

# Anticancer effect of deoxypodophyllotoxin induces apoptosis of human prostate cancer cells

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**Abstract.** Deoxypodophyllotoxin (DPPT) is extracted and separated from citrus-related plants, including *Podophyllum* (*P.*) *peltatum*, *P. pleianthum*, *P. emodi* (also called *P. hexandrum*) and *Diphylleia grayi*. DPPT has significant antitumor and antiviral activity. However, due to its strong toxicity and side effects, its use is limited in practical applications. The *in vitro* antitumor efficacy of DPPT on human prostate cancer (PCa) cells remains to be determined. The present study investigated the anticancer effect of DPPT on human PCa cells and its potential mechanism. The data revealed that DPPT markedly reduced cell proliferation and activated the caspase-3 expression level by an increase in apoptotic cell death in DU-145 cells. In addition, treatment with DPPT markedly downregulated the levels of phosphorylated Akt and activated the p53/B-cell lymphoma 2 associated X protein (Bax)/phosphatase and tensin homolog (PTEN) signaling pathway in DU-145 cells, suggesting that caspase-mediated pathways were involved in DPPT-induced apoptosis. The present study suggested the role of DPPT as a novel chemotherapeutic drug for human PCa, which may function through the Akt/p53/Bax/PTEN signaling pathway.

## Introduction

The prostate is the biggest subsidiary gonad organ in the male urogenital system, which is located in the male pelvic cavity and generally has a cone, chestnut-like shape (1). The prostate is situated between the bladder neck and the urogenital diaphragm, being its specific location below the pubic bone, under the bladder, on the upper urogenital diaphragm and before the rectum (2). The normal size of the prostate is as follows: The upper transverse diameter measures ~4 cm, the vertical diameter measures ~3 cm and the antero-posterior diameter

measures ~2 cm (3). Its surface comprises a thin layer of fiber muscular tissue called the prostate capsule, which is the outer sheath of the prostate (3). Morphologically, the prostate can be divided into three zones: The central zone, the peripheral zone and the transition zone (4). The production of prostate fluid is the main physiological function of the prostate, and a variety of enzymes and small molecular components in the prostate fluid are necessary to maintain normal sperm activity (4). The transitional zone is usually associated with hyperplasia of the prostate, while prostate cancer (PCa), the most popular malignant disease of the prostate, most commonly occurs in the peripheral zone (5). PCa is one of the most common tumors of the genitourinary system, and causes serious damage to men's health (2).

PCa is the most common malignant tumor in the male reproductive system (1). At present, PCa is acknowledged as one of the most important medical problems affecting old men (1). PCa is one of the most common solid tumors in Europe and the USA, and the number of cases of PCa has exceeded the number of lung and colorectal cancer cases, thus becoming the first type of tumor affecting men's health (3). According to the World Health Organization International Cancer Research Center GLOBOCAN 2008 estimates (6), there were ~900,000 new global PCa cases in 2008, with PCa incidence ranking in second place, behind lung cancer (7). The number of newly diagnosed PCa patients increased by 186,320 cases in the USA in 2008, and ~290,000 patients succumbed to PCa (8). In Europe, the PCa rates increased  $\leq 214/10$  (males) in 2008, with 2.6 million new cases being diagnosed each year (9). In China, the incidence of PCa is currently far lower than that in Europe, USA and other Western countries, and its morbidity and mortality rates are also far lower than the world average rates, being the global standardized incidence and the global standardized mortality rate 27.9/1,000,000 and 7.4/1,000,000, respectively, vs. 83.8/100,000 and 9.7/100,000 reported in the USA and other developed countries (8). However, with the increase in life expectancy, the westernization of the diet and the improvement in diagnostic technology, the morbidity and mortality rates of PCa are in clearly rising in China, particularly in patients with clinical stage T1 and T2, for whom a significant increase in detection rate has been reported (7,10).

Deoxypodophyllotoxin (DPPT) is extracted and separated from citrus-related plants, including *Podophyllum* (*P.*) *peltatum*, *P. pleianthum*, *P. emodi* (also

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known as *P. hexandrum*) and *Diphylleia grayi* with good antitumor activity (11). Due to the advantages of DPPT in terms of its small molecular weight and high activity, DPPT is expected to become a new type of high-efficient antitumor drug, whose research and development may provide a new road for antineoplastic drugs (12). The present study aimed to investigate the anticancer effect of DPPT on the induction of apoptosis in human PCa cells, and to further characterize its mechanism.

