of LNCaP, DU145, and MDA-MB231 cells present as xenografts in BALB/c nude mice without adverse effects. Further studies explored the molecular mechanism by which *Zanthoxyli* Fructus inhibited the proliferation of androgen-dependent human prostate cancer LNCaP cells because *Zanthoxyli* Fructus possessed the strongest anti-tumor activity against these cells. *Zanthoxyli* Fructus blocked androgen receptor (AR) signaling in conjunction with down-regulation of nuclear levels of AR and induced apoptosis of these cells, as measured by the reporter assay, Western blot analysis, and TUNEL assay, respectively. As expected, *Zanthoxyli* Fructus also decreased the level of the AR-target molecule, prostate-specific antigen in these cells. Furthermore, *Zanthoxyli* Fructus inhibited AKT kinase and down-regulated levels of cyclin D1 protein, as measured by the AKT kinase assay with GSK-3α/ß as a substrate and Western blot analysis, respectively. Taken together, *Zanthoxyli* Fructus might be useful as an adjunctive therapeutic agent for the treatment of individuals with a variety of cancer types.

Introduction

Zanthoxyli Fructus belongs to the family of oranges and has been used as a seasoning in Asian countries including Japan. Also, *Zanthoxyli* Fructus is one of the components of a three herbal mixture called Dai-kenchu-to, which stimulates intestinal motility and has been used for treatment of post-operative ileus in Japan. *Zanthoxyli* Fructus has been shown to increase contraction of the jejunum (1).

Androgens play an important role in the development and maintenance of the normal prostate as well as the initiation and progression of prostate cancer (2,3). Androgen deprivation therapy remains the mainstay of treatment for prostate cancer, once it has progressed outside the prostatic capsule (3). The androgen receptor (AR) belongs to the steroid hormone subfamily of nuclear hormone receptors. It is complexed in the cytoplasm to chaperone proteins that keep the receptor in a transcriptionally inactive form. Upon binding to androgens, AR dissociates from the chaperone and translocates to the nucleus where it binds to androgen response elements (AREs), recruits coregulators, and activates target genes such as prostate-specific antigen (PSA) (2,3). Recently, microarray analysis identified AR as the only up-regulated gene in androgen-independent prostate cancer cells compared to androgen-dependent prostate cancer cells (4). These data suggested that AR might be a promising molecular target for treatment of prostate cancer.

PSA belongs to the kallikrein-like serine protease family; it is produced almost exclusively by the prostate epithelial cells, and is used as a serum marker to help in the diagnosis and to monitor the progression of prostate cancer (5). The 5' upstream promoter and enhancer region of the *PSA* gene contains up to six AREs to which ligand activated AR binds and induces expression of PSA (5-8).

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AKT is a serine (Ser)/threonine (Thr) protein kinase and plays an important role in controlling cell growth and apoptosis (9). Phosphatidylinositol 3-kinase (PI3-K) is upstream of the AKT, activating AKT by phosphorylation of the protein at Ser473 and Thr308 (9). The activated AKT phosphorylates target molecules including Bad, forkhead transcriptional factor (FKHR), and glycogen synthase kinase-3ß (GSK-3ß), which cumulatively have anti-apoptotic effects (10-12). PTEN phosphatase is a major negative regulator of the PI3-K/AKT signal pathway (13,14). In many cancer types including those from prostate, PTEN is inactivated by several mechanisms such as either homozygous deletions, hemizygous deletion and mutation on the second allele, or methylation of its promoter region. Loss of expression of this phosphatase results in constitutive activation of AKT signaling (15,16).

The D-type cyclins, D1, D2 and D3, are closely related proteins whose expression is induced by mitogens and growth factors (17) and down-regulated by growth factor deprivation or by antimitogens (18). The D-type cyclins associate with cyclin-dependent protein kinase Cdk4 or Cdk6 to form an active complex that phosphorylates and inactivates the retinoblastoma protein, pRb, resulting in prolonged cell survival (19). Cyclin D1 is aberrantly overexpressed in many types of cancer including those from breast, colon and prostate (20-23). Previous studies showed that the level of cyclin D1 is regulated by GSK-3ß, which phosphorylates cyclin D1 on Thr286, leading to degradation via ubiqutin-dependent 26S proteasome (24). Thus, activated AKT inhibits GSK-3ß kinase and increases the level of cyclin D1 protein. This study found that *Zanthoxyli* Fructus inhibited growth of a wide variety of cancer cells *in vitro* and *in vivo*. It showed the strongest anti-tumor activity against androgen-dependent prostate cancer LNCaP cells. *Zanthoxyli* Fructus down-regulated levels of PSA and cyclin D1 in conjunction with blockade of signaling by AR and AKT.

Materials and methods

Cell line. Prostate (LNCaP, PC-3, DU145), breast (MCF-7, T47D, MDA-MB231), lung (NCI-H460 -520), myeloid leukemia (HL-60), and lymphoblastic T-cell leukemia (Jurkat) cells were obtained from American Type Culture Collection (Manassas, VA). NB4 cell line was a kind gift from M. Lanotte (St. Louis Hospital, Paris, France). The cells were maintained in RPMI-1640 with 10% fetal bovine serum (FBS).

Chemicals. *Zanthoxyli* Fructus (lot no. 251094010) was obtained from Tsumura (Tokyo, Japan). The purity of *Zanthoxyli* Fructus was confirmed by three-demensional (3D) chromatography, as previously described (25) (figure not shown). To prepare the extract, 100 mg of *Zanthoxyli* Fructus was added to 1 ml double distilled water and incubated at 40˚C for 1 h in a water bath with constant stirring. The mixture was further sonicated for 5 min, and undissolved solid residue was filtered through a 0.2-μm Millipore filter (Millipore Corporation, Bedford, MA). The filtrate was stored at 4˚C.

Trypan blue exclusion test. Leukemia cells (10^5/ml) and adherent cells (10^4/ml) were incubated with a variety of concentrations of *Zanthoxyli* Fructus for 2 days in 96-well plates (Flow Laboratories, Irvine, CA). After culture, cell number and viability were evaluated by staining with trypan blue and counting by light microscopy.

Colony-forming assay. Resuspended mononuclear bone marrow cells from healthy volunteers ($5x10^5$ cells/ml) were added 1:10 to methylcellulose medium H4534 (StemCell Technologies Inc., Vancouver, British Columbia, Canada) to yield a final concentration of 1% methylcellulose, 30% FCS, 1% BSA, 10^{-4} M mercaptoethanol, $2x10^{-3}$ M L-glutamine, 50 ng/ml stem cell factor, 10 ng/ml GM-CSF, and 10 ng/ml IL-3. Cells were plated in six-well plates in a volume of 1 ml. Before this step, control diluent or *Zanthoxyli* Fructus (2 or 4 μl/ml) was pipetted into the wells. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2, and 10 days later, colonies were counted. All experiments were done twice using duplicate plates per experimental point.

Assessment of apoptosis. Apoptotic cell death was examined by terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) method using the *in situ* cell death detection kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. For quantification, three different fields were counted under the microscope and at least 300 cells were enumerated in each field. All experiments were performed twice.

Plasmids. A 564-bp fragment of the PSA promoter with a 2.4-kb enhancer sequence (-5322 to -2925) cloned upstream of luciferase (PSA P/E-Luc) was used (26). Also, ARE4-E4 Lux, which is the multimerized four consensus AREs from the PSA promoter cloned upstream of the luciferase gene in the pGL3 vector (Promega, Chicago, IL) was utilized (26).