## Materials and methods

**Materials.** The RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA), while 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (St. Louis, MO, USA). DPPT was purchased from the Medicinal Chemical Institute of China Pharmaceutical University (Nanjing, China). Dimethyl sulfoxide (DMSO) was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Annexin V-FITC/PI Apoptosis Detection kit was purchased from BestBio. Co. Ltd. (Shanghai, China).

**Cell culture.** The human PCa cell line DU-145 was obtained from the American Type Culture Collection (Manassas, VA, USA), and was cultured in RPMI-1640 and 10% FBS, supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere.

**Cell proliferation analysis.** The anticancer effectiveness of DPPT on the cell viability of PCa cells was measured by MTT assay. DU-145 cells were plated into 96-well plates at a density of  $1.5\text{--}3 \times 10^3$  cells/well and then treated with DPPT (0, 50, 75 and 100 nM) for 12, 24 and 48 h. Next, 20 µl MTT (5 mg/ml) was added into each well, and incubated for 4 h at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. After treatment for 4 h, the medium was removed, and 150 µl DMSO was added to each well and agitated for 20 min. The optical density was measured at 490 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

**Apoptosis detection.** The anticancer effectiveness of DPPT on the apoptosis of PCa cells was measured by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay according to the manufacturer's protocol (BestBio. Co. Ltd.). DU-145 cells were plated into 6-well plates at a density of  $1\text{--}2 \times 10^6$  cells/well and then treated with DPPT (0, 50, 75 and 100 nM) for 24 h. Flow cytometry (FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA) was used to measure cell apoptosis of DU-145 cells using CellQuest Pro software (BD Biosciences).

**Caspase-3 activity assay.** The anticancer effectiveness of DPPT on the apoptosis of PCa cells was measured by Annexin V-FITC/PI assay according to the manufacturer's protocol (BestBio. Co. Ltd.). DU-145 cells were plated into 6-well plates at a density of  $1\text{--}2 \times 10^6$  cells/well and then treated with DPPT (0, 50, 75 and 100 nM) for 24 h. The proteins were extracted from cellular lysates dissolved with

radioimmunoprecipitation assay buffer, and the protein concentrations were determined with a Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent amounts of proteins (10–20 µg) were incubated with N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline for 12 h and measured at 405 nm with a microplate reader (Safire2™; Tecan; Thermo Fisher Scientific, Inc.).

**Western blot analysis.** DU-145 cells were plated into 6-well plates at a density of  $1\text{--}2 \times 10^6$  cells/well and then treated with DPPT (0, 50, 75 and 100 nM) for 24 h. The proteins were extracted from cellular lysates dissolved with buffer, and the protein concentrations were determined with a Protein Assay kit (Bio-Rad Laboratories, Inc.). Equivalent amounts of proteins (10–20 µg) were separated by 8–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The blots were incubated with the following antibodies: Anti-phosphorylated (p)-Akt (1:2,000; catalog no. sc-135650; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p53 (1:2,000; catalog no. sc-1311-R; Santa Cruz Biotechnology, Inc.), anti-B-cell lymphoma 2 (Bcl-2) associated X protein (Bax) (1:1,000; catalog no. sc-783; Santa Cruz Biotechnology, Inc.), anti-phosphatase and tensin homolog (PTEN) (1:1,500; catalog no. sc-9145; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:1,000; catalog no. sc-130656; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Then, the blots were washed with Tris-buffered saline with Tween-20 (TBST) and incubated with the appropriate secondary antibody (1:5,000; catalog no. A0208; Beyotime Institute of Biotechnology, Haimen, China) at room temperature for 2 h, prior to be developed with enhanced chemiluminescence western blot detection reagents (catalog no. P0018A; Beyotime Institute of Biotechnology).

**Statistical analysis.** All data are expressed as the mean ± standard deviation. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses, including one-way analysis of variance and Student's *t*-test. *P* < 0.05 was considered to indicate a statistically significant difference.