Transfections and luciferase assay. LNCaP cells were plated in 24-well plates and incubated until 60-80% confluency. Cells were transfected with the indicated plasmids using the GenePorter transfection reagent (Gene Therapy Systems, Inc., San Diego, CA). Following transfection, cells were incubated with 10% charcoal-stripped FBS RPMI-1640 either with or without DHT (10^{-8} M) and either with *Zanthoxyli* Fructus or control diluent for 24 h. Luciferase activity in cell lysates was measured by the Dual Luciferase assay system (Promega, Madison, WI); and this was normalized by Renilla activities. The results are presented as fold induction, which is the relative luciferase activity of the treated cells over that of control cells. All transfection experiments were carried out in triplicate wells and repeated separately at least 3 times.

Western blot analysis. LNCaP cells were plated on 60-mm plates and incubated until 60-80% confluency, then the media was replaced with RPMI-1640 containing 10% charcoal striped FBS either with or without DHT (10^{-8} M) and with either *Zanthoxyli* Fructus or control diluent. After incubation, cells were washed twice in PBS; whole cell lysates and nuclear extracts were prepared as previously described (26). Proteins were resolved by 4-15% SDS polyacrylamide gel, transferred to an immobilon polyvinylidene difluoride membrane (Amersham Corp., Arlington Heights, IL), and probed
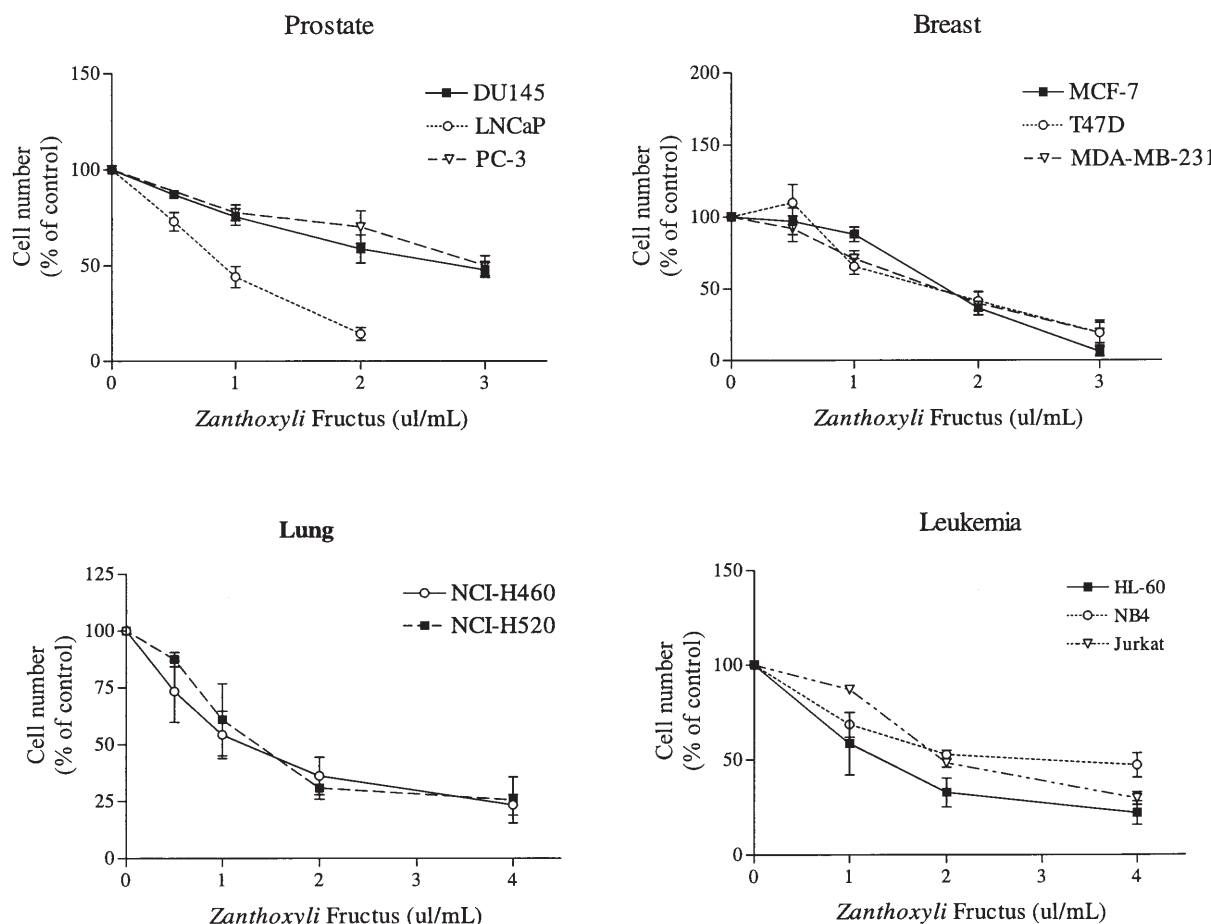
Figure 1. *Zanthoxyli* Fructus inhibits proliferation of a wide variety of cancer cells. (A) Prostate cancer (LNCaP, DU145, PC-3); (B) breast cancer (MCF-7, T47D, MDA-MB231); (C) non-small cell lung cancer (NCI-H460, NCI-H520); and (D) leukemia (HL-60, NB4, Jurkat) cells were plated in 96-well plates and cultured either with or without *Zanthoxyli* Fructus (0.5-4.0 μl/ml). After 2 days, cell number was counted by trypan blue exclusion test. Results represent the mean ± SD of three experiment done in triplicate.

sequentially with antibodies. Anti-AR N-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PSA C-19 (Santa Cruz), anti-cyclin D1 (Santa Cruz), anti-AKT (Cell Signaling, Beverly, MA), anti-p-AKT (Ser[473]) (Cell Signaling), anti-p-GSKα/ß (Ser[21/9]) (Cell Signaling), and anti-ß-actin (Santa Cruz) antibodies were used. The blots were developed using the enhanced chemiluminescence kit (Amersham Corp.).

AKT immunoprecipitation kinase assay. LNCaP cells were treated with either *Zanthoxyli* Fructus (0.5 μl/ml) or control diluent. After 24 h, cells were harvested and lysates were prepared. AKT kinase assay was performed using the AKT kinase assay kit (Cell Signaling), according to the manu-facturer's instructions. Briefly, 500 μg of cell lysates were incubated for 12 h with protein G-agarose beads bearing anti-AKT on a rotator at 4°C to immunoprecipitate Akt. This precipitate was next used to phosphorylate a specific substrate, the recombinant GSK-3α/ß protein expressed in *Escherichia coli*. Briefly, 1 μg of recombinant GSK-3α/ß were incubated with Akt-antibody-protein G-agarose complexes in the presence of magnesium/ATP mixture for 30 min at 37°C. Samples were boiled for 5 min, resolved on 10% SDS-PAGE, and transferred onto immobilon polyvinylidene difluoride membrane. The membranes were incubated sequentially with an anti-p-GSK-3α/ß (Ser[21/9]) and anti-AKT antibodies.

Small interfering RNA (siRNA) transfection. The Signalsilence Akt siRNA kit (Cell Signaling) was utilized to disrupt AKT signaling in LNCaP cells. In brief, LNCaP cells were transfected with either non-specific or AKT siRNA (final concentration of 100 nM) using transfection reagent (Cell Signaling). After 2 days, cells were harvested and subjected to Western blot analysis.

Mice. BALB/c nude mice at 6 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan) and were maintained in pathogen-free conditions with irradiated chow.

Treatment protocol. Animals were bilaterally, subcutaneously (s.c.) injected with 2x10^{6} DU145, LNCaP, or MDA-MB231 cells/tumor in 0.1 ml Matrigel (Collaborative Biomedical Products, Bedford, MA). These mice were divided randomly into 2 groups of 5 mice each: Group A, received 100 μl control diluent (control); Group B, received 100 μl water extract of *Zanthoxyli* Fructus every other day orally for 6 weeks. Tumors were measured every week with vernier calipers. Tumor size was calculated by the formula: a x b x c, where 'a' is the length and 'b' is the width and 'c' is the height in millimeters. At the end of the experiment, animals were sacrificed by CO_2 asphyxiation and tumor weights were measured after their careful resection.
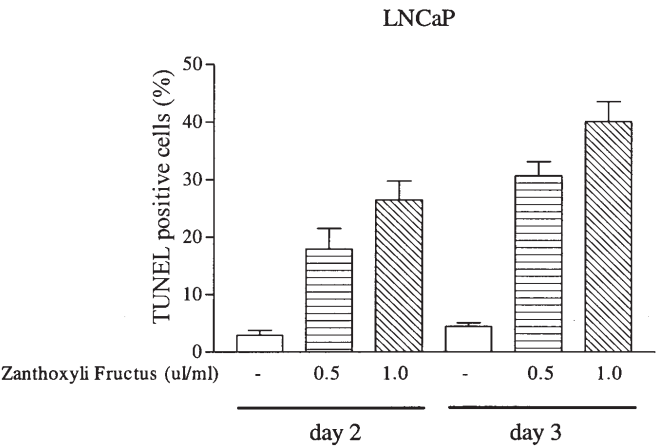
Figure 2. TUNEL assay. LNCaP cells were plated in 96-well plates and cultured either with or without *Zanthoxyli* Fructus (0.5 or 1.0 μl/ml); at 2 or 3 days later, apoptosis was measured by TUNEL assay. Results represent a mean ± SD of two experiments performed in triplicate.

Measurement of PSA in mice. Blood was withdrawn from mice several hours before sacrifice. Serum levels of PSA were measured by an immune assay employing the Hybritech Access (Beckman Coulter, Inc., Chaska, MN) according to the manufacturer's protocol.

Histology. Tumors were fixed for 12 h in 10% neutral-buffered formaldehyde after sacrifice, tissue blocks were embedded in paraffin, and hematoxylin and eosin-stained sections were examined by light microscopically.

Statistical analysis. Statistical significance of the differences was analyzed by the non-parametric Mann-Whitney U test.

Results

Effect of Zanthoxyli Fructus on the proliferation of cell lines from prostate, breast, and lung cancers as well as leukemia. Cells were cultured in the presence of various concentrations of *Zanthoxyli* Fructus (1-4 μl/ml) for 2 days. Dose-response curves were drawn (Fig. 1A-D), and the effective dose that inhibited 50% of target cells (IC_{50}) was determined. All cancer cell lines including those from prostate (LNCaP, DU145, PC3), breast (MCF-7, MDA-MB-231), non-small cell lung (NSCL) (NCI-H520, NCI-H460), acute myeloid leukemia (NB4, HL-60), acute lymphoblastic T-cell leukemia (Jurkat) were effectively inhibited in their proliferation in a dose-dependent manner. For example, IC_{50} of *Zanthoxyli* Fructus was 0.9, 1.8, and 1.3 μl/ml for LNCaP, T47D, and HL-60 cells, respectively. On the other hand, *Zanthoxyli* Fructus (2 or 4 μl/ml) did not affect growth of normal myeloid committed stem cells (CFU-GM) from healthy volunteers as measured by the clonogenic soft agar assay (data not shown).

Zanthoxyli Fructus induces apoptosis of LNCaP cells. In further experiments, we studied whether *Zanthoxyli* Fructus possessed proapoptotic effects against LNCaP cells by employing the TUNEL assay. *Zanthoxyli* Fructus induced apoptosis in a dose- and time-dependent manner. Exposure of LNCaP cells to either 0.5 or 1.0 μl of *Zanthoxyli* Fructus induced either 18±3% or 26±3% of these cells, respectively
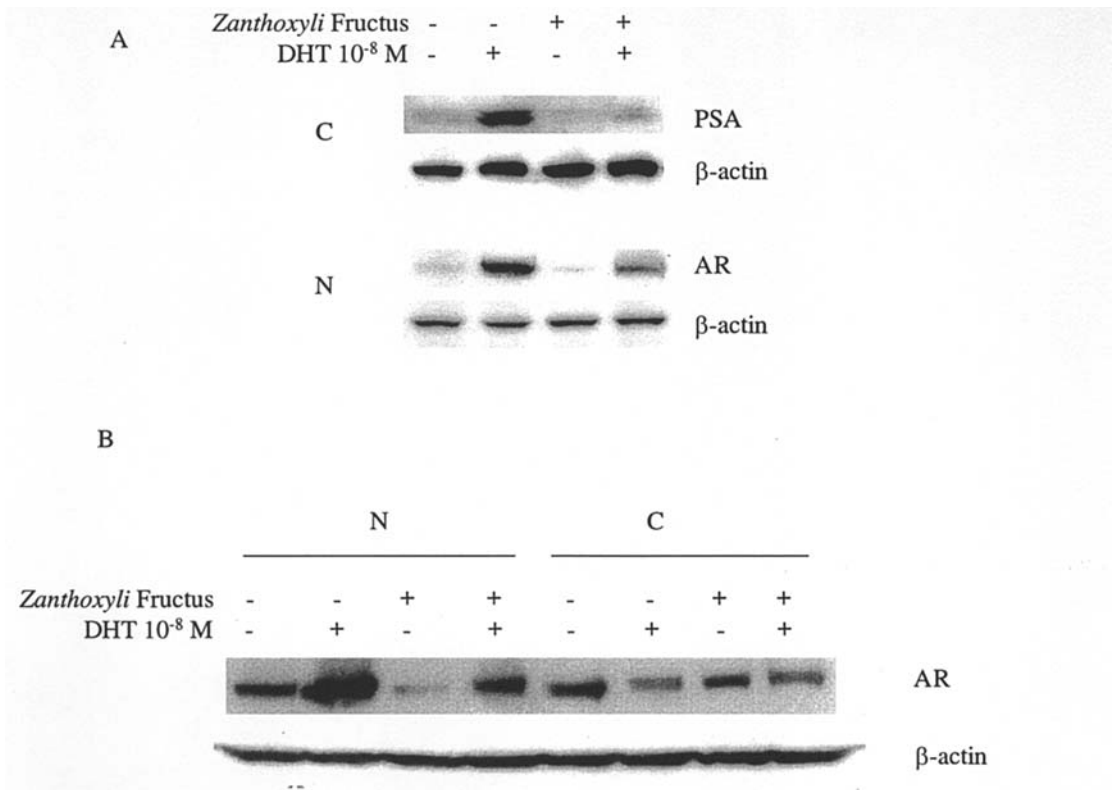