## Results

**Anticancer effect of DPPT reduces cell viability of human PCa cells.** The chemical structure of DPPT is shown in Fig. 1. To determine the anticancer effect of DPPT in terms of reducing the cell viability of human PCa cells, DU-145 cells were treated with DPPT (0, 50, 75 and 100 nM) for 12, 24 and 48 h, and cell proliferation was determined using MTT assay. Cell proliferation of DU-145 cells was effectively inhibited by treatment with DPPT in a dose- and time-dependent manner (Fig. 2).

**Anticancer effect of DPPT induces cell apoptosis of human PCa cells.** To explore the anticancer effect of DPPT in terms of inducing cell apoptosis of human PCa cells, DU-145 cells were treated with DPPT (0, 50, 75 and 100 nM) for 12, 24 and 48 h, and cell proliferation was determined by Annexin V-FITC/PI assay. As shown in Fig. 3, the apoptosis of DU-145 cells was induced by DPPT treatment in a dose-dependent manner (Fig. 3).

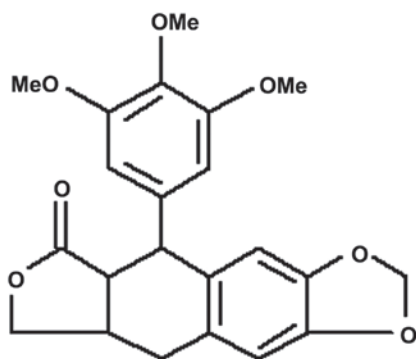


Figure 1. Chemical structure of deoxypodophyllotoxin.

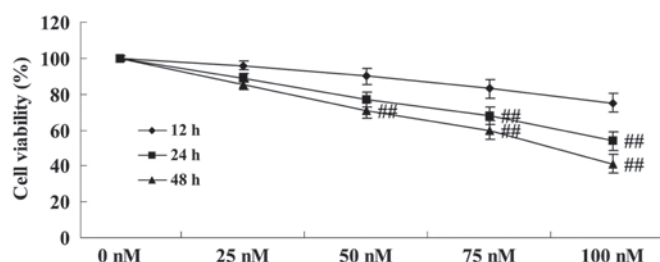


Figure 2. Anticancer effect of DPPT reduces cell viability of human prostate cancer cells. <sup>#</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin.

**Anticancer effect of DPPT induces caspase-3 activity of human PCa cells.** To explore the mechanism of DPPT by which it induces cell apoptosis in human PCa cells, the caspase-3 activity of DU-145 cells was evaluated following DPPT treatment. As expected, DPPT-treated cells exhibited a significant increase in caspase-3 activity compared with untreated cells in a dose-dependent manner (Fig. 4).

**Anticancer effect of DPPT reduces p-Akt protein expression in human PCa cells.** Compared with the blank control, the p-Akt/Akt rate was significantly inhibited in DU-145 cells treated with DPPT in a dose-dependent manner (Fig. 5).

**Anticancer effect of DPPT induces p53 protein expression in human PCa cells.** Treatment with DPPT (50 or 100 nM) for 24 h resulted in an increase in p53 protein expression in DU-145 cells compared with untreated cells (Fig. 6). This effect was also dose-dependent (Fig. 5).

**Anticancer effect of DPPT induces Bax protein expression in human PCa cells.** In order to characterize the anticancer mechanism of DPPT against DU-145 cells, the effects of DPPT on Bax protein expression were examined. Bax protein expression was markedly induced in DPPT-treated DU-145 cells compared with the blank control (Fig. 7).

**Anticancer effect of DPPT reduces PTEN protein expression in human PCa cells.** To further elucidate the mechanism involved in DPPT-mediated apoptosis, the effects of DPPT on PTEN protein expression were examined. Treatment with DPPT resulted in a dose-dependent increase in PTEN protein expression, compared with untreated control cells (Fig. 8).