Figure 3. Effect of *Zanthoxyli* Fructus on expression of PSA and AR. LNCaP cells were placed in culture media containing 10% charcoal stripped FBS for 24 h before the addition of either DHT (10^{-8} M) alone, or DHT (10^{-8} M) in combination with *Zanthoxyli* Fructus (0.5 μl/ml). After the addition of reagents, cells were cultured for either 24 h (A) or 6 h (B), cytoplasmic and nuclear proteins were extracted and subjected to Western blot analysis. The band intensities were measured by densitometry. PSA, prostate specific antigen; AR, androgen receptor; C, cytoplasmic protein; N, nuclear protein.
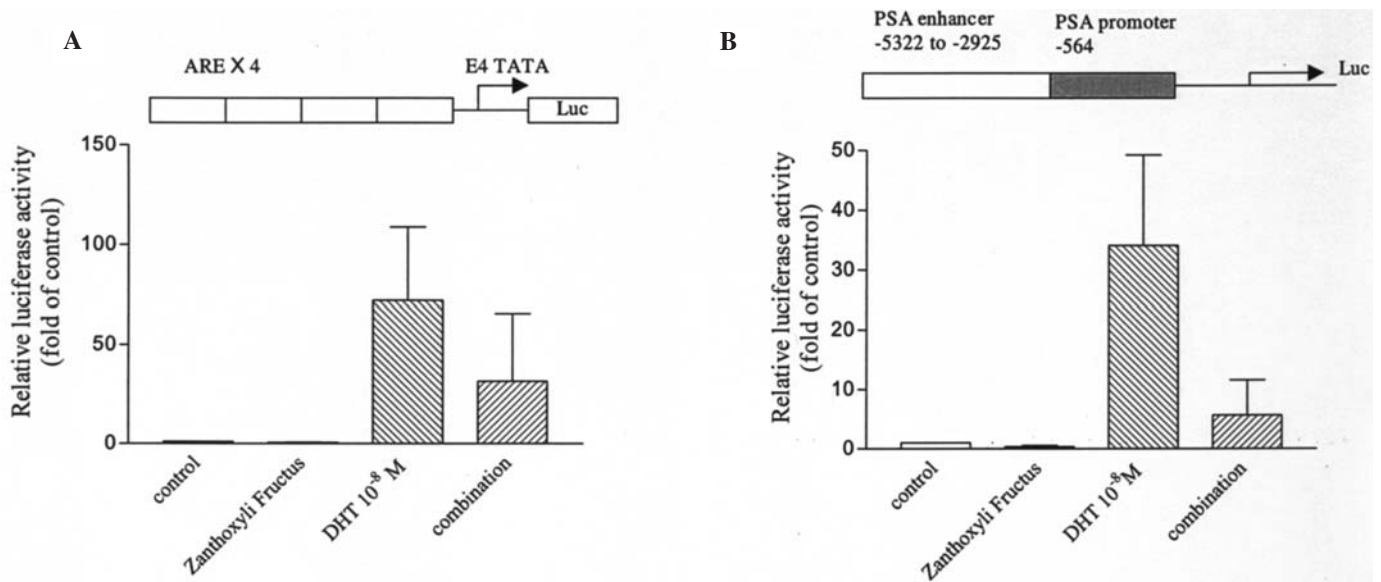
A

ARE X 4   E4 TATA

B

PSA enhancer   PSA promoter
-5322 to -2925   -564

Figure 4. (A) Effect of *Zanthoxyli* Fructus on ARE activation in LNCaP cells. Shown at the top is the construct (ARE4-E4Lux) containing the 4 concatemerized androgen receptor response elements (ARE) identical to those in the PSA enhancer, which is attached to the luciferase reporter. LNCaP cells were transfected with ARE4-E4Lux (0.8 μg). DHT (10^{-8} M) was added either with or without *Zanthoxyli* Fructus (0.5 μl/ml). (B) Effect of *Zanthoxyli* Fructus on the transcriptional activity of the PSA promoter/enhancer in LNCaP cells. The reporter construct (PSA P/E-Luc) is shown at the top. LNCaP cells were transfected with PSA P/E-Luc (0.8 μg), and DHT (10^{-8} M) either with or without *Zanthoxyli* Fructus (0.5 μl/ml) was added. pRL-SV40-Luciferase (Renilla luciferase) vector was cotransfected for normalization. Results represent the mean ± SD of three experiments done in triplicate.

to become apoptotic on the second day of culture, which increased to either 30±3% or 41±2%, respectively, on the third day of culture (Fig. 2). The proapoptotic effects of *Zanthoxyli* Fructus were also observed in NB4, HL-60, and Jurkat leukemia cells (data not shown).

Effect of Zanthoxyli Fructus on AR signaling. Level of PSA in the control LNCaP cells cultured with charcoal-stripped FBS (10%, 24 h) was negligible (Fig. 3A). DHT (10^{-8} M, 24 h) dramatically induced PSA expression in these cells. Exposure

of LNCaP cells to *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) almost completely blocked DHT-induced levels of PSA in these cells. (Fig. 3A). In addition, we explored the effect of *Zanthoxyli* Fructus on the nuclear level of AR. DHT (10^{-8} M, 24 h) increased the nuclear levels of AR by ~20-fold; and the addition of *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) decreased DHT-induced nuclear levels of AR by 95% (Fig. 3B). To study whether *Zanthoxyli* Fructus affects nuclear translocation of AR mediated by DHT, we analyzed levels of AR after a short exposure of the LNCaP cells to DHT (Fig. 3C). DHT (10^{-8} M,
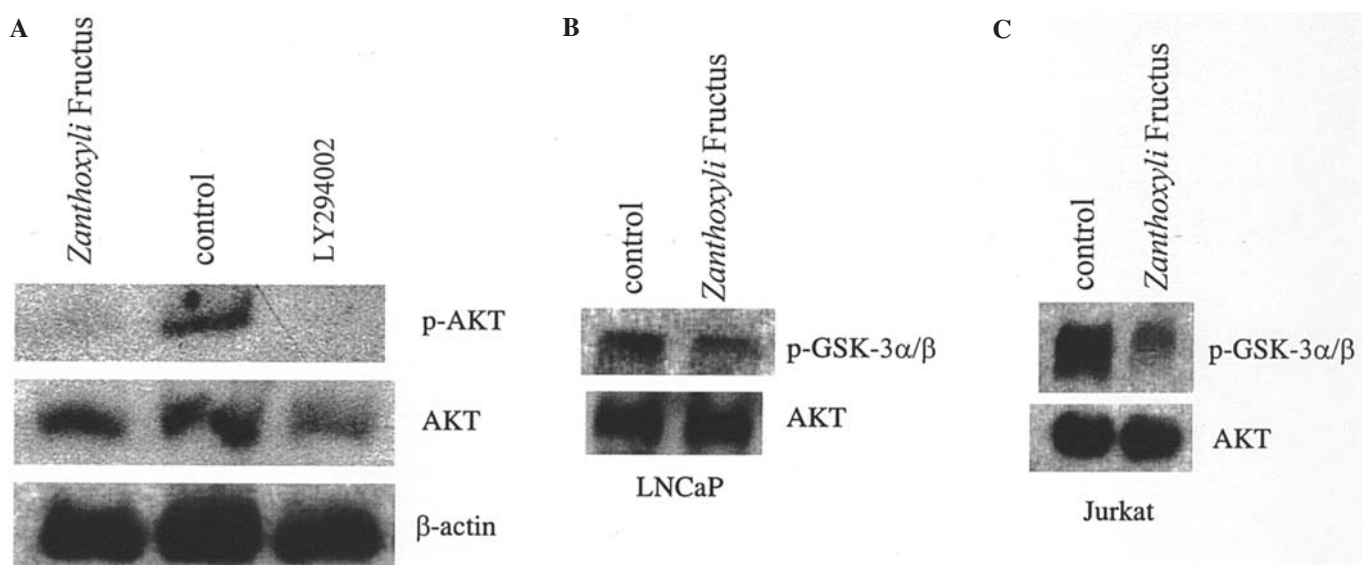
A