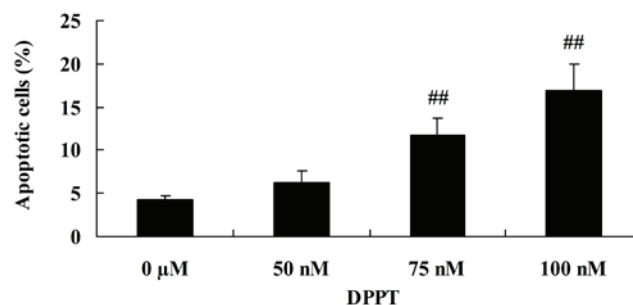


Figure 3. Anticancer effect of DPPT induces cell apoptosis in human prostate cancer cells. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin.

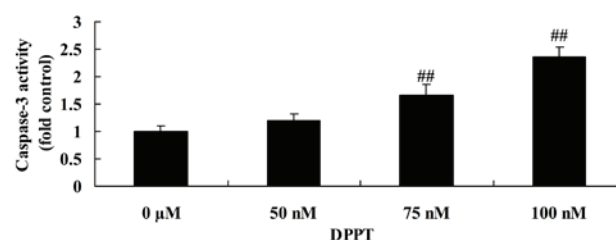


Figure 4. Anticancer effect of DPPT induces caspase-3 activity in human prostate cancer cells. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin.

## Discussion

In 2008, ~34,000 new PCa cases were reported in China (10). The standardized incidence of PCa is 4.3/100,000 of the world population, of which, 140,000 cases succumbed to disease, thus leading to a standardized mortality rate of 1.8/100,000 individuals (10). In Shanghai, the incidence of PCa increased from 12.6/100,000 (males) to 21.8/100,000 during 2000-2005, while in Beijing, the incidence of PCa increased 2.3 times during 1985-1995 (10). In 2004, the incidence of PCa in the Chinese Taiwan and Singapore areas increased 4.8 and 8.5 times, respectively, making the morbidity of PCa to be considered dangerous (10). Over the next 20 years, the incidence of PCa and the number of associated mortalities in China is expected to increase (13). It is estimated that by 2030, the average number of PCa cases and associated mortalities per year will increase from 106.4 to 111.4%, and from 4.8 to 5.1%, respectively (10,13). In the present study, DPPT significantly inhibited cell proliferation and cell apoptosis of DU-145 cells in a dose- and time-dependent manner. Several studies indicated that DPPT significantly induced cell apoptosis of HeLa cells (14), breast cancer cells (15) and non-small cell lung cancer cells (16). These results indicated that DPPT may be considered as a drug to target PCa.

The activation of the caspase family plays a key role in the process of cell apoptosis (17). Under normal conditions, caspases are synthesized and stored in the form of inactive precursors, and apoptosis signaling can initiate the caspase cascade (18). Caspase-3 is the main executing factor in the process of apoptosis, since the activation of caspase-3 has a strong effect on the induction of tumor cell apoptosis. Based on this, caspases inducing caspase-3/8/9 activation participate in the apoptosis program and cause cancer cell apoptosis (17,19).

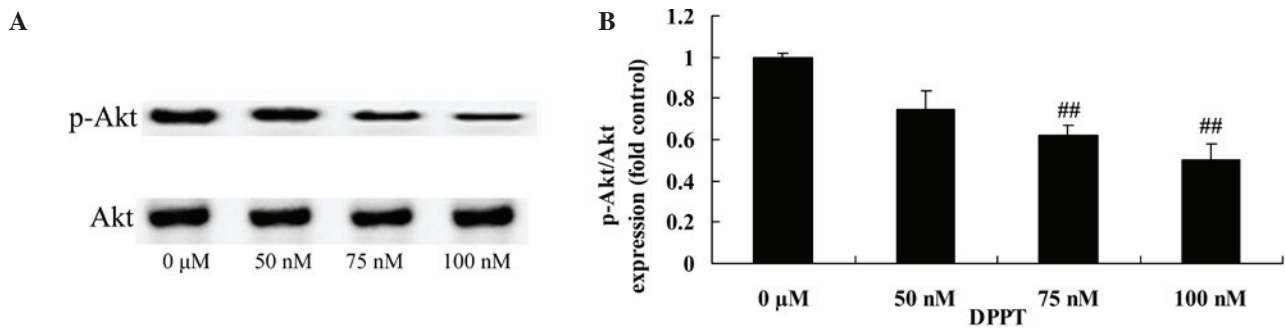


Figure 5. (A) Anticancer effect of DPPT reduces p-Akt protein expression in human prostate cancer cells, as indicated by western blot analysis. (B) Statistical analysis of p-Akt protein expression. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin; p-, phosphorylated.

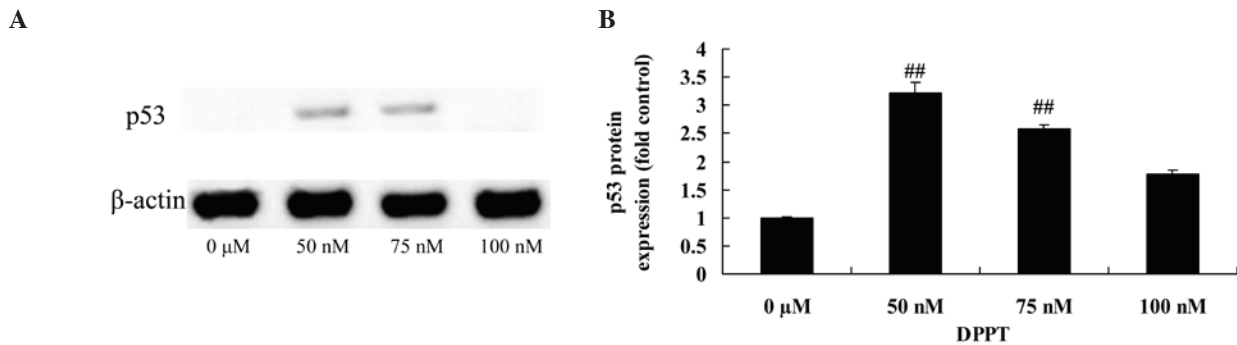


Figure 6. (A) Anticancer effect of DPPT induces p53 protein expression in human prostate cancer cells according to the results of western blot analysis. (B) Statistical analysis of p53 protein expression. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin.

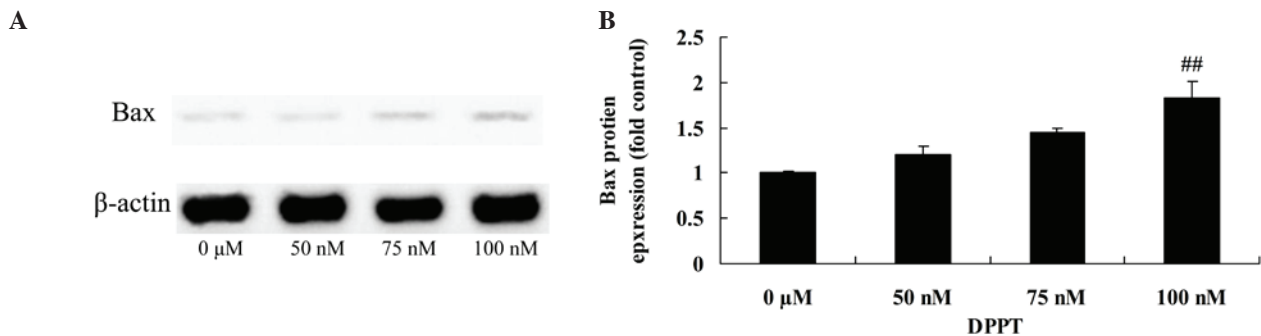


Figure 7. (A) Anticancer effect of DPPT induces Bax protein expression in human prostate cancer cells, as determined by western blotting. (B) Statistical analysis of Bax protein expression. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin; Bax, B-cell lymphoma 2 associated X protein.