B

LNCaP

C

Jurkat

Figure 5. Effect of *Zanthoxyli* Fructus on AKT activity. (A) Western blot analysis. LNCaP cells were cultured with either *Zanthoxyli* Fructus (0.5 μl/ml), LY294002 (2×10^{-5} M), or control diluent. After 24 h, cells were harvested and proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with anti-p-AKT (Ser^{473}), -AKT, and -ß-actin antibodies. (B and C) AKT kinase assay. LNCaP (B) or Jurkat (C) cells were cultured either with or without *Zanthoxyli* Fructus (0.5 μl/ml for LNCaP, 2.0 μl/ml for Jurkat cells). After 24 h, cells were harvested and proteins were extracted and subjected to the AKT kinase assay using recombinant GSK-3α/ß as a substrate. The polyvinylidene fluoride membrane was sequentially probed with anti-p-GSK-3α/ß (Ser$^{21/9}$) and -AKT antibodies.
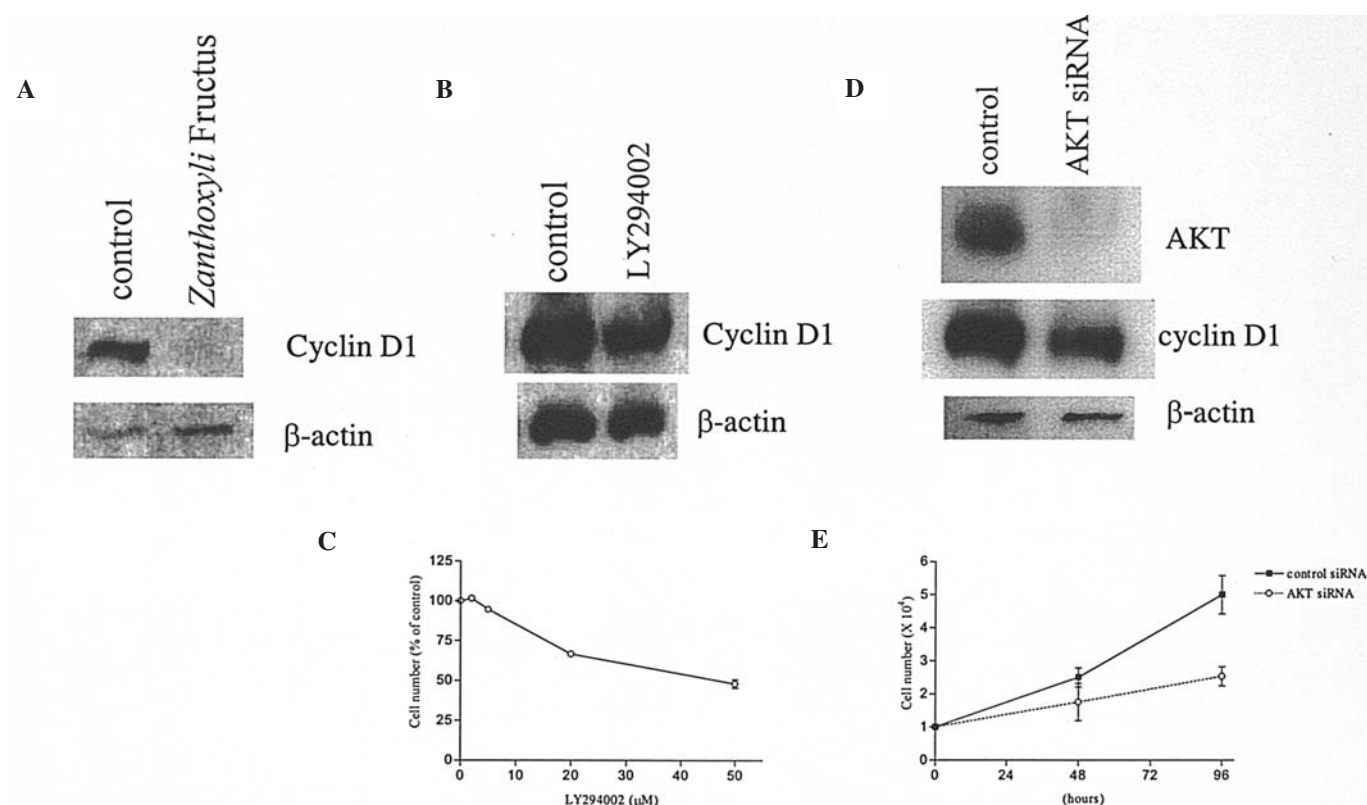
Figure 6. Effect of *Zanthoxyli* Fructus (A) or LY294002 (B) on cyclin D1 protein. Western blot analysis: LNCaP cells were cultured with either *Zanthoxyli* Fructus (A) (0.5 μl/ml) or LY294002 (B) (2x10^{-5} M). After 24 h, cells were harvested and proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with anti-cyclin D1 and -ß-actin antibodies. (C) Trypan blue exclusion test: LNCaP cells were plated in 96-well plate and cultured in the presence of LY294002 (2x10^{-6} M - 5x10^{-5} M). After 3 days, cell number was counted by trypan blue exclusion test. Results represent the mean ± SD of triplicate cultures done in duplicate experiments. (D) Transient transfection of AKT siRNA in LNCaP cells: Either control or AKT siRNA was tranfected into LNCaP cells. After 48 h, proteins were extracted and subjected to Western blot analysis. The polyvinylidene fluoride membrane was sequentially probed with anti-AKT, -cyclin D1, and -ß-actin antibodies. (E) Trypan blue exclusion test: Either control or AKT siRNA transfected cells were plated in 96-well plate and cultured for various durations as indicated. Viable cell numbers were evaluated by trypan blue exclusion test. Results represent the mean ± SD of triplicate cultures done in duplicate experiments.

6 h) increased the nuclear levels of AR by ~5-fold; *Zanthoxyli* Fructus (0.5 μl/ml, 6 h) decreased DHT-induced nuclear levels of AR by ~70% (Fig. 3D). At the same time, we extracted cytoplasmic protein and measured levels of AR. Exposure of LNCaP cells to DHT decreased levels of AR in cytoplasm by 50%; however, the addition of *Zanthoxyli* Fructus did not affect this DHT-induced down-regulation of AR (Fig. 3D), suggesting that *Zanthoxyli* Fructus did not interfere with DHT-induced nuclear translocation of AR.