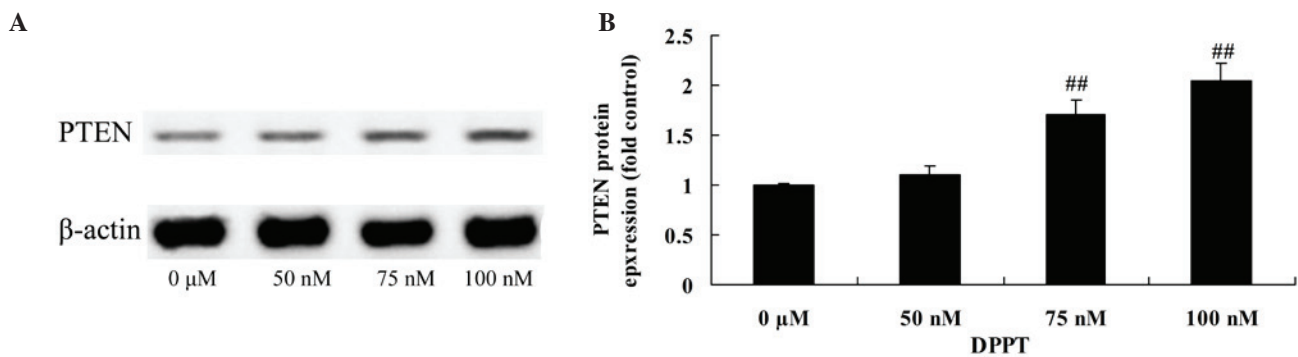


Figure 8. (A) Anticancer effect of DPPT increases PTEN protein expression in human prostate cancer cells, according to the results of western blotting. (B) Statistical analysis of PTEN protein expression. <sup>##</sup>P<0.01 vs. 0 nM DPPT-treated group. DPPT, deoxypodophyllotoxin; PTEN, phosphatase and tensin homolog.



In the present study, significant caspase-3 activity was observed in DU-145 cells upon treatment with DPPT. Sang *et al* (20) reported that DPPT inhibited microtubule formation, and induced caspase-3, Bax and p53 expression. Yong *et al* (21) indicated that DPPT inhibits tubulin polymerization and activates caspases-3/7 in HeLa human cervix carcinoma cells. The present results demonstrated that DPPT induces caspase-3 activity, and this pathway appears to be involved in the mechanism of DPPT-induced cell death.

In recent years, studies on molecular biology have demonstrated that cell proliferation and apoptosis are regulated by different genes; therefore, further studies on the regulation of genes at a molecular level will help to increase the understanding of the occurrence and development of PCa (22). Bcl-2, Bax and p53 affect the proliferation, apoptosis, occurrence and development of PCa (22,23). Among the genes involved in the regulation of cell apoptosis, Bcl-2 is currently recognized as an anti-apoptotic gene (24), which participates in the occurrence of tumors by inhibiting cell apoptosis and prolonging cell survival (25). The present results revealed that treatment with DPPT significantly reduced p-Akt protein expression, and increased p53 and Bax protein expression in DU-145 cells. Shin *et al* (14) suggested that DPPT induces G2/M cell-cycle arrest through inhibition of Akt and upregulation of the p53 and Bax signaling pathway in HeLa cells. Sang *et al* (20) demonstrated that DPPT induces cell apoptosis of non-small cell lung cancer A549 cells through p53/cell division cycle 2/Bax signaling.

Previous studies indicated that, in certain tumors such as lung cancer, malignant lymphoma and cancer of the liver, the expression of PTEN in cancerous tissues is significantly higher than that in the corresponding noncancerous tissues. High PTEN expression may be due to the cancer cell proliferation and DNA double chain fractures (26,27). PTEN is one of the key regulation factors in the process of DNA repair and the expression is then increased in order for it to participate in the process of cancer development (26,27). In previous studies, PTEN was tested in PCa and hyperplastic prostate tissues, and it was observed to be mainly expressed in PCa tissues and in the cell nuclei of hyperplastic prostate tissues (28,29). The expression of PTEN in PCa was significantly higher than that in prostate hyperplasia, which indicated that increased PTEN expression levels may be associated with the incidence of PCa (30,31). In the present study, treatment with DPPT significantly promoted PTEN protein expression in DU-145 cells. Shin *et al* (14) suggested that DPPT induces G2/M cell-cycle arrest through inhibition of Akt and activation of the PTEN signaling pathway in HeLa cells. Consequently, the present results provide the initial evidence that DPPT induces cell apoptosis via the PTEN signaling pathway.

Taken together, the results of the present study indicated that the anticancer effect of DPPT inhibited cell proliferation and induced cell apoptosis in human PCa cells through the Akt/p53/Bax/PTEN signaling pathway. Thus, the present study may provide experimental data for the use of DPPT in the treatment of human PCa.

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