Effect of Zanthoxyli Fructus on the promoter of PSA. To explore the effect of *Zanthoxyli* Fructus on the transcriptional activity of the ligand activated AR, the luciferase reporter construct in which the ARE of the PSA was concatemerized (ARE4-E4Lux) was utilized. LNCaP cells were transfected with ARE4-E4Lux and cultured with DHT (10^{-8} M) either with or without *Zanthoxyli* Fructus (0.5 μl/ml). The reporter activity increased ~70-fold when these cells were cultured with DHT (10^{-8} M, 18 h), as compared to the non-treated control LNCaP cells (Fig. 4C). When the cells were treated with the combination of DHT (10^{-8} M, 18 h) and *Zanthoxyli* Fructus (0.5 μl/ml, 18 h), luciferase activity was reduced by 55% compared with DHT alone (Fig. 4A).

We next analyzed the effect of *Zanthoxyli* Fructus on the ability of DHT to transactivate the PSA promoter/enhancer.

The LNCaP prostate cancer cells were cultured with DHT (10^{-8} M, 18 h) after they were transfected with the PSA promoter/enhancer-luciferase reporter vector. The reporter activity increased ~30-fold as compared with non-treated control LNCaP cells (Fig. 4B). When the cells were treated with the combination of DHT (10^{-8} M, 18 h) and *Zanthoxyli* Fructus (0.5 μl/ml, 18 h), luciferase activity was dramatically reduced by ~85% compared with DHT alone (Fig. 4B).

Effect of Zanthoxyli Fructus on AKT signaling. LNCaP cells constitutively expressed the phosphorylated form of AKT, and exposure of these cells to *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) completely blocked phosphorylation of AKT (Fig. 5A). We utilized PI3-K inhibitor LY294002 (2x10^{-5} M, 24 h) as a positive control, which also down-regulated levels of the phosphorylated form of AKT (Fig. 5A). The effect of *Zanthoxyli* Fructus on AKT signaling was further studied employing the AKT kinase assay with GSK-3α/ß as a substrate. *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) inhibited AKT kinase activity in LNCaP cells by ~50% (Fig. 5B). In addition, we studied the effect of *Zanthoxyli* Fructus on AKT signaling using Jurkat cells in which AKT signaling is activated because of loss of PTEN expression (27). Exposure of these cells to *Zanthoxyli* Fructus (2 μl/ml, 24 h) inhibited AKT kinase activity by ~80% (Fig. 5C).
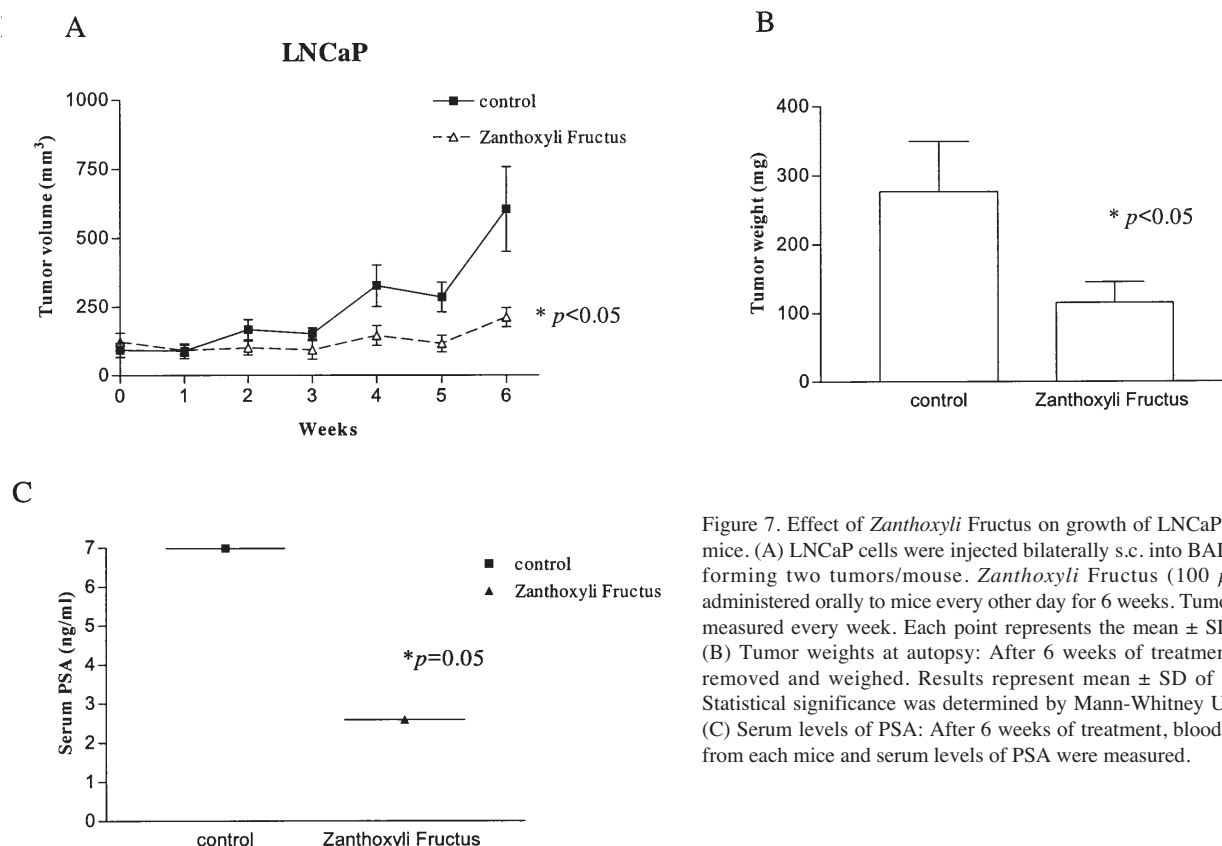
Figure 7. Effect of *Zanthoxyli* Fructus on growth of LNCaP tumors in nude mice. (A) LNCaP cells were injected bilaterally s.c. into BALB/c nude mice, forming two tumors/mouse. *Zanthoxyli* Fructus (100 μl/mouse) was administered orally to mice every other day for 6 weeks. Tumor volumes were measured every week. Each point represents the mean ± SD of 10 tumors. (B) Tumor weights at autopsy: After 6 weeks of treatment, tumors were removed and weighed. Results represent mean ± SD of tumor weights. Statistical significance was determined by Mann-Whitney U test. Bars, SD. (C) Serum levels of PSA: After 6 weeks of treatment, blood was withdrawn from each mice and serum levels of PSA were measured.

Effect of Zanthoxyli Fructus on expression of cyclin D1. LNCaP cells constitutively expressed cyclin D1 protein; exposure of these cells to *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) dramatically down-regulated levels of cyclin D1 (Fig. 6A). LY294002 (2×10^{-5} M, 24 h) also down-regulated levels of cyclin D1 and inhibited growth of LNCaP cells with an ED_{50} of 5×10^{-5} M on the third day of culture (Fig. 6B). Furthermore, we transiently transfected AKT siRNA to down-regulate AKT protein. Expression of cyclin D1 was decreased by ~60% in AKT siRNA transfected cells and their proliferation was slowed compared to non-specific siRNA transfected cells; the doubling time of control siRNA transfected LNCaP cells was ~30 h. On the other hand, the doubling time was prolonged to 98 h when AKT signaling was blocked by AKT siRNA (Fig. 6E). These results suggested that *Zanthoxyli* Fructus down-regulated levels of cyclin D1 in LNCaP cells via inhibition of AKT signaling, resulting in growth inhibition.

Effect of Zanthoxyli Fructus on LNCaP cells in vivo. We evaluated the effect of *Zanthoxyli* Fructus *in vivo* on LNCaP cells growing in BALB/c nude mice. *Zanthoxyli* Fructus was given by gavage every other day. Tumor volumes were measured weekly. All mice were euthanized after 6 weeks, and tumors were dissected and weighed. *Zanthoxyli* Fructus significantly suppressed both the growth of LNCaP tumors (p<0.05; Fig. 7A) and their mean weights (p<0.05) at autopsy (Fig. 7B), as compared with diluant controls.

Blood was withdrawn from mice several hours before they were sacrificed, and levels of PSA were measured. A mean serum levels of PSA in the mice who received *Zanthoxyli* Fructus was significantly lower than those who received diluent (2.6±1.3 ng/ml vs. 7.0±2.9 ng/ml, p=0.05) (Fig. 7C). Serum levels of PSA in normal mice at the same age were <0.03 ng/ml (figure not shown).

The tumors and organs of the mice were fixed, stained, and viewed by light microscopy. The tumors from control mice showed typical histological appearance of infiltrating, poorly differentiated adenocarcinomas of the prostate (Fig. 8A). Tumors from mice receiving *Zanthoxyli* Fructus had marked fibrosis (Fig. 8B). Some tumor cells treated with *Zanthoxyli* Fructus showed pyknotic nuclei and cytoplasmic shrinkage with eosinophilia, indicating that they were undergoing apoptosis (Fig. 8C). Organs from mice treated with *Zanthoxyli* Fructus did not show any changes compared to controls, including their livers, kidneys, spleens, lungs, and hearts. During the study, all the mice were weighed each week; the mean weights of the experimental groups were statistically the same as those of the control mice (data not shown).

Effect of Zanthoxyli Fructus on DU145 androgen-independent prostate and MDA-MB123 breast cancer cells in vivo. We also examined anti-tumor effects of *Zanthoxyli* Fructus *in vivo* using a more aggressive form of prostate cancer (DU145) as well as aggressive breast cancer MDA-MB123 cells. As shown in Fig. 9, *Zanthoxyli* Fructus significantly (p<0.05) slowed their growth (Fig. 9A and C) and significantly (p<0.03) reduced their tumor weights at autopsy (Fig. 9 B and D).

Discussion

Zanthoxyli Fructus possessed anti-tumor activity against a wide variety of cancer types. Among them, androgen-dependent human prostate cancer LNCaP cells were most sensitive to growth inhibition by *Zanthoxyli* Fructus. The proliferation of
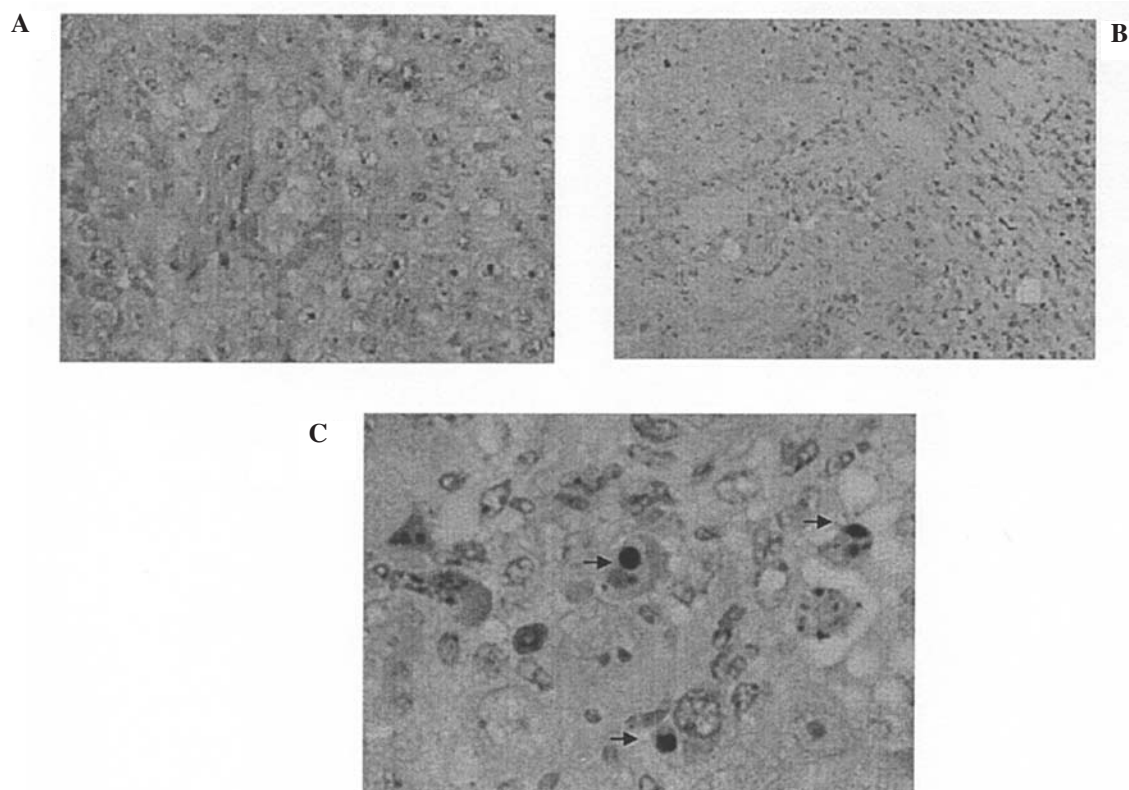
Figure 8. Histology of LNCaP human prostate tumors from mice treated with *Zanthoxyli* Fructus *in vivo*. After 6 weeks of growth in nude mice either with or without treatment with *Zanthoxyli* Fructus, LNCaP tumors were removed, fixed in formalin, and stained with H&E. (A) Control tumors from mice that received diluent displayed poorly differentiated adenocarcinoma (x200). (B) Tumors from mice treated with *Zanthoxyli* Fructus (100 μl/mouse) every other day for 6 weeks showed marked fibrosis (x200); and (C) a prominent population of these cells had pyknotic nuclei (arrows) and cytoplasmic eosinophilia, indicating apoptosis (x400).

LNCaP cells is under the control of AR signaling. In addition, AKT signaling is activated because of loss of PTEN expression, which contributes to their proliferation (16). Importantly, *Zanthoxyli* Fructus blocked both of these signal pathways. PTEN is also inactivated in PC-3 cells because of homozygous deletion, resulting in activation of AKT signaling (14). However, PC-3 cells are androgen-independent and their proliferation was not under the control of AR signaling. This could explain why PC-3 cells were less sensitive to growth inhibition by *Zanthoxyli* Fructus compared to LNCaP cells. DU145 prostate cancer cells are also androgen-independent. DU145 cells express PTEN protein and AKT signaling is inactivated and does not contribute to their proliferation (28). On the other hand, other survival factors such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) were activated in DU145 cells and regulated their proliferation (29). *Zanthoxyli* Fructus was not able to inhibit these cell survival factors (data not shown). At the present time, the molecular mechanism(s) by which *Zanthoxyli* Fructus inhibited the growth of DU145 cells remains unknown.

The activated AKT signaling is implicated in drug resistance of cancer cells. For example, forced expression of AKT resulted in enhanced drug resistance of NCI-H460 cells towards a panel of chemotherapeutic agents (30). Inhibition of the AKT signaling pathway sensitized cancer cells to the anti-cancer agents; for example, the PI3-K inhibitor LY294002 reduced resistance of HL-60 AR cells (PI3K/AKT signaling was constitutively activated) to retinoid, etoposide, and cytarabine (31). Moreovere, disruption of AKT signaling by the mTOR inhibitor rapamycin reversed chemoresistance in murine lymphoma cells *in vivo* (32). *Zanthoxyli* Fructus might sensitize cancer cells to chemotherapeutic agents via inhibition of AKT signaling.

Zanthoxyli Fructus decreased levels of cyclin D1 protein (Fig. 6A). We hypothesize that down-regulation of cyclin D1 by *Zanthoxyli* Fructus was mediated by inhibition of AKT kinase; our data suggested that inactivation of AKT stimulated GSK-3 kinase which probably phosphorylated cyclin D1 on Thr286 and shorten the half-life of cyclin D1 protein in LNCaP cells. Furthermore, when we disrupted AKT signaling in LNCaP cells by either the PI3-K inhibitor LY294002 or AKT siRNA, levels of cyclin D1 protein decreased associated with slowing cell growth (Fig. 6). These observations further augmented the hypothesis that *Zanthoxyli* Fructus down-regulated cyclin D1 via inhibition of AKT signaling.

AKT siRNA completely down-regulated levels of AKT, resulted in 40% reduction of cyclin D1 protein (Fig. 6D). *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) almost completely blocked expression of cyclin D1 protein, while inhibition of AKT kinase activity by *Zanthoxyli* Fructus (0.5 μl/ml, 24 h) was ~50% (Figs. 5B and 6A). AKT may not be the only target of *Zanthoxyli* Fructus. For example, activation of

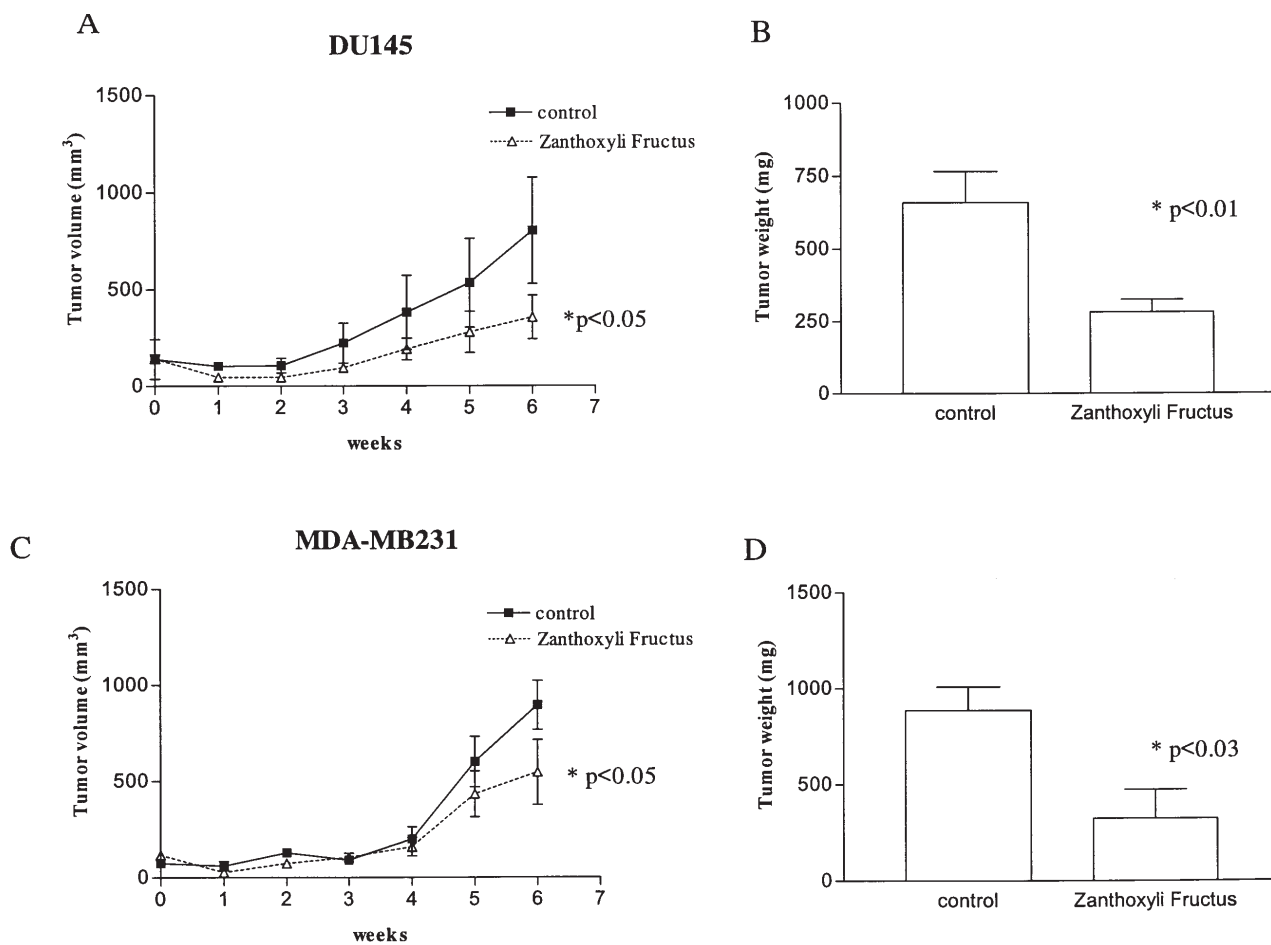A
DU145

B

C
MDA-MB231

D

Figure 9. Effect of *Zanthoxyli* Fructus on growth of tumors formed by DU145 prostate cancer cells (A and B) and MDA-MB123 (C and D) breast cancer cells in nude mice. DU145 (A) or MDA-MB231 (C) cells were injected bilaterally s.c. into BALB/c nude mice, forming two tumors/mouse. *Zanthoxyli* Fructus (100 μl/mouse) was administered to mice every other day for 6 weeks. Tumor volumes were measured every week. Each point represents the mean ± SD of 10 tumors. (B and D), Tumor weights at autopsy. After 6 weeks of treatment, DU145 (B) or MDA-MB123 (D) tumors were removed and weighed. Results represent mean ± SD of tumor weights. Statistical significance was determined by Mann-Whitney U test.

p21-activated kinase 1 (PAK1) and integrin-linked kinase (ILK) locate downstream of PI3-K and stimulates expression of cyclin D1 (33,34). *Zanthoxyli* Fructus could affect these kinases.

We always monitor purity of herb by 3D chromatography (25). A different batch of *Zanthoxyli* Fructus (lot no. 2041094010) showed an identical fingerprint pattern and similarly inhibited growth of LNCaP cells (data not shown).

Taken together, *Zanthoxyli* Fructus was active against a wide variety of cancer cell types. It showed the strongest anti-tumor activity against the LNCaP androgen-dependent prostate cancer cells in conjunction with blockade of AKT and AR signaling. *Zanthoxyli* Fructus might be useful as an adjunctive therapeutic agent for individuals with cancer. Further study will purify the active component(s) of *Zanthoxyli* Fructus.

Acknowledgements

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